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Fenugreek (*Trigonella foenum graecum*) seed polyphenols protect liver from alcohol toxicity: A role on hepatic detoxification system and apoptosis

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Received July 7, 2006, accepted August 15, 2006

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Pharmazie 62: 299–304 (2007) *doi: 10.1691/ph.2007.4.6648*

The present study investigates the hepatoprotective effect of fenugreek seed polyphenolic extract (FPEt) against ethanol-induced hepatic injury and apoptosis in rats. Chronic ethanol administration (6 g/kg/day × 60 days) caused liver damage that was manifested by the elevation of markers of liver dysfunction – aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), bilirubin and γ-glutamyl transferase (GGT) in plasma and reduction in liver glycogen. The effects on alcohol metabolizing enzymes such as alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) were studied and found to be altered in the alcohol-treated group. Ethanol administration resulted in adaptive induction of the activities of cytochrome p450 (cyt-p-450) and cytochrome-b_5 (cyt-b_5) and reduction in cytochrome-c-reductase (cyt-c-red) and glutathione-S-tranferase (GST), a phase II enzyme. Further, ethanol reduced the viability of isolated hepatocytes (*ex vivo*) as assessed by the trypan blue exclusion test and increased hepatocyte apoptosis as assessed by propidium iodide staining (PI). Treatment with FPEt restored the levels of markers of liver injury and mitigated the alterations in alcohol metabolizing and detoxification enzymes and the electron transport component cytochrome-c reductase. Increased hepatocyte viability and reduced apoptotic nuclei were observed in FPEt-treated rats. These findings demonstrate that FPEt acts as a protective agent against ethanol-induced abnormalities in the liver. The effects of FPEt are comparable with those of a known hepatoprotective agent, silymarin.

1. Introduction

Chronic alcohol consumption can induce dramatic changes in the physiological and biochemical processes in various organs and cells (You and Crabb 2004; Cook et al. 2004; Clemens and Jerrells 2004; Poschl and Seitz 2004; Oba et al. 2005). The effects of ethanol are related to its metabolism which takes place mainly in the liver. Liver offers three metabolic pathways catalysed by the cytosolic enzymes-alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH) and the microsomal ethanol oxidizing system (MEOS). ADH catalyzes the transformation of ethanol into acetaldehyde, which in turn is converted to acetate through ALDH. Both enzyme reactions generate an excess of reducing equivalents in the liver, primarily in the form of NADH resulting in redox imbalance (French 2000). Chronic alcohol intoxication is accompanied by a decrease in activity of the ethanol metabolizing enzymes ADH and ALDH and in consequence by increase in acetaldehyde accumulation (Puntarulo and Cederbaum 1989). The toxicity of the oxidation product acetaldehyde is due, in part, to its capacity to bind to sulfhydryl groups of peptides (primarily glutathione) and proteins, such as microtubules (Baraona et al. 1977), collagen (Ma et al. 1997) and microsomal protein (Nomura and Lieber 1981), including cytochrome-P450 (CYP2E1) (Behrens et al. 1988). Such reactions result in hepatomegaly and a score of toxic manifestations, including generation of free radicals, decreased GSH and impairment of other antioxidant mechanisms (Leiber 1992, 1998). The MEOS system requires the participation of the P-450 cytochrome which couples ethanol and nicotinamide adenine dinucleotide phosphate (NADPH) oxidation to the reduction of molecular oxygen to hydrogen peroxide. Induction of a cytochrome P-450 enzyme, CYP2E1 by ethanol increases superoxide generation (Lieber 2005).

Damage to the liver can be successfully prevented or controlled by supplementation with antioxidant substances of plant origin. Scientists and medical professionals have shown increased interest in this field as they recognize the true health benefits of these remedies. One kind of such potentially health promoting herb is the fenugreek (*Trigonella foenum graecum*), the seeds of which are commonly used as a spice in food preparations due to the strong flavor and aroma. Fenugreek is an annual herb that belongs to the family *Leguminosae*. The seeds are reported to have restorative and nutritive properties (Khosla et al. 1995). 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). Further-

more, the polyphenolic fraction of the seeds was found to inhibit peroxide-induced oxidative damage and prevent hemolysis of erythrocytes *in vitro* (Kaviarasan et al. 2004). In a previous study we found that administration of fenugreek seeds protects rat liver from ethanol-induced oxidative stress (Thirunavukkarasu et al. 2003). A study from our laboratory reported that fenugreek seed extract prevents ethanol-induced cytotoxicity and apoptosis in Chang liver cells (Kaviarasan et al. 2006).

The present study was designed to investigate the influence of FPEt *in vivo* on the markers of liver injury, alcohol metabolizing enzymes, detoxification enzymes, electron transport component (cytochrome-c-reductase) and glutathione-*S*-transferase of rats sub-chronically intoxicated with ethanol. In addition the effect of FPEt on hepatocyte viability and apoptosis was investigated.

2. Investigations, results and discussion

In the present investigation alcohol administration resulted in an increase in the activities of enzymes aminotranferases (AST and ALT), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (GGT), lactate dehydrogenase (LDH) and bilirubin (Table 1) as compared to control. Both FPEt and silymarin showed significant protection against liver injury as evidenced by a reduction in the rise in the enzyme markers. Ethanol administration resulted in decreased levels of liver glycogen content and treatment with FPEt/silymarin exerted significant protection and restored the levels to near normal range.

Alcohol is primarily metabolized in hepatocytes to acetaldehyde, a step that can be catalyzed by ADH and also by microsomal CYP2E1. Table 2 represents the percentage viability of hepatocytes isolated from control and experimental animals. Alcohol-treated rats showed reduced cell viability of isolated hepatocytes (about 43% of control) as tested by the trypan blue exclusion test. However the hepatocytes were viable after treatment with FPEt (81% of control) or silymarin (85% of control).

The Figure represents ethanol-induced apoptosis in isolated rat hepatocytes in control and experimental animals. Hepatocytes isolated from ethanol treated rats showed enhanced density and more apoptotic nuclei (B) when compared to control (A) and it was found to be reduced in FPEt and silymarin treated rats (C&D). Hepatocytes from normal rats treated with FPEt and silymarin (E&F) appeared similar to that of control.

Apoptosis is a form of cell death characterized by organized nuclear and ultimately cellular fragmentation. Increasing evidence suggests that apoptosis of hepatocytes plays an important role in the initiation of alcoholic liver injury (Ishii et al. 2003). Increased apoptosis of hepatocytes results in increased fibrosis in experimental models (Takehara et al. 2004). Furthermore, apoptosis of hepatocytes results in generation of apoptotic bodies, which can release lipid signals for their uptake by Kupffer cells and hepatic stellate cells.

Table 3 shows the reduction in activities of ADH and ALDH in ethanol treated animals. Treatment with FPEt and silymarin showed restoration of these two enzymes. This might be due to the increase in liver blood flow and probably through maintenance of the NAD^+/NADH ratio in the liver. The large amounts of reducing equivalents generated in the ADH reaction overwhelm the hepatocyte's ability to maintain redox homeostasis and a number of metabolic disorders ensue (Lieber 1992). The liver contains a large amount of ADH (about 3% of soluble protein). Chronic alcohol feeding decreased the activity of alcohol metabolizing enzymes (ADH) and aldehyde dehydrogenase (Hasumura 1975). Acetaldehyde, the metabolic product of ethanol, might damage the mitochondria thereby reducing the ADH enzyme which inturn impairs acetaldehyde metabolism. Changes in the redox potential could cause alterations in the metabolism of lipid, glycogen and protein (Matsuzaki and Leiber 1977).

Cytochrome c reductase activity was significantly decreased while that of cytochrome b_5 and cytochrome p-450 was increased after ethanol administration (Table 3). The en-

Table 1: Preventive effect of FPEt on biochemical serum and liver parameters against alcohol induced liver injury

Parameters	Group I	Group II	Group III	Group IV	Group V	Group VI
Plasma (IU/L)						
AST	84.65 ± 3.09[c]	152.77 ± 11.86[a, c]	107.33 ± 5.53[a, b]	104.08 ± 8.65[a, b]	82.98 ± 3.59[c]	85.27 ± 5.04[c]
ALT	92.89 ± 8.40[c]	143.10 ± 8.68[a, c]	112.88 ± 7.29[a, b]	110.69 ± 8.51[a, b]	89.97 ± 8.49[c]	93.23 ± 7.13[c]
ALP	85.66 ± 5.74[c]	197.01 ± 13.04[a, c]	127.08 ± 11.33[a, b]	121.86 ± 11.85[a, b]	87.51 ± 8.12[c]	85.81 ± 6.82[c]
GGT	11.23 ± 1.10[c]	48.05 ± 2.66[a, c]	24.16 ± 2.04[a, b]	22.35 ± 1.94[a, b]	12.11 ± 1.20[c]	11.58 ± 1.13[c]
LDH	326.28 ± 30.46[c]	649.21 ± 43.90[a, c]	439.80 ± 40.36[a, b]	416.84 ± 39.85[a, b]	309.91 ± 29.23[c]	323.27 ± 32.16[c]
Bilirubin (mg/dl)	0.93 ± 0.09[c]	2.10 ± 0.13[a, c]	1.21 ± 0.10[a, b]	1.15 ± 0.06[a, b]	0.98 ± 0.09[c]	0.99 ± 0.08[c]
Liver (mg/g tissue)						
Glycogen	43.29 ± 4.09[c]	23.66 ± 1.58[a, c]	31.37 ± 2.49[a, b]	34.87 ± 2.07[a, b]	43.54 ± 4.06[c]	42.85 ± 3.79[c]

Values are means ± SD of six animals. Group I-Control; Group II-Ethanol; Group III-Ethanol + FPEt; Group IV-Ethanol + Silymarin; Group V-Control + FPEt ; Group VI-Control + Silymarin
[a] significant as compared to Group I ($p < 0.05$, DMRT)
[b] significant as compared to Group II ($p < 0.05$, DMRT)
[c] significant as compared to Group III and IV ($p < 0.05$, DMRT)

Table 2: Protective effect of FPEt on viability of isolated rat hepatocytes against ethanol-induced toxicity

Parameters	Group I	Group II	Group III	Group IV	Group V	Group VI
Percentage Viability (Trypan blue exclusion test)	89.12 ± 3.03[c]	50.84 ± 2.45[a, c]	71.14 ± 6.22[a, b]	75.95 ± 5.91[a, b]	90.57 ± 4.71[c]	88.0 ± 4.86[c]

Values are means ± SD of six animals. Group I-Control; Group II-Ethanol; Group III-Ethanol + FPEt; Group IV-Ethanol + Silymarin; Group V-Control + FPEt ; Group VI-Control + Silymarin
[a] significant as compared to Group I ($p < 0.05$, DMRT)
[b] significant as compared to Group II ($p < 0.05$, DMRT)
[c] significant as compared to Group III and IV ($p < 0.05$, DMRT)

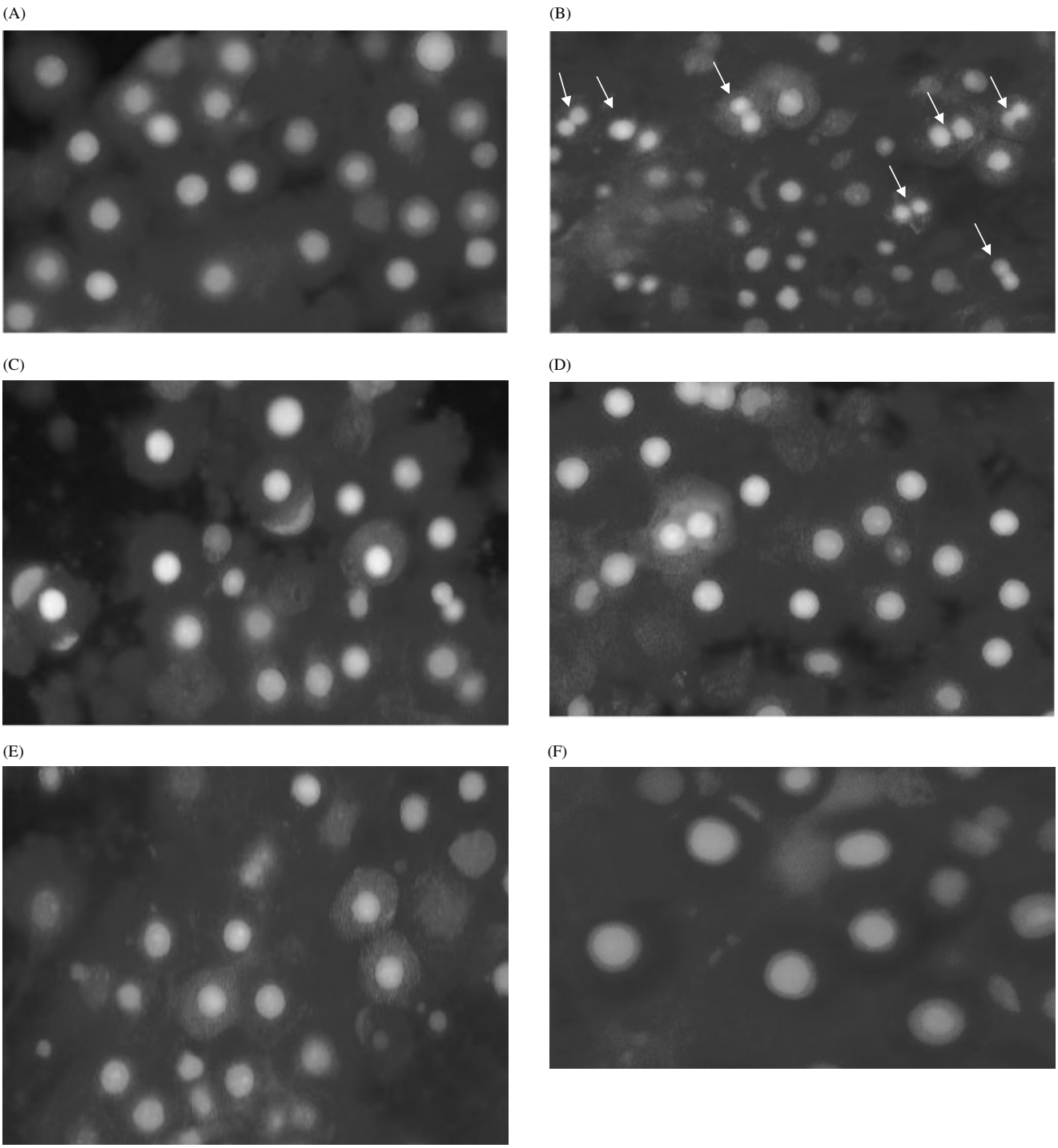

Fig.: (A–F) Propidium iodide staining of isolated rat hepatocytes. Apoptotic cells with fragmented nuclei (arrows) are seen in EtOH treated rat hepatocytes (magnification × 40) (Fig. B). Rats treated with ethanol and FPEt or silymarin (Fig. C and D) show similar hepatocyte morphology as that of control (Fig. A). Normal rats treated with FPEt or silymarin (Fig. E and F) showed similar morphology as that of control

hancement of cytochrome b_5 content in ethanol-fed rats may be due to the increase in electron transport to cytochrome p-450 the content of which is increased in relation to ethanol oxidation (Sinclair et al. 1981). CYP2E1 induction results in oxidative stress and cell injury in the liver. Induction of CYP2E1 may predispose to the membrane permeability transition (MPT) through an increase in the production of ROS, 1-hydroxy ethyl radical and products such as acetaldehyde. The MPT is the regulatable opening of a large, nonspecific pore across the outer and inner mitochondrial membrane. MPT development is followed by cytochrome c release into the cytosol, caspase activation and apoptosis (Higuchi et al. 2001). Damage to the mitochondrial membrane has been proposed to be a key mechanism by which CYP2E1-dependent lipid peroxidation causes loss in cell viability (Wu and Cederbaum 2002).

Under the present experimental conditions, a significant reduction in the activity of a phase II enzyme GST in the liver of ethanol-treated rats was observed. The action of phase II enzymes on the substrates generated by the action of phase I enzymes leads to their solubilization and excretion. GST is a critical detoxification enzyme that primarily functions in conjugating toxic metabolites with endogenous ligands (reduced glutathione) favouring their elimination.

Table 3: Effect of FPEt on alcohol metabolizing enzymes and detoxification enzymes in liver against chronic ethanol administered rats

Parameters	Group I	Group II	Group III	Group IV	Group V	Group VI
ADH[1]	0.75 ± 0.07^{c}	$0.47 \pm 0.03^{a,c}$	$0.62 \pm 0.04^{a,b}$	$0.63 \pm 0.03^{a,b}$	0.74 ± 0.07^{c}	0.75 ± 0.07^{c}
ALDH[1]	1.05 ± 0.05^{c}	$0.66 \pm 0.07^{a,c}$	$0.87 \pm 0.08^{a,b}$	$0.91 \pm 0.09^{a,b}$	1.04 ± 0.05^{c}	1.02 ± 0.05^{c}
Cytochrome P-450[2]	0.91 ± 0.07^{c}	$1.30 \pm 0.10^{a,c}$	$1.11 \pm 0.11^{a,b}$	$1.07 \pm 0.08^{a,b}$	0.99 ± 0.03^{c}	0.94 ± 0.05^{c}
Cytochrome b_5[2]	0.49 ± 0.03^{c}	$0.62 \pm 0.02^{a,c}$	$0.57 \pm 0.03^{a,b}$	$0.55 \pm 0.01^{a,b}$	0.48 ± 0.02^{c}	0.50 ± 0.03^{c}
Cytochrome c reductase[3]	1.11 ± 0.10^{c}	$0.81 \pm 0.07^{a,c}$	$0.96 \pm 0.06^{a,b}$	$0.99 \pm 0.05^{a,b}$	1.10 ± 0.03^{c}	1.13 ± 0.05^{c}
Glutahione-S-transferase[4]	5.47 ± 0.33^{c}	$2.81 \pm 0.26^{a,c}$	$4.32 \pm 0.36^{a,b}$	$4.5 \pm 0.42^{a,b}$	5.51 ± 0.26^{c}	5.55 ± 0.32^{c}

Values are means ± SD of six animals. Group I-Control; Group II-Ethanol; Group III-Ethanol + FPEt;
Group IV-Ethanol + Silymarin; Group V-Control + FPEt ; Group VI-Control + Silymarin
[a] significant as compared to Group I ($p < 0.05$, DMRT)
[b] significant as compared to Group II ($p < 0.05$, DMRT)
[c] significant as compared to Group III and IV ($p < 0.05$, DMRT)
[1] μmol NAD/min/mg protein; [2] nmol/mg protein; [3] μmol/min/mg protein; [4] μmoles of CDNB-GSH/min/mg protein

A substantial body of evidence has revealed that treatment with fenugreek seed prevents hyperlipidemia, atherosclerosis (Sharma et al. 1996), diabetes (Sharma et al. 1996), cancer (Sur et al. 2001) and ulcer (Suja pandian et al. 2002) in experimental animals. Although the mechanism of action in these studies has been ascribed to its role as antioxidant, no previous studies have evaluated its influence on the detoxification mechanisms.

Identification of active components in FPEt is ongoing. 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). Later seven compounds, *N*,*N*′-dicarbazyl, glycerol monopalmitate, stearic acid, beta-sitosteryl glucopyranoside, ethyl-alpha-D-glucopyranoside, D-3-*O*-methyl-chiroinositol and sucrose were identified (Shang et al. 1998). Dixit et al. (2005) reported the presence of gallic acid, *o*-coumaric acid, *p*-coumaric acid, rutin and caffeic acid in aqueous extract of fenugreek seeds.

Quercetin, one of the constituents of the extract, has been reported to prevent 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 cells (V79) (Nakayama et al. 1993) and glucose oxidase-mediated apoptosis in mouse thymocytes (Lee et al. 2003). The protective effect 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 NF-κB transcriptional activation, and inhibits DNA strand breaks produced by H_2O_2 (Musonda and Chipman 1998).

The present paper reveals that in rats intoxicated with ethanol, FPEt restored the liver function parameters, modulates the metabolism of alcohol and detoxification system. Further FPEt offers protection to the liver from ethanol-induced apoptosis. These effects could be related to the presence of a wide variety of active ingredients. Thus, fenugreek could be of use in the treatment of alcoholic liver disease, and warrants further detailed evaluation.

3. Experimental

3.1. Animals and chemicals

Healthy male albino Wistar rats (150–170 g) purchased from Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical Collage, Annamalai University were housed in polypropylene rat cages in a room with controlled temperature (24 ± 2 °C) and light (lights on 0600 to 1800). They were fed with standard pellet diet (Agro Corporation Private Limited, Bangalore, India) and water *ad libitum*. The experimental and animal-handling procedures were approved by the Institutional Animal Ethics Committee (IAEC). Silymarin was purchased from Hunan Kinglong Bio-Resource Co., Ltd (China). Trypan blue was obtained from Himedia Pvt Ltd (India). Alcohol dehydrogenase, cytochrome c, propidium iodide and type IV collagenase were obtained from Sigma (St. Louis, MO, USA). All other chemicals and solvents were of analytical grade.

3.2. Preparation of fenugreek seed extract (FPEt)

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 temperature and the combined ethyl acetate layer was concentrated (Xia et al. 1998). The residue was lyophilised 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 by the method of Singleton and Rossi (1965).

3.3. Study design

The animals were randomly divided into six groups of six rats in each. Alcoholic rats (toxicity control) received ethanol (6 g/kg) as an aqueous solution for 60 days by intragastric intubation. Normal control rats received glucose solution equivalent to the calorific value of ethanol (5 ml of 40% glucose solution/100 g). After the induction of toxicity (i.e. initial 30 days), treatment groups received FPEt (200 mg/kg/day) and silymarin (SM) (100 mg/kg/day) for the next 30 days along with ethanol (Table 4). The total experimental duration was 60 days. Rats in all the groups were sacrificed by decapitation 24 h after the last treatment. Blood was collected

Table 4: Experimental design

Group	Treatment and duration	
	1–30 days	31–60 days
I	Isocaloric glucose (40% stock) twice a day (09.00 a.m. and 5.00 p.m)	
II	Ethanol (3 g/kg twice a day i.e 6 g/kg/day)	
III	Ethanol (6 g/kg/day)	Ethanol + FPEt (100 mg/kg twice a day) i.e. 200 mg/kg/day
IV	Ethanol (6 g/kg/day)	Ethanol + SM (50 mg/kg twice a day) i.e. 100 mg/kg/day
V	Glucose (40% stock)	Glucose (40% stock) + FPEt (100 mg/kg twice a day) i.e. 200 mg/kg/day
VI	Glucose (40% stock)	Glucose (40% stock) + SM (50 mg/kg twice a day) i.e. 100 mg/kg/day

with heparin as anticoagulant. Plasma was separated by centrifugation at 2000 rpm. The liver tissue was sliced into pieces and homogenized in cold 50 mM phosphate buffer (pH 7.4) to give 10% homogenate (w/v). The homogenate was centrifuged at 1000 rpm for 10 min at 0 °C in a cold centrifuge. The supernatant was separated and used for various estimations.

3.4. Biochemical estimations

The activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) was assayed by the method of Reitman and Frankel (1957), that of alkaline phosphatase (ALP) according to Kind and King (1954). Serum bilirubin was estimated by the method of Malloy and Evelyn (1937). GGT was assayed by the method of Oralowsky and Meister (1963). Lactate dehydrogenase (LDH) activity was measured by the method of King (1965). Estimation of liver glycogen was done by the method of Montgomery (1957). Alcohol metabolizing enzymes such as ADH and ALDH activities were assayed by the method of Agarwal and Goedde (1990). Glutathione-*S*-transferase (GST) was assayed by the method of Habig et al. (1974). Protein content was determined by the method of Lowry et al. (1951).

3.5. Analysis of hemoprotein and electron transport component content

Cytochrome P-450 was assayed by the method of Omura and Sato (1964) using carbon monoxide difference spectra of dithionite-reduced microsomes. The extinction difference of the hemoprotein was taken as 91 $mM^{-1} \cdot cm^{-1}$ (450 minus 490 nm). Cytochrome b_5 was determined according to Omura and Sato (1964) by measuring the reduced minus oxidized difference spectrum by the addition of NADH, taking the extinction coefficient of the cytochrome between 424 and 409 nm as 185 $mM^{-1} \cdot cm^{-1}$. Electron transport component cytochrome c reductase activity was determined by the method of Umeki et al. (1984).

3.6. Isolation of rat hepatocytes

Hepatocytes were isolated according to the method of Berry and Friend (1969), as modified by Seglen (1976). Rats were fasted for 24 h prior to the hepatocyte isolation. The rats were anesthetized with sodium pentobarbital (62.5 mg/kg subcutaneous) and hepatocytes were isolated by collagenase perfusion technique. Cell viability of isolated hepatocytes, from both control and experimental rats, was measured by the Trypan blue exclusion test.

3.7. Trypan blue exclusion test

One drop of hepatocyte stock suspension ($1-1.2 \times 10^6$ cells/ml) was mixed with three drops of Trypan blue solution (0.2%). The unstained viable cells were distinguished visually from the blue stained dead cells and protection was expressed in terms of percent viable cells (Moldeus et al. 1978).

3.8. Propidium Iodide staining

Cells isolated from rat liver were washed in PBS thrice and incubated for another 10 min with 50 µl of propidium iodide (5 mg/ml), mounted in slides and examined by fluorescence microscopy (Olympus BX51). Quantification of data was obtained by examination of a defined area of each slide.

3.9. Statistical analysis

Statistical evaluation was done using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). Values are expressed as means ± SD of six rats in each group. A probability of $p < 0.05$ was considered as significant.

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