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Research Article
Biochemical and Molecular Investigation of Antioxidant Enzymes in Liver Tissue of Rats Intoxicated with Carbon Tetrachloride and Treated with Aqueous Extract of Fenugreek (*Trigonella foenum-graecum* L.)

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Abstract

Background and Objective: The investigation of antioxidant effect of fenugreek at molecular level in rats intoxicated with carbon tetrachloride (CCl_4) are not completely elucidated. Therefore, the present study aimed to investigate the protective effect of aqueous extract of fenugreek (*Trigonella foenum-graecum* L.) against CCl_4 hepatotoxicity in rats. **Materials and Methods:** Twenty four rats were allocated into four groups. Rats in groups 1-4 were injected with paraffin oil (control), subjected to oral administration of aqueous extract of fenugreek, injected with CCl_4 diluted with paraffin oil 1:1 (1 mL kg^{-1} b.wt.) for 2 executive days and a combination of group 2 and 3, respectively. **Results:** Liver injury and oxidative stress were observed in untreated CCl_4-intoxicated rats as reflected on histopathological picture, increase in hepatic transaminases, increase in lipid peroxidation (thiobarbituric acid reactive substances, TBARS), decrease of reduced glutathione (GSH) concentration, decrease in the activities of antioxidant enzymes namely catalase (CAT), total superoxide dismutase (SOD), glutathione peroxidase (GPX) and glutathione-s-transferase (GST) as well as down-regulation of gene expression of these enzymes compared to control. Administration of aqueous extract of fenugreek attenuated the detrimental effects of CCl_4 via an up-regulation of gene expression and activities of antioxidant enzymes with increase in GSH concentration. **Conclusion:** Aqueous extract of fenugreek ameliorated CCl_4-induced hepatotoxicity in rats. Aqueous extract of fenugreek exerted its protective effect against CCl_4-induced toxicity by modulating the extent of lipid peroxidation and enhancing the antioxidant defence system at the activity and gene expression levels.

Key words: Fenugreek, carbon tetrachloride, antioxidants, gene expression, rats, liver

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Carbon tetrachloride (CCl_4) is a chemical agent used for induction of experimental hepatotoxicity with subsequent elevation of hepatic transaminases and necrosis to liver tissues of rats[1]. Trichloromethyl radical is produced during oxidative stress and responsible for the toxic effect[2] of CCl_4. The efficiency of chemical drug to relief injured liver still limited[3]. Phytochemicals that present in medicinal plants have been used for treatment of many chronic diseases[4]. The medicinal plants are safer and cheaper alternative to conventional chemical drugs[5]. Medicinal plants have been used to ameliorate hepatic toxicity due to their known antioxidant properties[6,7]. Fenugreek (*Trigonella foenum-graecum*) is one of the most traditional herbs[8]. The protective effect of fenugreek against ethanol[9], thioacetamide[10] and aluminum chloride[11] induced hepatotoxicity in rats has been documented. The ameliorative effect of fenugreek against carbendazim[12] and adriamycin[13] induced testicular damage has been also reported. The above mentioned antitoxic effects of fenugreek have been attributed to its antioxidant effect. Fenugreek administration decreased the lipid peroxidation level, increased the concentration of reduced glutathione and increased the elevated activities of antioxidant enzymes like SOD, CAT, GST, GPX and Glutathione Reductase (GR) to scavenging generated free radicals[10-15]. However, effect of fenugreek on differential mRNA levels, gene expression of these antioxidant enzymes has not been completely verified so far. Therefore, in the current study intraperitoneal injection of CCl_4 was used to generate free radicals in liver tissues with subsequent liver damage. The antioxidant capacity of aqueous extract of fenugreek (*Trigonella foenum-graecum* L.) has been examined at biochemical and molecular levels in liver of rats against CCl_4 induced hepatoxicty.

MATERIALS AND METHODS

Chemicals and kits: Paraffin oil and CCl_4 has been purchased from Spectrosol® BHD chemicals Ltd., pool, England. Ethylene Glycol Tetraacetic Acid (EGTA), ethylenediaminetetraacetic acid (EDTA), hydrogen peroxide (H_2O_2), sucrose, butanol, tris, metaphosphoric and mannitol were purchased from Sigma Chemical Co. (St., Louis, MO, USA). Radioimmunoprecipitation assay (RIPA) buffer (Cayman chemical company, USA) and kits for serum alanine transaminase (ALT) and aspartate transaminase (AST) were supplied from united diagnostic industry (UDI, Dammam, Saudi Arabia).

Preparation of aqueous extract of fenugreek (*Trigonella foenum-graecum* L.): Fenugreek aqueous extract made by soaking 150 g of fenugreek seeds in 900 mL of boiling de-ionized water for 1 h[16]. The extract left all night and then filtered and filtrate was dried under low pressure. A yellowish residue was obtained. A solution containing[17] 20 mg mL^{-1} of extract was prepared in distilled water for oral administration to the animals in the experimental groups for 5 weeks immediately after CCl_4-intoxication as a sole source of drinking water.

Experimental intoxication of liver by CCl_4: The toxicity of the liver was induced by i.p injection of CCl_4 (1 mL kg^{-1} b.wt.), 1:1 diluted with paraffin oil, for 2 successive days of the experiment[1,18].

Experimental design: Twenty four Albino rats (180-200 g) were acclimated for 10 days before the beginning of the experiment. All animals (6 rats per cage) fed standard laboratory diet and tap water *ad libitum*. The experimental animals were housed in air-conditioned rooms at 20-24°C and 60-65% of relative humidity and kept on a 12 h light/12 h dark cycle. All experimental procedures and management conditions used in this study were approved by the King Faisal University Animal Care and Use Committee (KFU-ACUC), Saudi Arabia. Rats were divided into 4 groups (6 rats for each) during 5 weeks experimental period. Group 1: Control rats fed basal diet and injected i.p., with paraffin oil. Group 2: Rats fed basal diet, injected i.p., with paraffin oil and treated with fenugreek aqueous extract (20 mg mL^{-1})[17] as their sole source of drinking water. Group 3: Rats fed basal diet and intoxicated with CCl_4 (1 mL kg^{-1} b.wt.), 1:1 diluted with paraffin oil on first 2 days of the experiment[1,18]. Group 4: Like group 3 but treated immediately with fenugreek aqueous extract as their sole source of drinking water.

Sampling and biochemical analysis: Blood samples were collected by cardiac puncture after diethyl ether anesthesia at the end of the experiment and the harvested serum stored frozen at -30°C until the time of biochemical analysis of ALT and AST using provided diagnostic assay kits according to the manufacturer's instruction. In addition, liver tissues were removed and divided into three portions. The first portion of the liver tissue was used for histopathological examination[19]. The extent of CCl_4-induced necrosis was estimated by assessing the changes in the liver sections stained with

Table 1: Details giving primer sequences for the genes amplified

Gene	Forward primer sequence	Reverse primer sequence
β-actin	5′-AGC CAT GTA CGT AGC CAT CC-3′	5′-CTC TCA GCT GTG GTG GTG AA-3′
Total SOD	5′-AGG ATT AAC TGA AGG CGA GCA T-3′	5′-TCT ACA GTT AGC AGG CCA GCA G-3′
CAT	5′-ACG AGA TGG CAC ACT TTG ACA G -3′	5′-TGG GTT TCT CTT CTG GCT ATG G-3′
GPX	5′-AAG GTG CTG CTC ATT GAG AAT G-3′	5′-CGT CTG GAC CTA CCA GGA ACT T-3′
GST	5′-GCT GGA GTG GAG TTT GAA GAA-3′	5′-GTC CTG ACC ACG TCA ACA TAG-3′

SOD: Superoxide dismutase, CAT: Catalase, GPX: Glutathione peroxidase, GST: Glutathione-s-transferase

haematoxylin and eosin (H and E) using standard techniques. The second portion of the liver tissue was immediately frozen at -30°C and used for analysis of activities of antioxidant enzyme[18]. The last portion of the liver tissue was immediately frozen in liquid nitrogen and stored at -80°C for analysis of gene expression of antioxidant enzymes[6,18,20]. The sequences of used primers[6,18,20] are illustrated in Table 1.

Statistical analysis: Data are presented as the mean±standard error of the mean using one way analysis of variance (ANOVA) by using a statistical analysis system program[21]. The relative gene expression of target genes compared to the β-actin reference gene was calculated using the bio-rad CFX manager 3.0 software of the C1000 touch thermal cycler-CFX96 real-time PCR (Bio-rad, Foster city, California, USA).

RESULTS

The biochemical estimation of liver injury as reflected on activities of AST and ALT has been described in Table 2. The current findings indicated a significant increase ($p<0.05$) in serum AST and ALT activities of untreated CCl_4 intoxicated rats (group 3) compared to controls (group 1 and 2). These values have been decreased significantly ($p<0.05$) when these CCl_4 intoxicated rats treated with aqueous extract of fenugreek (group 4) compared to untreated CCl_4-intoxicated rats (group 3). The serum of rats treated with aqueous extract of fenugreek only did not show any significant changes in hepatic transaminases compared to the control. Histopathological analysis revealed that, hepatocytes, portal triads and vasculature appeared within normal limit in control and fenugreek treated rats (group 1 and 2, Fig. 1a). The liver of CCl_4-intoxicated rats showed fatty change, necrosis and apoptosis (Fig. 1b) as well as hepatitis which characterized by mononuclear cells infiltration of lymphocytes and macrophages around central veins and in portal areas (Fig. 1c). The liver of CCl_4-intoxicated rats and treated with aqueous extract of fenugreek characterized by clear hepatic recovery and the hepatic tissue appeared more or less normal in most cases (Fig. 1d). The effect of fenugreek on hepatic lipid peroxidation, GSH and hepatic antioxidant enzyme activities (CAT, total SOD, GPX and GST) are summarized in Table 3. The TBARS level was increased significantly ($p<0.05$) in the liver of CCl_4-intoxicated rats compared to the control. Treatment of CCl_4-intoxicated rats with aqueous extract of fenugreek significantly reduced ($p<0.05$) TBARS level of liver tissue compared to untreated CCl_4-intoxicated rats. Administration of aqueous extract of fenugreek alone only did not significantly affect ($p>0.05$) the level of TBARS in the rat liver compared to the control. The GSH concentration was significantly decreased ($p<0.05$) in CCl_4-intoxicated rats compared to the control. Aqueous extract of fenugreek administration returned GSH values in CCl_4-intoxicated rats near to the values of untreated CCl_4-intoxicated rats. Administration of CCl_4 significantly ($p<0.05$) reduced the activities of all studied hepatic antioxidant enzymes compared to the control, however, the activities of these enzymes showed a significant ($p<0.05$) recovery in response to administration of aqueous extract of fenugreek compared to untreated CCl_4-intoxicated rats. The activities of hepatic antioxidant enzymes of rats treated with aqueous extract of fenugreek only were comparable to that of the control (Table 3). The real-time RT-PCR of total SOD, CAT, GPX and GST genes revealed dawn-regulated of all of the these enzymes ($p<0.05$) in CCl_4-intoxicated rats compared to control (Fig. 2). However, the expression of these enzymes was significantly up-regulated ($p<0.05$) in CCl_4-intoxicated rats treated with aqueous extract of fenugreek compared to untreated CCl_4-intoxicated rats. The expression of hepatic antioxidant enzymes of rats treated with aqueous extract of fenugreek only was comparable to that of the control (Fig. 2).

Table 2: Effect of CCl_4 and/or oral aqueous extract of fenugreek for 5 weeks on serum biomarker enzymes activities of AST (U L^{-1}) and ALT (U L^{-1})

	Experimental groups			
Parameters	Control	FG	CCl_4	CCl_4+FG
AST	117.6±4.0	128.9±3.0	137.3±4.0*	115.9±2.2**
ALT	30.0±0.2	27.4±1.1	50.2±2.0*	39.0±3.1**

Values are expressed as Mean±SEM, n = 6 for each group, significance was calculated at $p<0.05$, FG: Fenugreek, CCl_4: Carbon tetrachloride, AST: Alanine transaminase, AST: Aspartate transaminase, *Significant as compared to control animals, **Significant as compared to CCl_4 treated animals

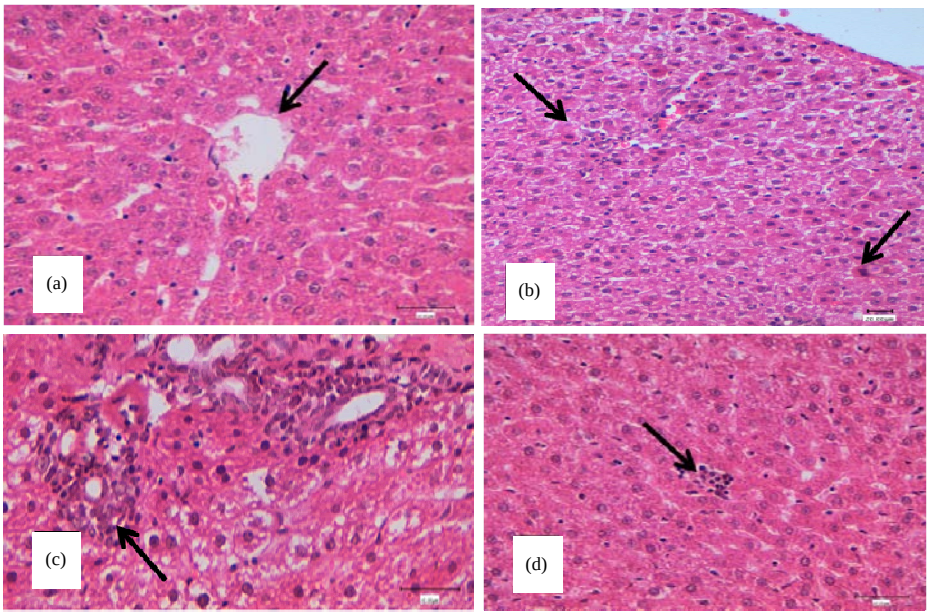

Fig. 1(a-d): Histopathological assessment of liver damage in (a) Liver of normal control or aqueous extract of fenugreek treated rats showing normal central veins and normal hepatocytes (arrow), H and E, (b) Liver of carbon tetrachloride-intoxicated rats showing massive number of apoptotic hepatocytes (arrows), H and E, (c) Liver of carbon tetrachloride-intoxicated rats showing mononuclear cells infiltration in portal area (arrow), H and E and (d) Liver of aqueous extract of fenugreek+CCl_4 treated rats showing few number of mononuclear cells around central veins, H and E

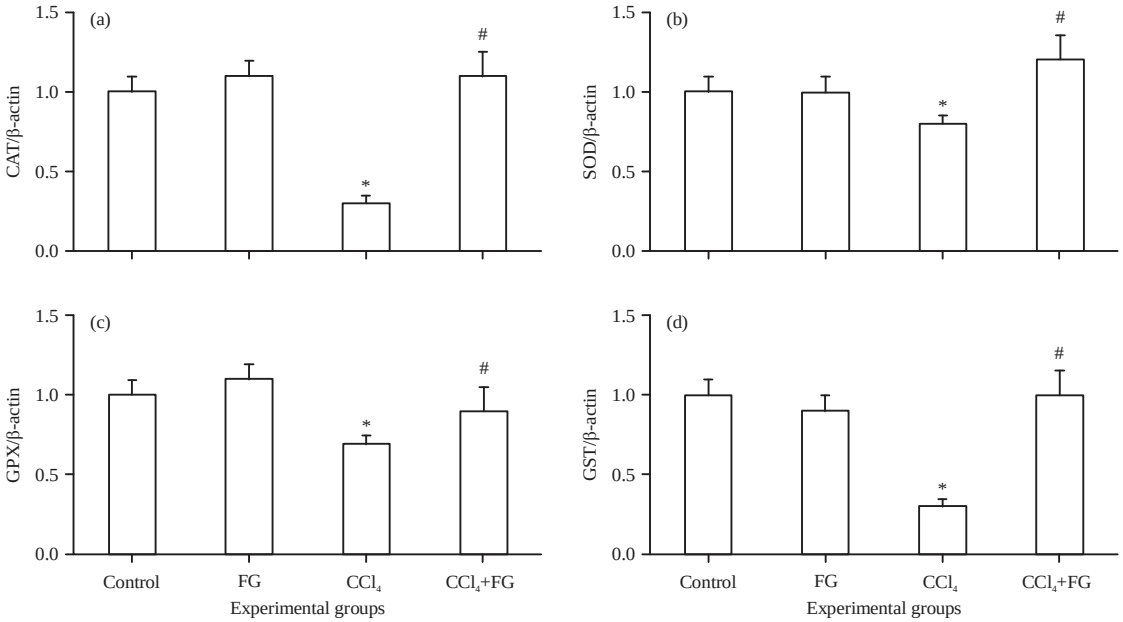

Fig. 2(a-d): Real time RT-PCR analysis of (a) CAT, (b) SOD, (c) GPX and (d) GST in liver tissues of control non-treated, aqueous extract of fenugreek treated (FG), carbon tetrachloride treated (CCl_4) and carbon tetrachloride+aqueous extract of fenugreek treated rats (CCl_4+FG). Values are expressed as Mean±SEM, *Values are significantly different ($p<0.5$) compared to control, #Values are significantly different ($p<0.5$) compared to CCl_4 intoxicated group

Table 3: Effect of CCl_4 and/or oral aqueous extract of fenugreek for 5 weeks on levels of TBARS (μM) and GSH (μM) and activities of CAT (nmol min^{-1} g^{-1} tissue), total SOD (U g^{-1} tissue), GPX (nmol min^{-1} g^{-1} tissue) and GST (nmol min^{-1} g^{-1} tissue) in rats liver tissues

	Experimental groups			
Parameters	Control	FG	CCl_4	CCl_4+FG
TBARS	29.1±1.3	28.3±1.1	37.6±1.2*	30.1±1.5**
CAT	29.1±1.3	28.6±1.1	12.1±1.2*	27.9±2.3**
Total SOD	8.0±0.02	7.0±0.03	4.0±0.05*	7.3±0.05**
GPX	303.1±1.4	297.5±2.6	266.3±1.5*	296.4±4.4**
GST	201.5±1.2	195.2±1.1	100.6±1.2*	190.7±1.5**
GSH	7.2±0.20	6.9±0.10	5.4±0.10*	6.8±0.40**

Values are expressed as Mean±SEM, n = 6 for each group, significance was calculated at $p<0.05$, TBARS: Thiobarbituric acid reactive substances, CAT: Catalase, SOD: Superoxide dismutase, GPX: Glutathione peroxidase, GST: Glutathione-s-transferase, GSH: Reduced glutathione, *Significant as compared to control animals, **Significant as compared to CCl_4 treated animals

DISCUSSION

The significant elevation in hepatic ALT and AST activities in rats intoxicated with CCl_4 compare to control were parallel to the previous studies[1,18,22,23]. The observed recovery of the examined ALT and AST activities and the ameliorative effect of fenugreek aqueous extract against liver damage induced by CCl_4 in rats has been supported by other reports[13,22,23].

The active metabolite (CCl_3) is assumed to be the main inducer of lipid peroxidation and subsequent hepatotoxic injures caused[24] by CCl_4. However, the protective effect of fenugreek aqueous extract against CCl_4-induced oxidative stress in rats may attributed to the documented antioxidant effect of fenugreek[13] due to its content of flavonoids which able to scavenge the produced free radicals[13,25,26]. The observed reduction of lipid peroxidation biomarker (TBARS) and significant elevation of GSH and antioxidant enzymes activities (CAT, GPX, SOD and GST) in liver of CCl_4-intoxicated rats treated with aqueous extract of fenugreek confirms the antioxidant ability of fenugreek aqueous extract. In the same direction, previous study[23] demonstrated that aqueous extract of fenugreek attenuated the level of lipid peroxidation and activated hepatic CAT and GPX antioxidant activities.

The current biochemical findings were supported by histopathological findings which indicated degenerative changes in the liver of rats intoxicated with CCl_4. Similarly degenerative changes in the liver of rats intoxicated with CCl_4 have been reported[1]. The significant relieve of hepatic tissues in rats treated with combination of CCl_4 and fenugreek aqueous extract (Fig. 1d) comes in accordance with earlier reports in rats intoxicated with ethanol[9], adriamycin[13], aluminium chloride[11] and thioacetamide[10]. In addition, improvement of injured kidney[27] and testis[12] by administration of fenugreek has been reported in rats.

The significant increase of TBARS in liver of untreated CCl_4-intoxicated rats considered a bio-marker for production of free radicals, higher level of lipid peroxidation and inhibition of antioxidants mechanisms[24]. The present findings indicated a significant decrease in both the GSH content and the activities of antioxidant enzymes (total SOD, GPX, CAT and GST) in CCl_4-intoxicated rats compared to the control, which induced an elevation of TBARS levels. These findings were consistent with same studies in the livers of rats intoxicated with CCl_4[18,23,28,29]. The protective effect of fenugreek aqueous extract against CCl_4-induced lipid peroxidation as observed in the present study was consistent with previous reports[13,23].

The depletion of hepatic GSH in CCl_4 intoxicated rats may be attributed to the conjugation of GSH with electrophilic metabolites of CCl_4, a reaction that is catalysed by GST. Similar depletion in hepatic GSH of CCl_4 intoxicated rats was reported[18]. The ability of aqueous extract of fenugreek to scavenge free radicals and restore the antioxidant status was the reason behind its ameliorative effect[13,25,26]. The active hydrogen donating ability of the hydroxyl substitutions of phenolic contents[30] and flavonoids[25,26] in fenugreek were responsible for its free radicals scavenging activity.

Generally, the imbalance between prooxidants and antioxidants in biological systems produced a case termed as oxidative stress. Therefore, lower level of lipid peroxidation may owed to lower activities of enzymatic antioxidants (CAT, SOD, GPX and GST) as well as non-enzymatic antioxidants (GSH) in the liver of CCl_4-intoxicated rats when compared to the control. Superoxide radical is converted to H_2O_2 by SOD. Furthermore, H_2O_2 is transferred to molecular oxygen and water by CAT and GPX. Therefore, SOD, CAT, GPX and GST constitute the main components of the antioxidant system and their inhibition induces a case of oxidative stress. Therefore, the significant decrease in the activities of these enzymes in liver of CCl_4-intoxicated rats is the reason stand behind the observed higher lipid peroxidation level as reflected on high level of TBARS along with low level of GSH in these animals compared to the control during CCl_4-induced hepatotoxicity. Significant lower activities of GPX, SOD and CAT have been reported in CCl_4-intoxicated rat liver[18], kidney[31] and lung[31]. It has been reported that, aqueous extract of fenugreek is a potent inducer of detoxifying enzymes and thereby attenuates the hepatotoxicity induced by ethanol[9], adriamycin[13], aluminium chloride[11], thioacetamide[10] and CCl_4[23].

Molecular investigation of gene expression of antioxidant enzymes revealed down-regulation of all examined antioxidant enzymes (SOD, CAT, GPX and GST) in rats intoxicated with CCl_4. Similar down-regulation of the same antioxidant enzymes were obtained in CCl_4-induced liver

fibrosis in mice[32]. The present study reported that, aqueous extract of fenugreek up-regulated the gene expression of antioxidant enzymes (CAT, SOD, GPX and GST) (Fig. 2). As the publications regarding the effect of fenugreek on gene expression levels of antioxidant enzymes are lack and the results of the present study may be the first report that discussed this issue. The conjugation of reactive metabolites with GSH as reported above is an important step in detoxification process. This conjugation is mediated by GST. The CCl_4 depleted the GSH through conjugation process. The down regulation of GST gene expression in CCl_4-intoxicated rats compare to control as presented in the present study (Fig. 2c) may reduce the ability of hepatic tissues to conjugate the reactive metabolites with GSH. However, aqueous extract of fenugreek may alleviated this effect via up-regulation of GST gene expression.

Taken together, oral administration of aqueous extract of fenugreek induced up-regulation of gene expression and higher activities of antioxidant enzymes and increase in GSH concentration in CCl_4-intoxicated rats compared to untreated CCl_4-intoxicated rats. These effects induced significant lower in lipid peroxidation level (TBARS) and amelioration to CCl_4 hepatotoxicity in rats. However, the mode of action of up-regulation of hepatic gene expression of antioxidant enzymes by aqueous extract of fenugreek has not been identified whether the cause of activation of gene expression was due to a direct effect of aqueous extract of fenugreek in the studied antioxidant enzymes or due to the overall effect of aqueous extract of fenugreek that leads to a cascade of reactions that resulting in the observed changes. Therefore, additional studies in this field are essentially required and large scale study has to be designed to evaluate the effect of different concentrations of aqueous extract of fenugreek on gene expression of antioxidant enzymes.

CONCLUSION

The present study concluded that, aqueous extract of fenugreek ameliorated CCl_4-induced hepatotoxicity in rats. Aqueous extract of fenugreek exerted its protective effect against CCl_4-induced toxicity by modulating the extent of lipid peroxidation and enhancing the antioxidant defence system at the activity and gene expression levels.

SIGNIFICANCE STATEMENT

The present study reported for the 1st time that, protective effect of aqueous extract of fenugreek against CCl_4-induced hepatotoxicity in rats exerted via modulating the extent of lipid peroxidation, increasing the activity of antioxidant enzymes and up-regulation of gene expression of antioxidant enzymes.

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