Introduction

Liver fibrosis is one of the most prevalent chronic diseases in the world. Liver fibrosis and nodular regeneration leads to cirrhosis. Cirrhosis is a serious and generally irreversible disease and is the tenth leading cause of death, with an age adjusted death rate of 9.2 per 100,000 per year in USA alone. Liver fibrosis is the wound healing response of an organ by means of formation of fibrous tissue as reparative or reactive process in response to an injury. During liver fibrosis there is an excessive production and deposition of collagen which makes major alterations in both quality and quantity of hepatic extracellular matrix (ECM) and activation of hepatic stellate cells (HSC), which are the major producers of fibrotic neomatrix.

HSCs reside in the Disse’s space, the narrow space separating the sinusoidal wall from the liver cell plates and stores vitamin A as retinyl esters. Due to liver fibrosis, HSCs proliferate, loose their vitamin A and undergo a major phenotypical transformation to smooth muscle, alpha-actin positive myofibroblasts (activated HSC) which produces a variety of collagenous and noncollagenous ECM proteins. Matrix metalloproteinase (MMP), a family of zinc dependent endoproteinase, have capability to degrade the ECM components. But there is an expression of tissue inhibitors of metalloproteinases (TIMP) in fibrotic liver, which inhibits the MMP. The removal or inactivation of activated HSC formed in liver is therefore, likely to be a key process for the recovery from liver fibrosis. The degree of TIMP–I expression correlates with the extent of fibrosis, which is assessed by hydroxyproline (HP) content.

Trigonella foenum-graecum Linn. (Fenugreek) of the family Fabaceae is a well known plant in Ayurvedic and Unani medicine. In the present study an attempt was made to explore the antifibrotic effect of ethanolic extract of this plant. Liver fibrosis was induced in rats by CCl4 (carbon tetrachloride) orally 1 ml/kg for 28 days. The extent of liver fibrosis was assessed by measuring the level of liver hydroxyproline (HP), serum enzymes and total bilirubin (TBL) levels due to deposition of collagen. The liver weight of the animals was increased following CCl4 administration. The administration of ethanolic extract reduced the liver weight of CCl4 treated animals and treatment with ethanolic extract of the plant reduced significantly the HP, serum enzyme and TBL level and inhibited liver fibrosis induced by CCl4.
A detailed review of literature has afforded no information about its antifibrotic effect on liver. In the present study an attempt is made to explore the anti-liver fibrotic activity of the plant on liver fibrosis. Liver fibrosis is one of the most prevalent chronic diseases in the world hence the investigation for an efficient hepatoprotective drug from the natural source is an urgent need.

**Materials and Methods**

**Collection and preparation of whole plant extract**

The plant was authenticated by Prof. Madhava Chetty, Department of Botany, S.V. University, Tirupati. The specimens were shade dried, powdered coarsely and extracted by Soxhlet extraction method using ethanol. The drug solvent ratio of 1:2 was maintained. The yield was 14% w/w. Preliminary phytochemical qualitative analysis was carried out by standard chemical tests.

**Experimental animals**

Albino Wistar rats of either sex, weighing 180-200g, were housed under standard environmental conditions of temperature, humidity and were provided with standard rat chow and water ad libitum. The experimental procedures were carried out in strict compliance with the institutional animal ethical committee regulations.

**Study protocol**

A pilot study was conducted to study the dose response relationship of the plant extract, a dose of 100 mg/kg per orally was selected and used throughout the study.

**Induction of liver fibrosis by Carbon tetrachloride**

The rats were subjected to oral administration of 1ml/kg CCl₄ mixed with an equal volume of liquid paraffin, twice a week for 28 days. Three days after the last dose, the rats were sacrificed under ether anaesthesia. Blood and liver samples were collected for biochemical and histopathological studies.

**Treatment protocol**

The animals were divided into four groups of six animals each and treated orally as per the protocol given below:

- **Control (G₁)** — The animals were given 2 ml/kg gum tragacanth (1% w/v) orally for 28 days.
- **Extract control (G₂)** — The animals were given orally 100mg/kg of ethanolic extract of the plant daily (extract alone), suspended in 1% w/v gum tragacanth for 28 days.
- **Fibrotic control (G₃)** — The animals were given CCl₄ twice a week for 28 days (1ml/kg CCl₄ mixed with equal quantity of liquid paraffin), the remaining days they received saline.
- **Treatment group (G₄)** — The animals were given CCl₄ twice a week for 28 days (1ml/kg CCl₄ mixed with equal quantity of liquid paraffin). The animals also received 100mg/kg body weight of ethanolic extract daily, suspended in 1% w/v gum tragacanth orally. After 28 days the animals were left free for 3 days and then the animals were sacrificed.

The animals were made to fast overnight after the experimental period. They were anaesthetized with ether and blood was collected by carotid artery bleeding. Blood samples were kept for 30 minutes in dry test tubes without any disturbance. Then the supernatant layer was centrifuged for 10 minutes at 3000 rpm to separate the serum. Livers were dissected out, weighed and preserved for histopathological studies. The livers from different groups were weighed and differences in weights were noted.

**Histopathological studies**

Liver sections were preserved in 10% formalin. They were stained with haematoxylin and eosin, the stained sections were observed under the microscope to estimate the extent of liver fibrosis.

**Biochemical tests**

The serum was subjected to various biochemical tests to assess liver function, viz. aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) and total bilirubin (TBL) levels. The livers were homogenized to estimate the levels of hydroxyproline (HP).

(i) **Estimation of serum transaminases**

Buffered substrate (1ml) containing L-aspartic acid and α-ketoglutaric acid was taken in a test tube and 0.2 ml of serum was added to it. Standard pyruvate 2mM solution was added to standard and 0.2 ml of distilled water was added to blank, incubated at 37°C for about one hour to estimate AST. Serum (0.2ml) was added at the end of incubation period to control. DNPH (2, 4-Dinitrophenyl hydrazine) was added to all tubes, mixed and allowed to stand for 20 minutes at room temperature. Finally 10 ml of NaOH was added. After 20 minutes the readings were taken at 505 nm. The same procedure was followed...
using 2mM solution of DL-alanine instead of aspartic acid to estimate ALT.

(ii) Estimation of alkaline phosphatase
One ml of 1M buffer was mixed with one ml of phenyl phosphate substrate in the test tube and incubated at 37ºC for 3 minutes. Serum (0.1ml) was added and incubated for 15 minutes. The reaction was stopped by the addition of 0.8ml of 0.5N NaOH and estimated colorimetrically.

(iii) Estimation of total bilirubin
Serum (0.2ml) was diluted to 2ml by the addition of 1.8ml of distilled water. Diazo reagent (0.5ml) and 2.5ml of methanol were added to it. A blank was also prepared similarly except 0.5ml of diazo reagent. After 30 minutes, optical densities were read at 540nm. The concentration of bilirubin was read directly from standard curve.

(iv) Estimation of hydroxyproline
Liver (0.5g) was homogenised in 10 ml of 3% aqueous sulphosalicylic acid. The homogenate was filtered through Whatmann No.2 filter paper. The filtrate (2ml) was taken in a test tube to which 2ml of acid ninhydrin was added and heated in the boiling water bath for an hour. The reaction was terminated by placing the test tube in ice bath. Toluene (4ml) was added to the reaction mixture and stirred well for 2 minutes. The toluene layer was separated and warmed to room temperature. The intensity of red colour was measured at 520 nm.

Results

Phytochemical analysis
Qualitative phytochemical analysis of the ethanolic extract of the plant indicated the presence of flavonoids, alkaloids, steroids, proteins, saponins, glycosides, gums, mucilages and some sugars. Results are summarised in Table 1.

Biochemical parameters
The animals treated with saline had shown normal biochemical parameters i.e. normal AST, ALT, ALP, TBL and HP levels. The same observation was observed in the group which was treated

Table 1: Qualitative chemical analysis of the ethanolic extract of Fenugreek plant

<table>
<thead>
<tr>
<th>Tests for phytochemicals</th>
<th>Alcoholic extract of whole plant</th>
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<tbody>
<tr>
<td>1. Test for carbohydrates</td>
<td></td>
</tr>
<tr>
<td>a. Molisch test</td>
<td>+</td>
</tr>
<tr>
<td>b. Fehling’s test</td>
<td>+</td>
</tr>
<tr>
<td>2. Test for gum and mucilage</td>
<td>+</td>
</tr>
<tr>
<td>3. Test for proteins and amino acids</td>
<td></td>
</tr>
<tr>
<td>a. Millon’s test</td>
<td>+</td>
</tr>
<tr>
<td>b. Biuret test</td>
<td>+</td>
</tr>
<tr>
<td>c. Nihydrin test</td>
<td>+</td>
</tr>
<tr>
<td>4. Test for fixed oils and fats</td>
<td></td>
</tr>
<tr>
<td>a. Spot test</td>
<td>+</td>
</tr>
<tr>
<td>b. Saponification</td>
<td>+</td>
</tr>
<tr>
<td>5. Test for alkaloids</td>
<td></td>
</tr>
<tr>
<td>a. Dragendorff’s test</td>
<td>+</td>
</tr>
<tr>
<td>b. Mayer’s test</td>
<td>+</td>
</tr>
<tr>
<td>c. Wagner’s test</td>
<td>+</td>
</tr>
<tr>
<td>d. Hager’s test</td>
<td>+</td>
</tr>
<tr>
<td>6. Test for glycosides</td>
<td></td>
</tr>
<tr>
<td>a. Legal’s test</td>
<td>+</td>
</tr>
<tr>
<td>b. Balget’s test</td>
<td>+</td>
</tr>
<tr>
<td>c. Borntrager’s test</td>
<td>+</td>
</tr>
<tr>
<td>7. Test for flavonoids</td>
<td></td>
</tr>
<tr>
<td>a. Ferric chloride test</td>
<td>+</td>
</tr>
<tr>
<td>b. Shindona’s test</td>
<td>+</td>
</tr>
<tr>
<td>c. Fluorescence test</td>
<td>+</td>
</tr>
<tr>
<td>d. Reaction with alkali and acid</td>
<td>+</td>
</tr>
<tr>
<td>8. Test for saponins</td>
<td>+</td>
</tr>
<tr>
<td>9. Test for steroidal sapogenins</td>
<td>+</td>
</tr>
</tbody>
</table>

+ = Presence of phytochemicals
with extract alone. The treatment with CCl₄ has increased the level of AST, ALT, ALP, TBL and HP significantly. Treatment with ethanolic extract of the plant significantly reduced the elevated enzyme levels, but the values did not reach the normal level as shown in Table 2.

**Histopathological studies**
The results of histopathological studies are shown in Figs. 1-3

**G₁-Control** — The sections from control group showed the structure of liver with normal hepatocytes, normal liver parenchyma as shown in Figs. 1.

**G₂-Extract control** — The section treated with the plant extract showed liver parenchyma with sheets of hepatocytes showing hydropic change and mild fatty change, areas of mild necrosis were seen and sinusoids showed dilation. The picture was suggestive of very mild hepatic injury as shown in Fig. 3.

**G₃-Fibrotic Control** — The sections from CCl₄ treated group showed liver parenchyma with sheets of hepatocytes showing hydropic change, fatty change and areas of severe necrosis. There was dense periportal inflammatory infiltrate and peripoortal fibrosis, the picture was suggestive of severe hepatic injury and early hepatic fibrosis as shown in Fig. 2.

From the histopathological studies the sections of liver from treatment groups showed improvements when compared with liver damage induced in fibrotic control group. The livers of different groups were weighed and compared. The weights were similar in G₁ and G₂ (Control and extract control) whereas they were increased significantly in G₃ (Fibrotic control). The weights of livers in G₄ (Treatment group) was near to the normal. The liver weights are shown in Table 2.

**Discussion**
The statistically significant increase in biochemical parameters observed in G₃ indicates that hepatic damage was induced by CCl₄. After treatment with 100 mg/kg of ethanolic extract, all the elevated parameters were brought down significantly. These findings were further supported by histopathological studies, which clearly indicated that the hepatic fibrosis occurred in G₃ and increased liver weights and HP content were also seen in G₃. HP content is a good marker of fibrosis. Treatment with ethanolic extract of the plant markedly reduced the increased liver weights.

In the absence of reliable liver protective drugs in allopathic medical practice, liver diseases remain as one of the serious health problems. A number of herbs possess hepatoprotective activity hence can be used for the management of various liver disorders. The CCl₄ has been used as a tool to induce hepatotoxicity in experimental animals. In experimental hepatopathy, the toxin CCl₄ is biotransformed by cytochrome P₄₅₀ to produce the trichloromethyl free radical, which caused peroxidative degradation in the adipose tissue resulting in fatty infiltration of the hepatocytes. Trichloromethyl free radicals elicit lipid

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### Table 2: Effect of CCl₄ and Fenugreek on the serum enzymes, total bilirubin, hydroxyproline levels and liver weight

<table>
<thead>
<tr>
<th>Groups</th>
<th>AST (IU/l)</th>
<th>ALT (IU/l)</th>
<th>ALP (IU/l)</th>
<th>TBL (mg/dl)</th>
<th>HP (µg/g)</th>
<th>Liver weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G₁ Control</td>
<td>45.08 ± 1.225</td>
<td>41.58 ± 0.780</td>
<td>214.36 ± 1.326</td>
<td>0.69 ± 0.040</td>
<td>53.72 ± 0.834</td>
<td>3.48 ± 0.05</td>
</tr>
<tr>
<td>G₂ Extract Control</td>
<td>47.75 ± 1.371</td>
<td>39.35 ± 0.493</td>
<td>233.8 ± 16.55</td>
<td>0.59 ± 0.042</td>
<td>44.18 ± 0.837</td>
<td>3.44 ± 0.06</td>
</tr>
<tr>
<td>G₃ Fibrotic Control</td>
<td>70.53 ± 3.335 *</td>
<td>67.73 ± 2.213 *</td>
<td>404.75 ± 24.16 *</td>
<td>1.35 ± 0.102 *</td>
<td>135.91 ± 0.710 *</td>
<td>4.36 ± 0.1</td>
</tr>
<tr>
<td>G₄ Treatment Group</td>
<td>56.43 ± 1.784 *</td>
<td>55.46 ± 2.93 *</td>
<td>256.19 ± 30.98 *</td>
<td>0.89 ± 0.041 *</td>
<td>87.45 ± 0.608 *</td>
<td>3.52 ± 0.03</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SEM of 6 animals; *P<0.05 vs Control group; *P<0.05 vs fibrotic control
peroxidation of membrane lipids in presence of oxygen generated by metabolic leakage from mitochondria. All these events culminate in loss of integrity of cell membranes and damage of hepatic tissue. The increase in the levels of serum bilirubin reflected the jaundice and the increase in transaminases and alkaline phosphatases was the clear indication of cellular leakage and loss of functional integrity of the cell membrane. Assessment of liver function can be made by estimating the activities of serum AST and ALT, which are originally present in higher concentrations in cytoplasm. In hepatopathy, these enzymes leak into blood stream in conformity with the extent of liver damage. The elevated levels of marker enzymes (AST, ALT and ALP), TBL and HP in CCl₄ treated rats in the present study corresponded to the extensive liver damage, jaundice and fibrosis induced by the CCl₄, respectively. The extract by itself was not found to alter function as shown by normal values of these parameters in the extract control group G2. Pretreatment with the 100mg/kg ethanolic extract of the plant significantly reduced the elevated liver enzymes, TBL and HP levels (P<0.05), indicating hepatoprotective and antifibrotic action. Comparative histopathological study of the liver from different groups of rats corroborated the hepatoprotective efficacy of the plant. Various pathological changes like hydropic change, steatosis, vacuolisation, fatty change, areas of severe necrosis, periportal inflammatory infiltrate and periportal fibrosis, associated with liver fibrosis were prevented to moderate extent in treatment group (G4).

Hepatotoxic effect of CCl₄ is due to oxidative damage by free radical generation and antioxidant property is claimed to be one of the mechanisms of hepatoprotective drugs. Many phytochemical reports revealed that the ethanolic extract of the plants were found to contain higher concentration of flavonoids and glycosides. The qualitative phytochemical investigations of the ethanolic extract of the plant showed positive for the presence of flavonoids and glycosides. It has been reported that the flavonoid constituents of plant possess antioxidant properties by free radical scavenging. Flavonoids are phenolic
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compounds widely distributed in plants, which have been reported to exert multiple biological effects, including antioxidant and free radical scavenging abilities\(^\text{32,33}\). Overexpression of fibrogenic cytokines as well as increased transcription and synthesis of collagen can be down regulated, at least in experimental models, by the use of antioxidants\(^\text{34}\) and a study has demonstrated that natural phenolics inhibit stellate cell activation by perturbing signal transduction pathway and cell protein expression\(^\text{35}\).

The co-administration of hepatoprotective agents may induce the hepatocytes to resist the toxic effects of CCl\(_4\). The results indicate that the ethanolic extract of the plant has significant hepatoprotective activity may be due to the presence of flavonoids.

References


