

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

SUBRAMANIAN KAVIARASAN¹, NALINI RAMAMURTY², PALANI GUNASEKARAN²,
ELANGO VARALAKSHMI² and CARANI VENKATRAMAN ANURADHA^{1*}

¹Department of Biochemistry, Annamalai University, Annamalai Nagar 608 002, Tamil Nadu, India and ²Department of Virology, King Institute of Preventive Medicine, Guindy, Chennai 600 014, Tamil Nadu, India

(Received 4 January 2006; first review notified 9 February 2006; in revised form 13 February 2006; accepted 15 February 2006; advance access publication 30 March 2006)

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 thiobarbituric 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.

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 and excessive generation of free radicals during the course of its metabolism among others (Cederbaum, 2001).

Fenugreek (*Trigonella foenum graecum*) is an annual herb that belongs to the family Leguminosae. It has a long history as both a culinary and medicinal herb. The seeds of fenugreek are commonly used as a spice in food preparations due to the strong flavour and aroma. The seeds are reported to have restorative and nutritive properties (Khosla *et al.*, 1995). Fenugreek seeds are used in remedies for diabetes and hypercholesterolaemia in Indian, Arabic and Chinese medicine. Its utility has been proved experimentally in diabetic humans (Sharma and Raghuram, 1990).

Damage to the liver can be successfully prevented or controlled by supplementation with antioxidant substances of plant origin. Fenugreek seeds have antioxidant activity and have been shown to produce beneficial effects such as neutralization of free radicals and enhancement of antioxidant apparatus (Anuradha and Ravikumar, 1998, 2001). Furthermore, the polyphenolic fraction of the seeds was found to inhibit peroxide-induced oxidative damage and prevent haemolysis of erythrocytes *in vitro* (Kaviarasan *et al.*, 2003). In a previous study, we found that the administration of fenugreek seeds protects rat liver from EtOH-induced oxidative stress (Thirunavukkarasu *et al.*, 2003). To further explore the

protective role of the seeds in detail, we evaluated the effect of the polyphenolic extract of the seeds on EtOH-induced cytotoxicity in Chang liver cells and compared it with a standard hepatoprotective drug silymarin. We evaluated this by measuring cell viability, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) metabolism, lactate dehydrogenase (LDH) leakage, the ratio of reduced glutathione and oxidized glutathione (GSH/GSSG), thiobarbituric acid reactive substances (TBARS) formation, intracellular reactive oxygen species (ROS) production and apoptosis in Chang liver cells exposed to EtOH in the presence and absence of FPet and or silymarin.

MATERIALS AND METHODS

Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM) from Biobrom (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 80% methanol and kept at room temperature for 5 days. 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

*Author to whom correspondence should be addressed at: Tel.: +914144-238343; Fax: +914144-239141; E-mail: cvaradha@hotmail.com

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 $\mu\text{g/ml}$ penicillin and 100 $\mu\text{g/ml}$ streptomycin. Cells were incubated in 25 cm^2 tissue culture flasks at 37°C in a humidified atmosphere (5% CO_2). 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% CO_2 . On attaining 75–80% confluency the cells were treated with either EtOH (30 mM) or EtOH + FPEt (20, 40, 60 $\mu\text{g/ml}$) or EtOH + silymarin (30 $\mu\text{g/ml}$) 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 10^4 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 media, 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 haemocytometer 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 microtitre plate (2×10^4 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 2×10^5 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 2×10^5 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 10^6 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 H_2O_2 . 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 2×10^5 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 1×10^6 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 μ l of 0.1 M PBS, 0.5 mM EDTA, pH 7.5, 50 μ l of 10 mM dithionitro benzoic acid and 100 μ l of GR (4 U/ml). The reaction was initiated by the addition of 50 μ l 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 μ l of the cellular extract was incubated with an equal volume of 40 mM *N*-ethyl maleimide (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 \pm 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

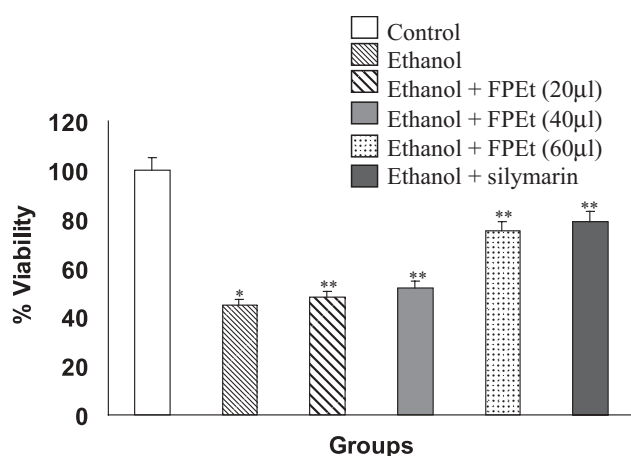


Fig. 1. Effect of EtOH treatment on the Chang liver cell viability. The columns represent the percent viability of Chang liver cells. FPEt increased cell viability in a dose-dependent manner in EtOH-treated Chang liver cells. Data are means \pm SD ($n = 3$). *Significantly different from control ($P < 0.05$). **Significantly different from EtOH-treated cells ($P < 0.05$).

EtOH, only 45% of cells were viable. Simultaneous incubation of the same dose of EtOH along with 20, 40, and 60 μ g/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.

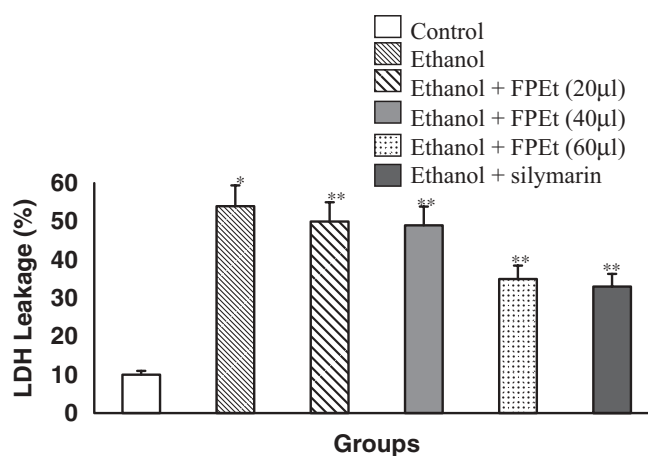


Fig. 2. Dose-dependent effect of FPEt extract on EtOH-induced LDH leakage in human Chang liver cells. Data are the means \pm SD ($n = 3$). *Significantly different from control ($P < 0.05$). **Significantly different from EtOH-treated cells ($P < 0.05$).

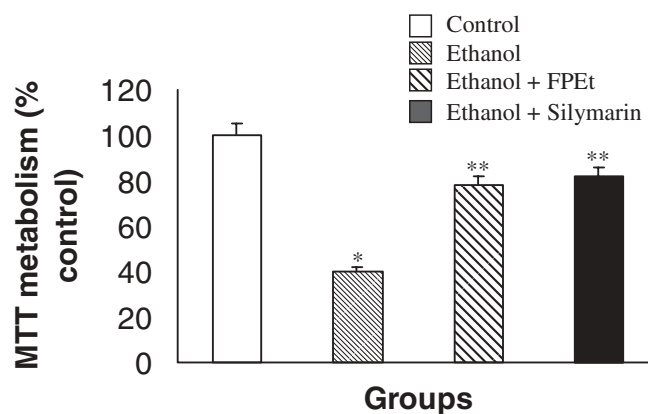


Fig. 3. Mitochondrial function as assessed by MTT metabolism in Chang liver cells. Cells were incubated for 24 h with 30 mM EtOH alone or in combination with either FPEt or silymarin. Data are the means \pm SD ($n = 3$). *Significantly different from control ($P < 0.05$). **Significantly different from EtOH-treated cells ($P < 0.05$).

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 $\mu\text{g}/\text{ml}$. 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 antidiabetic (Sharma *et al.*, 1996), antiatherosclerotic (Sharma *et al.*, 1996), anti-inflammatory (Thakur *et al.*, 1994), antinociceptive (Puri, 1998), antiulcerogenic (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

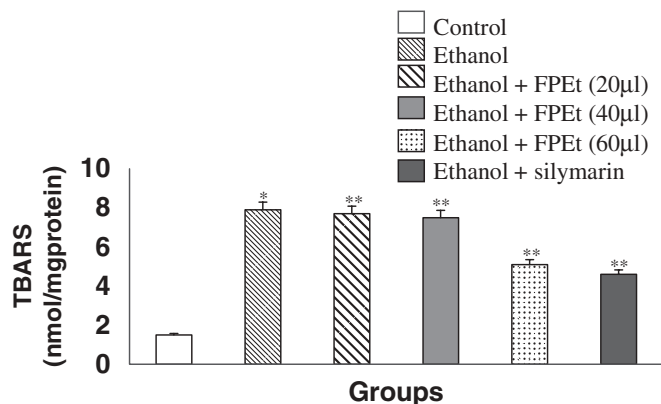


Fig. 4. Dose-dependent inhibition of FPEt on EtOH-induced TBARS production in Chang liver cells. Chang liver cells were treated with EtOH in the presence and absence of varying concentrations of FPEt or silymarin. Data are means \pm SD ($n = 3$). *Significantly different from control ($P < 0.05$). ** Significantly different from EtOH-treated cells ($P < 0.05$).

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

Parameters	Control	EtOH	EtOH + FPEt	EtOH + silymarin
ROS (mean fluorescence intensity)	386 \pm 12.16	669 \pm 20.18*	455 \pm 14.20**	410 \pm 9.11**
GSH (nmol/mg protein)	50.6 \pm 2.07	27.5 \pm 1.78*	31.50 \pm 1.55**	41.49 \pm 1.95**
GSSG (nmol/mg protein)	4.15 \pm 0.19	14.09 \pm 0.69*	7.12 \pm 0.34**	5.92 \pm 0.27**
GSH/GSSG ratio	12.29 \pm 0.78	2.15 \pm 0.17*	4.57 \pm 0.22**	7.13 \pm 0.37**

Values are expressed as means \pm SD.

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

**Significantly different from EtOH-treated cells ($P < 0.05$).

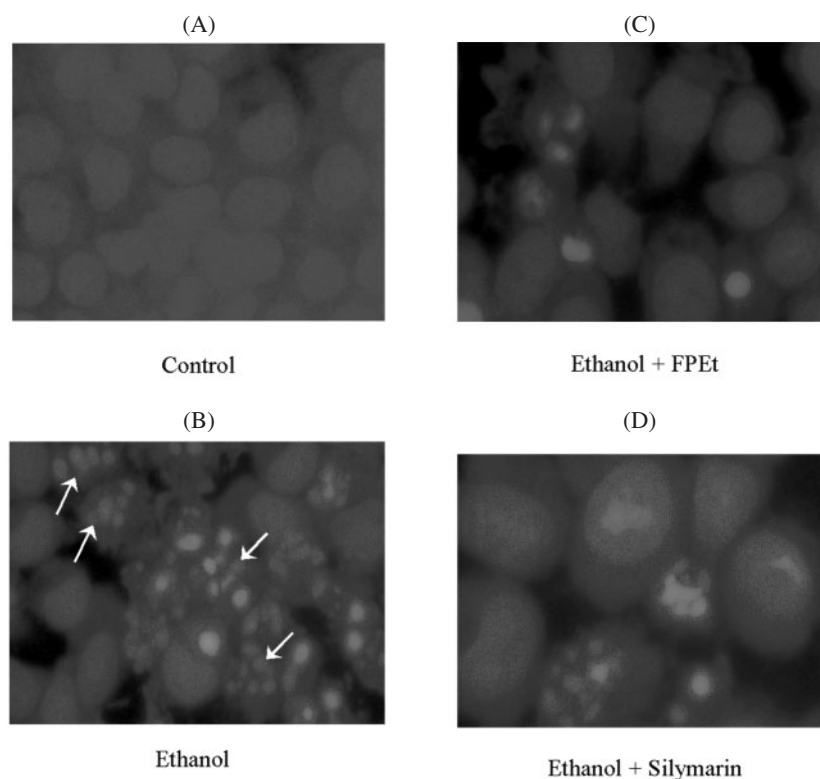


Fig. 5. (A–D) PI staining of nuclei in EtOH-treated Chang liver cells in the absence and presence of FPEt or silymarin. Apoptotic cells with fragmented nuclei (arrows) are seen in EtOH-treated cells (magnification 40 \times). Cells treated with FPEt/silymarin (C and D) show similar morphology as that with control.

accumulation of cells in the sub- G_1 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 (Nanji, 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, tricetin, naringenin, quercetin, and tricetin-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_2O_2 -induced

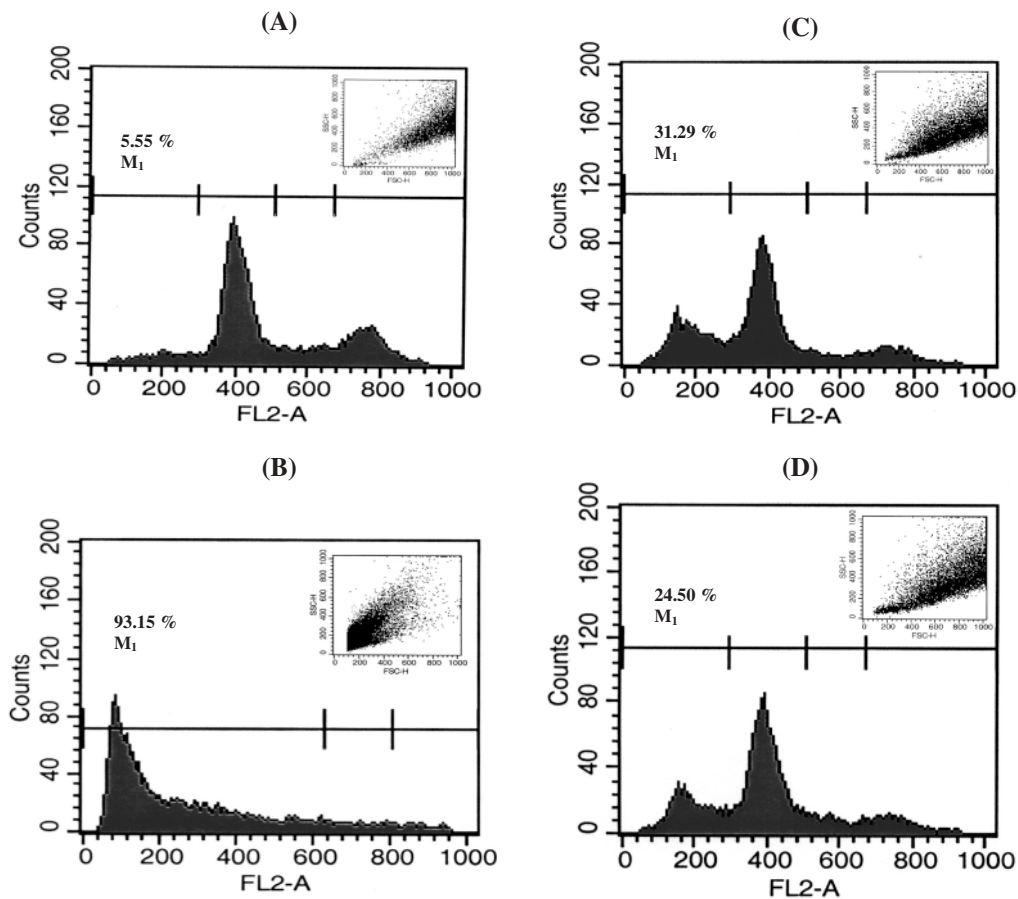


Fig. 6. (A–D) Flow cytometry analysis of PI-stained Chang liver cells treated with EtOH alone or in the presence of FPEt/silymarin. (A) Control (B) EtOH (C) EtOH + FPEt (D) EtOH + silymarin. Insets in figures show the scatter analysis.

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

Groups	Percentage of cells		
	Sub-G ₁	S	G ₂ /M
Control	5.55 ± 0.27	64.5 ± 2.28	21.50 ± 1.05
EtOH	93.15 ± 0.19	5.19 ± 0.69	2.45 ± 0.34
EtOH + FPEt	31.29 ± 1.78	57.15 ± 2.17	7.57 ± 0.22
EtOH + silymarin	24.5 ± 1.54	62.15 ± 1.98	7.80 ± 0.35

Values are means ± SD of three independent experiments.

NF-κB transcriptional activation, and inhibits DNA strand breaks produced by H₂O₂ (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.

Acknowledgements—Financial support to S.K., one of the authors, in the form of Senior Research Fellowship (SRF) by the Indian Council of Medical Research (ICMR), New Delhi, is gratefully acknowledged. The authors also thank the Director, King Institute of Preventive Medicine, Chennai for his permission to carry out the work at their institute.

REFERENCES

- Adachi, M., Higuchi, H., Miura, S. *et al.* (2004) Bax interacts with the voltage-dependent anion channel and mediates ethanol-induced apoptosis in rat hepatocytes. *American Journal of Physiology* **287**, 695–705.
- Anuradha, C. V. and Ravikumar, P. (1998) Anti lipid peroxidative activity of seeds of fenugreek (*Trigonella foenum graecum*). *Medical Science Research* **26**, 317–321.
- Anuradha, C. V. and Ravikumar, P. (2001) Restoration of tissue antioxidants by fenugreek (*Trigonella foenum graecum*) seeds in alloxan-diabetic rats. *Indian Journal of Physiology and Pharmacology* **45**, 408–420.
- Arti, A., Byren, T. M., Nair, M.G. *et al.* (2000) Modulation of liposomal membrane fluidity by flavonoids and isoflavonoids. *Archives of Biochemistry and Biophysics* **373**, 102–109.
- Bors, W., Heller, W., Michel, C. *et al.* (1996) Flavonoids and Polyphenols: chemistry and biology. In *Handbook of Antioxidants*. Cadenas, E. and Parker, L. eds, pp. 398 and 409. Marcel Dekker, New York.

- Cameron, R. G., Neuman, M. G., Shear, N. H. *et al.* (1998) Modulation of liver specific cellular response to ethanol *in vitro* in HepG₂ cells. *Toxicology in vitro* **12**, 111–122.
- Cederbaum, A. I. (2001) Alcohol, oxidative stress and cell injury—a serial review. *Free Radical Biology and Medicine* **31**, 1524–1526.
- Feher, J., Deak, G., Muzes, G. *et al.* (1989) Liver-protective action of silymarin therapy in chronic alcoholic liver diseases. *Orvosi Hetilap* **130**, 2723–2727.
- Higuchi, H., Adachi, M., Miura, S. *et al.* (2001) The mitochondrial permeability transition contributes to acute ethanol-induced apoptosis in rat hepatocytes. *Hepatology* **34**, 320–328.
- Kavirasan, S., Vijayalakshmi, K. and Anuradha, C. V. (2004) Polyphenol-rich extract of fenugreek seeds protect erythrocytes from oxidative damage. *Plant Foods for Human Nutrition* **59**, 143–147.
- Khosla, P., Gupta, D. D. and Nagpal, R. K. (1995) Effect of *Trigonella foenum graecum* (Fenugreek) on serum lipids in normal and diabetic rats. *International Journal of Pharmacology* **27**, 89–93.
- Lee, J. C., Kim, J., Park, J. K. *et al.* (2003) The antioxidant, rather than pro-oxidant, activities of quercetin on normal cells: quercetin protects mouse thymocytes from glucose oxidase-mediated apoptosis. *Experimental Cell Research* **291**, 386–397.
- Lowry, O. H., Rosenbrough, N. J., Parr, A. L. *et al.* (1951) Protein measurement with folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.
- Musonda, C. A. and Chipman, J. K. (1998) Quercetin inhibits hydrogen peroxide (H₂O₂)-induced NF-KappaB DNA binding activity and DNA damage in HypG₂ cells. *Carcinogenesis* **19**, 1583–1589.
- Nakayama, T., Yamada, M., Osawa, T. *et al.* (1993) Suppression of active oxygen-induced cytotoxicity by flavonoids. *Biochemical Pharmacology* **45**, 265–267.
- Nanji, A.A. (1998) Apoptosis and alcoholic liver disease. *Seminars in Liver Disease* **18**, 187–190.
- Negre-Salvayre, A. and Salvayre, R. (1992) Quercetin prevents the cytotoxicity of oxidised LDL on lymphoid cell lines. *Free Radical Biology and Medicine* **12**, 101–106.
- Puri, D. (1998) Therapeutic potential of fenugreek. *Indian Journal of Physiology and Pharmacology* **42**, 423–424.
- Sasaki, N., Toda, T., Kaneko, T. *et al.* (2002) Flavonoids suppress the cytotoxicity of linoleic acid hydroperoxide toward PC12 cells. *Biological Pharmaceutical Bulletin* **25**, 1093–1096.
- Shang, M., Cais Han, J., Li, J. *et al.* (1998). Studies on flavonoids from fenugreek (*Trigonella foenum graecum* L). *Zhongguo Zhong Yao Za Zhi* **23**, 614–639.
- Sharma, R. D. and Raghuram, T. C. (1990) Hypoglycaemic effect of fenugreek seeds in non-insulin dependent diabetic subjects. *Nutrition Research* **10**, 731–739.
- Sharma, R. D., Sarkar, A., Hazra, D. K. *et al.* (1996) Hypolipidaemic effect of fenugreek seeds: a chronic study in non-insulin dependent diabetic patients. *Phytotherapy Research* **10**, 332–334.
- Shen, H. M., Shi, C. Y. and Ong, C. N. (1996) Detection of elevated reactive oxygen species level in cultured rat hepatocytes treated with aflatoxin B1. *Free Radical Biology and Medicine* **21**, 139–146.
- Singleton, S. L. and Rossi, J. A. (1965) Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture* **16**, 144–158.
- Sugihara, N., Arakawa, T., Ohnishi, M. *et al.* (1999) Anti- and pro-oxidant effects of flavonoids on metal-induced lipid hydroperoxides dependent lipid peroxidation in cultured hepatocytes loaded with alpha-linoleic acid. *Free Radical Biology and Medicine* **27**, 1313–1323.
- Suja Pandian, R., Anuradha, C. V. and Viswanathan, P. (2002) Gastroprotective effect of fenugreek seeds (*Trigonella foenum graecum*) on experimental gastric ulcer in rats. *Journal of Ethnopharmacology* **81**, 393–397.
- Sur, P., Das, M., Gomes, A. *et al.* (2001) *Trigonella foenum graecum* (Fenugreek) seed extract as an antineoplastic agent. *Phytotherapy Research* **15**, 257–259.
- Thakur, A. M., Sarvaiya, J. G., Bhavsar, S. K. *et al.* (1994) Studies on anti-inflammatory activities of trigonella in rats. In Update Ayurveda 75, Bombay, India.
- Thirunavukkarasu, V., Anuradha, C. V. and Viswanathan, P. (2003) Protective effect of Fenugreek (*Trigonella foenum graecum*) seeds in experimental ethanol toxicity. *Phytotherapy Research* **17**, 737–743.
- Tietze, F. (1969) Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Analytical Biochemistry* **27**, 502–522.
- Walker, F., Elmslie, N., Fraser, R. A. *et al.* (1974) Cytotoxic effect of alcohol on liver cells and fibroblast *in vitro*. *Scotland Medical Journal* **19**, 125–127.
- Wu, D. and Cederbaum, A. I. (1996) Ethanol cytotoxicity to a transfected HepG₂ cell line expressing human cytochrome P4502E1. *Journal of Biological Chemistry* **39**, 23914–23919.
- Wu, D. and Cederbaum, A. I. (1999) Ethanol-induced apoptosis to stable HepG₂ cell lines expressing human cytochrome P-4502E1. *Alcoholism Clinical Experimental Research* **23**, 67–76.
- Xia, J., Allenbrand, B. and Sun, G.Y. (1998) Dietary supplementation of grape polyphenols and chronic ethanol administration on LDL oxidation and platelet function in rats. *Life Science* **63**, 383–390.