**INTRODUCTION**

Chronic ethanol (EtOH) consumption is a medical problem with important socio-economic repercussions worldwide. EtOH is metabolized in the liver during which it is first converted into acetaldehyde, a toxic metabolite, and then into acetate. The mechanisms by which EtOH causes cell injury are many. Some of the leading hypotheses suggested to play a role in EtOH-induced cell injury are redox state changes, damage to mitochondria, direct effects of EtOH on membrane reactive oxygen species (ROS) production and apoptosis in Chang liver cells exposed to EtOH in the presence and absence of sub-G1 phase cells. The cytoprotective effects of FPEt were comparable with those of a positive control silymarin, a known hepatoprotective agent. The findings suggest that the polyphenolic compounds of fenugreek seeds can be considered cytoprotective during EtOH-induced liver damage.

**MATERIALS AND METHODS**

**Chemicals and reagents**

Dulbecco’s modified Eagle’s medium (DMEM) from Biochrom (Germany), fetal bovine serum, glutathione reductase (GR), propidium iodide (PI) and RNase A from Sigma (St Louis, MO, USA) and 2’,7’-dichlorofluorescein diacetate (DCF-DA) from Molecular probes (Eugene, OR, USA) were purchased. Trypan blue and MTT were obtained from Himedia Pvt Ltd (India). LDH assay kit was obtained from Merck (India) while silymarin was obtained from Hunan Kinglong Bio-Resource Co., Ltd (China). All other chemicals and solvents were of analytical grade.

**Preparation of fenugreek seed extract**

Fenugreek seeds (100 g) were finely powdered, mixed with ethyl acetate containing glacial acetic acid (10 ml/l). Petroleum ether was obtained. The lower layer was then treated with ethyl acetate containing glacial acetic acid (10 ml/l). Extraction of polyphenols was carried out for 36 h at room temperature. After 5 days it was filtered and the solvent was evaporated. The residue was dissolved in water and the aqueous layer was washed with petroleum ether several times until a clear upper layer of petroleum ether was obtained. The lower layer was then treated with ethyl acetate containing glacial acetic acid (10 ml/l). Extraction of polyphenols was carried out for 36 h at room temperature.

**FENUGREEK (TRIGONELLA FOENUM GRAECUM) SEED EXTRACT PREVENTS ETHANOL-INDUCED TOXICITY AND APOPTOSIS IN CHANG LIVER CELLS**

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**Abstract** — The protective effect of a polyphenolic extract of fenugreek seeds (FPEt) against ethanol (EtOH)-induced toxicity was investigated in human Chang liver cells. Cells were incubated with either 30 mM EtOH alone or together in the presence of seed extract for 24 h. Assays were performed in treated cells to evaluate the ability of seeds to prevent the toxic effects of EtOH. EtOH treatment suppressed the growth of Chang liver cells and induced cytotoxicity, oxygen radical formation and mitochondrial dysfunction. Reduced glutathione (GSH) concentration was decreased significantly (P < 0.05) while oxidized glutathione (GSSG) concentration was significantly elevated in EtOH-treated cells as compared with normal cells. Incubation of FPEt along with EtOH significantly increased cell viability in a dose-dependent manner, caused a reduction in lactate dehydrogenase leakage and normalized GSH/GSSG ratio. The extract dose-dependently reduced thiorbarbituric acid reactive substances formation. Apoptosis was observed in EtOH-treated cells while FPEt reduced apoptosis by decreasing the accumulation of sub-G1 phase cells. The cytoprotective effects of FPEt were comparable with those of a positive control silymarin, a known hepatoprotective agent. The findings suggest that the polyphenolic compounds of fenugreek seeds can be considered cytoprotective during EtOH-induced liver damage.

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temperature and the combined ethyl acetate layer was concentrated (Xia et al., 1998). The residue was lyophilized and stored at -70°C. This yielded about 6–8 g per 100 g of seed powder. An aqueous extract (FPEt) was prepared and used for the studies. The polyphenolic content of the extract was assayed using the method of Singleton and Rossi (1965).

**Cell culture and treatment**

Monolayer cultures of human Chang liver cells (NCCS, Pune) were grown in DMEM growth medium containing 10% heat inactivated fetal calf serum, 100 μg/ml penicillin and 100 μg/ml streptomycin. Cells were incubated in 25 cm² tissue culture flasks at 37°C in a humidified atmosphere (5% CO₂). Cells were plated at different seeding densities in complete medium and allowed to settle for at least 4 h. The cultures were washed twice in warm phosphate-buffered saline (PBS) and incubated at 37°C in a humidified atmosphere: 5% CO₂. On attaining 75–80% confluency the cells were treated with either EtOH (30 mM) or EtOH + FPEt (20, 40, 60 μg/ml) or EtOH + silymarin (30 μg/ml) for 24 h. Preliminary studies were done to fix the time of exposure and dosage of EtOH, FPEt and silymarin using cell viability and MTT assay (data not given). At the end of the incubation, cells were used for the various assays described below.

**Cell viability**

Cell viability was measured by the ability of living cells to exclude trypan blue vital dye. Cells were seeded in 96-well microplates at a density of 10⁵ cells/well and were treated with EtOH in the presence and absence of FPEt/silymarin for 24 h. After this, the cells were trypsinized from the culture plates, combined with any floating cells present in the medium, and pelleted by centrifugation at 1000 g for 10 min at 4°C. Cells were washed twice with PBS, pH 7.2–7.4, and trypan blue was added at a final concentration of 0.2%. Living cells were counted in a haemacytometer and expressed as the percentage of the total count in untreated control. A dose-dependent study was carried out for FPEt to find out maximum inhibition.

**MTT metabolism**

Cell mitochondrial function was assayed by following the conversion of MTT to a purple formazan product. Briefly, cells were seeded in a 96-well microplate (2 x 10⁴ cells/well in 100 μl of complete medium) and then incubated as mentioned above. After 24 h of exposure to the additives, 50 μl of MTT (5 mg/5 ml) was added to each well, and the cells were incubated in the dark at 37°C for an additional 4 h. Thereafter, the medium was removed, the formazan crystals were dissolved in 200 μl of dimethyl sulfoxide, and the absorbance was measured at 570 nm.

**LDH leakage**

The release of LDH from cells over the course of the experiment was used as a measure of membrane damage. Briefly, cells were seeded in 24-well plates at a density of 2 x 10⁵ cells/well in 500 μl of complete medium and incubated with additives. At the end of the treatment, one aliquot of medium (0.2 ml) was taken out for extracellular LDH activity analysis. The total LDH activity was determined after the cells were disrupted thoroughly by sonication. The percentage of LDH released into the medium against the total activity present in the hepatocytes was then calculated to reflect the cytotoxicity.

**Lipid peroxidation**

Lipid peroxidation was measured in terms of TBARS production. Cells were seeded in 24-well plates at a density of 2 x 10⁵ cells/well in 500 μl of complete medium and incubated as mentioned above. After 24 h, the cells were treated with 1 ml of 0.5 M KCl in 10 mM Tris–HCl, mixed and then treated with 0.5 ml of 30% trichloroacetic acid (TCA), and 0.5 ml of 52 mM TBA and heated in a water bath at 90°C for 30 min. After cooling, the mixtures were centrifuged at 3000 g for 10 min. The absorbance of the supernatant was measured at 532 nm using a UV–VIS spectrophotometer. The levels of TBARS were expressed as nmol/mg protein.

**PI staining**

Cells were seeded upon slides kept in 6-well plates at a density of 10⁶ cells/well in 2 ml of complete medium and treated with EtOH with or without FPEt and silymarin for 24 h. After exposure for 24 h, cells on slides were washed in PBS and fixed with 4% paraformaldehyde for 10 min and incubated for another 10 min with 50 μl of PI (5 mg/ml). After washing, the slides were dried and examined by fluorescence microscopy (Olympus BX51). Quantification of data was obtained by examination of a defined area of each slide.

**Measurement of intracellular ROS**

Intracellular ROS was estimated by using the fluorescent probe, DCFH-DA (Shen et al., 1996). DCFH-DA diffuses through the cell membrane readily and is enzymatically hydrolysed by intracellular esterases to nonfluorescent DCFH, which is then rapidly oxidized to highly fluorescent DCF in the presence of ROS, especially H₂O₂. The DCF fluorescence intensity thus parallels the amount of ROS formed intracellularly. Cells were collected using a cell scraper and washed twice with PBS. Each fluorescence cuvette contained 2 x 10⁵ cells in 3 ml of PBS. Incubation of cells was done with EtOH, EtOH + FPEt or EtOH + silymarin along with 5 μM DCFH-DA for 4 h at 37°C. The fluorescence intensity was detected by a luminescence spectrometer (Perkin-Elmer LS-5B) with excitation wavelength at 485 nm and emission wavelength at 530 nm.

**GSH/GSSG assay**

The cells were seeded at densities 1 x 10⁶ cells/well in 6-well plates in 3 ml complete medium. After incubation with the additives, the cells were rinsed with PBS twice. The cells were collected by scraping, and 250 μl of ice-cold 10% TCA and 0.01 N HCl were added to the cell pellets. The tubes were immediately agitated with a vortex mixer, kept on ice for 15 min and centrifuged at 12 000 g for 20 min at 4°C. The resulting supernatants were extracted six times with diethyl ether to remove TCA. The aqueous phase was divided into two parts and used for the total GSH and GSSG measurements (Tietze, 1969). To determine the total GSH concentration, 100 μl of the cellular extract, appropriately diluted with 0.01 N HCl, was mixed with the following reaction
mixture: 700 μl of 0.1 M PBS, 0.5 mM EDTA, pH 7.5, 50 μl of 10 mM dithionitro benzoic acid and 100 μl of GR (4 U/ml). The reaction was initiated by the addition of 50 μl of 4 mM NADPH in 5% NaHCO₃. The total GSH concentration was determined by measuring the change in optical density per minute at 412 nm. To determine the GSSG concentration, 150 μl of the cellular extract was incubated with an equal volume of 40 mM N-ethyl malemide (NEM) for 1 h at room temperature. Excess NEM was then removed by 10 extractions with diethyl ether and the aqueous phase was used to measure the GSSG concentration.

Flow cytometry
Flow cytometry was used to detect the presence of apoptotic cells and the cell-cycle distribution. After cultivation in the medium alone or in a medium containing 30 mM EtOH in the presence and absence of FPEt or silymarin, the cells were harvested and washed with PBS. Then, they were fixed with ice-cold 70% EtOH at 4°C, stained with PI (5 mg/ml) and treated with RNase A (100 mg/l). The cell-cycle distribution was measured with FACScan Flow Cytometry (Becton–Dickinson) and analysed by Cell Quest software.

Estimation of protein
Protein content of the cells was determined using the method of Lowry et al. (1951).

Statistical analysis
Each experiment was performed in triplicate and the results are presented as means ± SD. The data were analysed for statistical significance using Student’s t-test. A value of \( P < 0.05 \) was considered significant.

RESULTS
Figure 1 shows the viability of cells calculated as a percentage of the total count in control cells. In the presence of 30 mM EtOH, only 45% of cells were viable. Simultaneous incubation of the same dose of EtOH along with 20, 40, and 60 μg/ml FPEt, increased the cell viability to 48, 52, and 75%, respectively while silymarin treatment increased the cell viability to 79%.

EtOH caused cytotoxicity in Chang liver cells, as demonstrated by the release of the intracellular enzyme, LDH. Incubation with FPEt significantly reduced EtOH-induced LDH leakage in a dose-dependent manner (\( P < 0.05 \)) (Fig. 2). Significant inhibition of EtOH-induced membrane leakage occurred with silymarin.

Mitochondrial function was assessed by the ability to metabolize MTT. Figure 3 shows that cells exposed to EtOH for 24 h showed decreased MTT metabolism (40% of control, \( P < 0.05 \)), whereas cells exposed to FPEt or silymarin along with EtOH showed a significant increase in MTT metabolism.
It is seen from Figure 4 that treatment of cells with EtOH led to significantly higher release of TBARS as compared with untreated control cells. FPEt caused a dose-dependent reduction of TBARS production. Among the three concentrations tested the reduction was maximum at the concentration of 60 \( \mu \)g/ml. Addition of silymarin also reduced TBARS production in EtOH-treated Chang liver cells.

Table 1 gives the DCF fluorescence intensity, levels of GSH, GSSG, and GSH/GSSG ratio in Chang liver cells. Treatment with EtOH decreased cellular redox status in Chang liver cells by decreasing the level of GSH and by increasing the level of GSSG. The decline in GSH and GSH/GSSG ratio was restored in cells, which were concomitantly treated with FPEt or silymarin. There was a significant increase (57%) in fluorescent intensity of cells exposed to EtOH as compared with control. However, the increase in intensity was not observed in cells treated with FPEt or silymarin with EtOH.

Figure 5A–D show the cell morphology after PI staining. The nuclei of EtOH-treated cells (Fig. 5B) were highly fluorescent, condensed, and fragmented as compared with the intact round and healthy nuclei of unexposed control cells (Fig. 5A). The cells treated with EtOH and FPEt or silymarin showed regular morphology with round contours and reduced uptake of the dye (Fig. 5C and D).

Figure 6A–D shows the cell-cycle progression of Chang liver cells stained with PI after treatment with EtOH alone or with FPEt/silymarin. Apoptotic cells produced a broad sub-G1 peak on EtOH treatment (Fig. 6B), while on treatment with FPEt/silymarin the percentage of apoptotic cells were reduced (Fig. 6C and D). Insets in the figures show the scatter analysis of EtOH-induced apoptosis in Chang liver cells. The apoptotic cells displayed enhanced light scatter in a lateral direction.

Table 2 indicates the alterations in the percentage of cells in each stage of the cell cycle: sub-G1, S and G2/M phase. In cells exposed only to EtOH, the percentage of cells at S phase and G2/M phase were decreased (5.19 and 2.45%), corresponding to an increase in the percentage of cells at sub-G1 phase (93.15%) (Fig. 6B). In control, FPEt or silymarin-treated cells, an increase in the percentage of cells in the G2/M and S phase with a decrease in sub-G1 phase was observed.

**DISCUSSION**

Literature survey revealed that fenugreek seeds possess a plethora of benefits under various experimental conditions. The seeds possess significant anti-diabetic (Sharma et al., 1996), antiatherosclerotic (Sharma et al., 1996), anti-inflammatory (Thakur et al., 1994), antinoceptive (Puri, 1998), antiallergicogenic (Suja Pandian et al., 2002) and anti-neoplastic effects (Sur et al., 2001). However, there is limited information concerning its protection against EtOH toxicity. In a previous study, we reported that fenugreek seed administration reduced the increase in the levels of biomarkers of oxidative stress in the liver of rats dosed with EtOH (Thirunavukkarasu et al., 2003). Other than this, there are no reports concerning the effects of fenugreek seeds on the cytotoxicity induced by EtOH. This study provides evidence that fenugreek seeds offer a strong protective effect against EtOH-induced cytotoxicity in Chang liver cells.

We used Chang liver cells as a non-malignant cell model for studying EtOH toxicity. Cameron et al. (1998) reported that hepatoblastoma cells (HepG2) exposed to 60–80 mM EtOH for 24 h show cytotoxicity, which was dose-dependent. Walker et al. (1974) obtained cytotoxicity in normal human liver cells after exposure to 69–174 mM EtOH. In our study, the cytotoxic effect in Chang liver cells was obtained after 24 h exposure to 30 mM EtOH. The cytotoxic dose of EtOH to hepatocytes differs between the cell strains and can probably be accounted for by a variety of mechanisms, which are due to the differences in the metabolism of EtOH by alcohol dehydrogenase and induction of cytochrome P450 (Wu and Cederbaum, 1996).

EtOH markedly inhibited the viability of Chang liver cells and this was a consequence of the induction of apoptosis as ascertained by flow cytometry. EtOH treatment caused

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**Table 1. Effect of FPEt on EtOH-induced changes in ROS production and cellular redox state in Chang liver cells**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>EtOH</th>
<th>EtOH + FPEt</th>
<th>EtOH + silymarin</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROS (mean fluorescence intensity)</td>
<td>386 ± 12.16</td>
<td>669 ± 20.18*</td>
<td>455 ± 14. 20**</td>
<td>410 ± 9.11**</td>
</tr>
<tr>
<td>GSH (nmol/mg protein)</td>
<td>50.6 ± 2.07</td>
<td>27.5 ± 1.78*</td>
<td>31.50 ± 1.55**</td>
<td>41.49 ± 1.95**</td>
</tr>
<tr>
<td>GSSG (nmol/mg protein)</td>
<td>4.15 ± 0.19</td>
<td>14.09 ± 0.69*</td>
<td>7.12 ± 0. 34**</td>
<td>5.92 ± 0.27**</td>
</tr>
<tr>
<td>GSH/GSSG ratio</td>
<td>12.29 ± 0.78</td>
<td>2.15 ± 0.17*</td>
<td>4.57 ± 0.22**</td>
<td>7.13 ± 0.37**</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD.

*Significantly different from control (\( P < 0.05 \)).

**Significantly different from EtOH-treated cells (\( P < 0.05 \)).
accumulation of cells in the sub-G1 peak, which is the hallmark of apoptosis (Wu and Cederbaum, 1999). Cells treated with EtOH displayed enhanced density and more apoptotic nuclei than the control cells.

Acute EtOH intoxication induces oxidative stress and apoptosis in primary cultured hepatocytes (Higuchi et al., 2001). Both oxidative stress and cytokines have been suggested to play a crucial role in hepatocyte apoptosis in alcoholic liver disease (Adachi et al., 2004). Cellular GSH is a major component of the intracellular reducing factor and a critical determinant of apoptosis. Excessive ROS formation can impair cells and thus become important regulators of cell-cycle arrest and apoptosis (Higuchi et al., 2001). The cells exposed to EtOH showed marked increases in the production of ROS and TBARS, and profound alterations in redox status suggesting oxidative stress.

It has also been demonstrated that EtOH metabolism in the hepatocyte causes depolarization of the mitochondrial membrane, which results in a mitochondrial permeability transition followed by cytochrome c release, caspase activation, and apoptosis (Nanj, 1998). Mitochondrial dysfunction and membrane damage are evident in EtOH-treated cells in this study.

Treatment with FPEt resulted in an increase of cell population as compared with EtOH. The data further show that FPEt caused a dose-dependent reduction in TBARS formation and LDH leakage. Interestingly, FPEt reduced ROS formation and abolished the deleterious effects on mitochondrial function. FPEt also abolished the formation of apoptotic nuclei. These data suggest that the active components, the polyphenols in the extract, might act as cytoprotective agents.

Until now five different flavonoids namely vitexin, tricin, naringenin, quercetin, and tricin-7-O-β-D-glucopyranoside are reported to be present in fenugreek seeds (Shang et al., 1998). Polyphenolic flavonoids have been shown to protect various cell types from oxidative stress-mediated cell injury. For example the phenolic antioxidant probucol protects human umbilical vein endothelial cells and rat neuronal cells (PC12) exposed to highly toxic lipid peroxide, linoleic acid hydroperoxide (Sasaki et al., 2002). Quercetin, one of the constituents of the extract, has been reported to prevent the cytotoxicity of oxidized low-density lipoproteins in human lymphoid cell lines (Negre-Salvayre and Salvayre, 1992), suppress the cytotoxicity of hydrogen peroxide towards Chinese hamster cell (V79) (Nakayama et al., 1993), glucose oxidase-mediated apoptosis in mouse thymocytes (Lee et al., 2003), and metal-induced lipid hydroperoxide-dependent lipid peroxidation in α-linoleic acid-loaded rat hepatocytes (Sugihara et al., 1999). The scavenging activities of the phenolic substances are attributed to the active hydrogen donating ability of the hydroxyl substitutions (Bors et al., 1996).

Further, the polyphenolic structures of flavonoids partition into the hydrophobic core of the membrane similar to cholesterol and cause a modulation in lipid fluidity (Arti et al., 2000). These substances, therefore, could react with the deeper membrane domains and intracellular structures, and protect the cells from oxidant injury.

The protective action of polyphenols may also be related to their influence on the regulation of gene expression. It has been shown that quercetin inhibits the H_{2}O_{2}-induced
NF-κB transcriptional activation, and inhibits DNA strand breaks produced by H$_2$O$_2$ (Musonda and Chipman, 1998). These findings clearly show that the fenugreek seeds in a dose-dependent manner could protect cell structure and function from the toxic effects of EtOH. FPEt has a similar action as that of the standard hepatoprotective agent, silymarin (Feher et al., 1989). These findings offer evidence for the utility of fenugreek seeds in the treatment of alcoholic liver disease and indicate a need for further studies to get an insight into the molecular mechanisms of its protection.

**Table 2.** Cell distribution in Chang liver cells analysed by FACS after EtOH treatment in the presence and absence of FPEt or silymarin

<table>
<thead>
<tr>
<th>Groups</th>
<th>Sub-G$_1$</th>
<th>S</th>
<th>G$_2$/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.55 ± 0.27</td>
<td>64.5 ± 2.28</td>
<td>21.50 ± 1.05</td>
</tr>
<tr>
<td>EtOH</td>
<td>93.15 ± 0.19</td>
<td>5.19 ± 0.69</td>
<td>2.45 ± 0.34</td>
</tr>
<tr>
<td>EtOH + FPEt</td>
<td>31.29 ± 1.78</td>
<td>57.15 ± 2.17</td>
<td>7.57 ± 0.22</td>
</tr>
<tr>
<td>EtOH + silymarin</td>
<td>24.5 ± 1.54</td>
<td>62.15 ± 1.98</td>
<td>7.80 ± 0.35</td>
</tr>
</tbody>
</table>

Values are means ± SD of three independent experiments.

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