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Ethanolic extract of *Trigonella Foenum Graecum* attenuates cisplatin-induced nephro- and hepatotoxicities in rats

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Abstract

Nephro-and hepatotoxicities are important complications in cancer patients undergoing cisplatin (CP) therapy. We aimed to study the protective effect of fenugreek (FG) on CP induced renal and hepatic injuries in rats. Cisplatin intoxication resulted in structural and functional renal and hepatic impairments, which were revealed by massive histopathological changes and elevated kidney and liver function tests. However, it was associated with oxidative stress and lipid peroxidation as evident by increased reactive oxygen species (ROS) and malondialdehyde (MDA) with decreased levels of total antioxidant activity. Cisplatin administration triggered inflammatory responses and apoptosis in rat livers and kidneys as evident by increased expression of pro-inflammatory cytokine, tumor necrosis factor- α (TNF-α) and apoptotic marker p38 mitogen-activated protein kinase (p38 MAPK) as results of overproduction of ROS. FG significantly attenuated the cisplatin-induced biochemical and histopathological alterations, inflammation and apoptosis in rat livers and kidneys. Results suggested that fenugreek co-administration has a powerful antioxidant effect and may serves as a novel and promising preventive strategy against cisplatin-induced nephron- and hepatotoxicities.

Key words: Cisplatin; fenugreek, hepatotoxicity, nephrotoxicity.

Introduction

Cisplatin (cis-dichlorodiammineplatinum (II), CP) is a synthetic anticancer drug extensively used clinically for the treatment of various human solid tumors such as ovarian cancer, non-small-cell lung carcinoma and head and neck cancer, both as a single agent and in combination with other agents (1). CP is generally considered to exert its cytotoxic effect by binding to DNA, resulting in mutagenesis (2). Although higher doses of cisplatin are more efficacious for the suppression of cancer, the clinical use of CP is limited because of its unwanted side effects such as nephrotoxicity (1), neurotoxicity (3), ototoxicity (4) and hepatotoxicity (5).

The anticancer property of CP comes from its ability to bind to N-7 of purine bases of cellular DNA leading to formation of mono adducts which are later transformed into inter- and intra-strand cross links by reaction of second reactive site of the drug with the second nucleobase (6). This is inhibitory to fundamental cellular processes including replication, transcription, translation and DNA-repair in many cell types (7). Besides, CP generates oxidative and nitrosative stresses (8), because of depletion or inhibition of antioxidant enzymes and proteins which results into nephrotoxicity and hepatotoxicity as major side effects of the drug (9).

Since ancient times, herbal products have been used to cure human diseases. These products are receiving more attention due to their low toxicity and high efficacy. The use of plants and their extracts in medicinal purposes has been rapidly increasing worldwide. Fenugreek (*Trigonella foenum graecum*) is a herb belongs to family leguminosa. It has been used as a medicinal herb (10). Its seeds are used in many oriented countries as a spice in food preparations due to their strong flavor and aroma (11). It is used as an herbal medicine for its carminative, tonic and aphrodisiac effects (12). Fenugreek seeds exhibit hypoglycemic, hypolipidaemic, antifertilitic, antiandrogenic, antinociceptive, antidepressant and wound healing properties and are good sources of dietary fibers (13).

Fenugreek seed contains 45-60% carbohydrates, mainly mucilaginous fiber (galactomannans); 20-30% proteins high in lysine and tryptophan and 5-10% fixed oils (lipids). The seed also contains pyridine-type alkaloids, mainly trigonelline (0.2-0.36%), choline (0.5%), gentianine and carpaine; in addition to the flavonoids such as apigenin, luteolin, orientin, quercetin, vitexin, isovitexin and free amino acids such as 4- hydroxyisoleucine (0.09%); arginine, histidine and lysine. Moreover calcium and iron; saponins (0.61.7%); glycosides; cholesterol and sitosterol; vitamins A, B1, C and nicotinic acid; coumarin compounds and 0.015% volatile oils were all found in fenugreek seed (14, 15).

The present study was undertaken to demonstrate the effect of fenugreek seeds extract on CP induced renal and hepatic injuries in rats.

Materials and methods

Drugs and chemicals

Cisplatin and thiobarbituric acid (TBA) reagent were purchased from Sigma–Aldrich Co, St Louis, MO, USA. All kits were supplied by Biodiagnostic Co., Egypt. All other chemicals unless mentioned were of analytical grade.

Extraction

The fresh seeds of *T. foenum graecum* were air-dried and crushed into powder in a grinding machine. The powder (1.5kg) was extracted in Erlenmeyer flasks with 90% ethanol at room temperature. The maceration was

carried out five times**,** each in 48 hrs with occasional shaking and stirring. The whole extract was combined, filtered (Whatman filter paper No.1) and concentrated at 40°C in vacuum and finally the extract was freeze-dried to get 150 gm of crude extract (16).

Experimental animals

Male Wister rats weighing between 200–220 g were purchased from the Egyptian Company for vaccines and medicines. The animals were housed under standard conditions of light and dark cycle with free access to food and water. All animals were acclimatized for 7 days before the beginning of the experiment. The experimental protocols were approved by the Institutional Ethical Committee of Faculty of Science, Ain Shams University, Egypt.

Experimental design

The animals were randomly divided into four groups, containing ten rats in each. Based on a previous study, hepato and nephro− toxicities were induced by intraperitoneal (i.p) administration of cisplatin dissolved in normal saline at the dose of 7.5 mg/kg body weight (17). Experimental design was performed as followings:

(1) Placebo group (N). Rats were injected with a single i.p dose of 0.5 ml isotonic saline on 5th day.

(2) Fenugreek control group (FG). Rats were given a suspension of fenugreek (1g/kg/day) orally for 10 days (16).

(3) Cisplatin control group (CP). Rats were injected with a single i.p dose of cisplatin (7.5 mg/kg) dissolved in normal saline on 5th day.

(4) Fenugreek + Cisplatin group (FG+CP). Rats were given a suspension of fenugreek (1 g/kg/day) orally for 10 days and a single dose of cisplatin (7.5 mg/kg, i.p.) on 5th day, 1 hour after fenugreek dose.

Sampling

Animals' body weights were recorded on the first and last day of the experimentation. At the end of the experiment, blood was collected (for serum isolation) and rats were sacrificed by carotid bleeding under ether anesthesia. Liver and kidney were excised, weighed and divided into two parts. One part was fixed in 10% formalin for histopathological examination**,** while the rest were used for RNA isolation.

Determination of kidney and liver functions

As indicators of kidney function, serum creatinine and blood urea nitrogen (BUN) levels were measured. Serum alanine transaminase (ALT), aspartate transaminase (AST), total protein and total bilirubin levels were measured to evaluate the liver function using commercially available kits (Biodiagnostic Co., Egypt).

Assessment of oxidative stress markers

Lipid peroxidation was estimated as the amount of thiobarbituric acid reactive substances (TBARS) determined by the reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA) as described (18). Total antioxidant activity (TAA) was determined (19).

RNA isolation and real-time PCR

Total RNA was extracted from liver and kidney tissues using the Thermo Scientific GeneJETRNA Purification Kit (Thermo Scientific, Waltham, MA, USA). Total RNA (1μg) was treated with DNase I to eliminate genomic DNA contamination, followed by synthesis of the first strand using the Thermo Scientific RevertAid^{TM} First Strand cDNA synthesis kit (Fermentas life science Co.).

Reverse transcription was carried out as follows: 42°C for 30 min, 95°C for 5 min, and 4°C for 5 min (one cycle). Real-time PCR was performed to examine the relative expression levels of mRNA encoding TNF-α, P38 MAP kinase and β-actin using the Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix (2X), according to manufacturer's protocol, and the results were computerized using Stratagene (Mx3000P^{TM}) machine. Primer sequences were: 5'-TGACTTGCTTCCCTGTTCTTGA -3' (sense) and 5'-TTTGGAAATGTGTCCACAGAGG -3' (anti-sense) for p38 MAPK,5'-ACT GAACTTCGGGGTGATTG -3' (sense) and 5'-GCTTGGTGGTTTGCTACGAC -3' (anti-sense) for TNF-α, and 5'-GTCAGGTCATCACTATCGGCAAT-3' (sense) and 5'-AGAGGTCTTTACGGATGTCAACGT-3' (anti-sense) for β-actin. The expression levels of TNF-α and p38 MAPK were normalized to β-actin and presented as fold change relative to untreated control group **(20).** The cycles for PCR were as follows: 95°C for 7 min, 40 cycles of 95°C for 20 s, 54°C for 30 s, and 72°C for 30 s.

Histopathological examination

Pieces of liver and kidney from each group were fixed immediately in 10% neutral formalin for a period of at least 24 h, dehydrated in xylene and embedded in paraffin at 56^{o}C in hot air oven for 24 hours. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns by sledge microtome. The obtained tissue sections were collected on glass slides, deparaffinized and stained by hematoxylin and eosin (H&E) stains (21) for histopathological examination using the electric light microscope (400x magnification).

Statistical analysis

All data were expressed as means ± S.E.M. and statistically analyzed using SPSS 17.0 for Windows (SPSS Inc, Chicago, IL). Statistical significance of differences among different study groups was evaluated by one-way analysis of variance (ANOVA). Post hoc testing was performed for intergroup comparisons using the Least Significant Difference (LSD) test, and a P value <0.05 was considered significant.

Results

Effect of fenugreek on body weights

At the end of the experimental period, the body weight was depressed in all animals treated with CP. Although administration of FG with CP prevented the reduction in the body weights to some extent, the body weights of the FG+CP group was still decreased compared to the placebo group. However, there were no statistically significant differences between the groups ($P>0.05$) (Table1).

Table 1. Effect of FG on CP-induced changes on different biochemical parameters.

	N	CP	FG	FG+CP
Body weight (g)	266 ± 32.4	219 ± 21.9	255 ± 22.2	235 ± 9.6
Liver function tests				
ALT (U/L)	37.7 ± 1.1[b]	68.1 ± 8.01[a]	34.8 ± 2.51[b]	43.2 ± 2.91[a,b]
AST (U/L)	36.5 ± 0.80[b]	65.5 ± 3.4[a]	34.6 ± 3.4[b]	41.4 ± 3.16[a,b]
Total bilirubin mg%	0.8 ± 0.05[b]	1.1 ± 0.08[a]	0.75 ± 0.03[b]	0.7 ± 0.04[a,b]
Total protein mg%	8.13 ± 1.4[b]	6.35 ± 0.35[a]	8.12 ± 0.56[b]	8.62 ± 0.96[a,b]
Kidney function tests				
Creatinine (mg/dl)	0.58 ± 0.1[b]	0.82 ± 0.1[a]	0.53 ± 0.1[b]	0.75 ± 0.1[a,b]
BUN (mg/dl)	48 ± 3.84[b]	241 ± 18.3[a]	45 ± 3.1[b]	60 ± 4.6[a,b]
Oxidative stress markers				
MDA(nmol/ml)	171.13±4.75[b]	263.7±10.64[a]	149.13±13.42[a, b]	187.7 ± 6.27[a,b]
TAA (mmole/L)	2.5 ±0.12[b]	0.86 ±0.05[b]	2.3 ±0.34[a]	1.6 ±0.58[a,b]

N: Placebo, CP, cisplatin; FG: Fenugreek: AST, aspartate aminotransferase; ALT, alanine aminotransferase. MDA: malondialdehyde: TAA: total antioxidant activity. Values are mean ± SE for 10 animals in each group.

a: Sgnificant difference at $p< 0.05$ compared with placebo group.

b: Significant difference at $p< 0.05$ compared with CP treated animals.

The effect of fenugreek on cisplatin-induced kidney and liver dysfunction

Cisplatin caused a marked reduction in kidney functions, as characterized by a significant increase in serum BUN ($p=0.000$, $p<0.001$) and creatinine ($p=0.019$, $p<0.05$) levels. Cisplatin also caused a significant increase in serum ALT ($p=0.000$, $p<0.001$), AST ($p=0.000$, $p<0.001$), total bilirubin levels ($p=0.000$, $p<0.001$) and a significant decreased in serum total protein level ($p=0.044$, $p<0.05$) when compared to placebo levels (Table1). Thus, these data indicated that a single intravenous injection of 7.5 mg kg^{-1} cisplatin impaired both kidney and liver functions.

Administration of FG for 10 days markedly reversed cisplatin-induced increases in serum creatinine ($p=0.043$, $p<0.05$) and BUN ($p=0.000$, $p<0.001$) levels. FG was also found to be effective to reverse cisplatin-induced changes in serum ALT ($p=0.000$, $p<0.001$), AST ($p=0.000$, $p<0.001$), total bilirubin ($p=0.000$, $p<0.001$) and total protein ($p=0.016$, $p<0.05$) levels when compared to CP group.

The effect of fenugreek on oxidative stress markers

In order to evaluate the protective effects of FG against CP induced oxidative stress in rats, the levels of both MDA and TAA were determined. As shown in Table (1), the MDA level was significantly increased ($p=0.000$, $P < 0.001$) among rats of the CP group compared to those of the placebo group. Concurrently, a significant decrease ($p=0.000$, $P < 0.001$) in the level of TAA was observed among rats of CP group compared to the placebo group. Notably, co-treatment of rats with FG, along with CP significantly suppressed the CP -induced elevation of serum MDA ($p=0.000$, $p<0.001$) and restored control levels of TAA ($p=0.029$, $p<0.05$), as compared to the CP group. In contrast, no significant changes in the serum levels of TAA ($p=0.495$, $p>0.05$) and significant decrease in serum levels of MDA ($p=0.021$, $p<0.05$) were observed among rats receiving FG alone. These results demonstrated the antioxidant and protective effects of FG against CP -induced oxidative stress and their ability to augment cellular antioxidant defenses.

The effect of fenugreek on histological changes in kidney and liver samples

In cisplatin-injected animals, there was cellular damage in both kidney and liver samples accompanied by severe degeneration in glomeruli and tubuli (both proximal and distal tubuli). The liver morphology was characterized by focal necrotic area in hepatic parenchyma, degenerated hepatocytes and moderate enlarge-

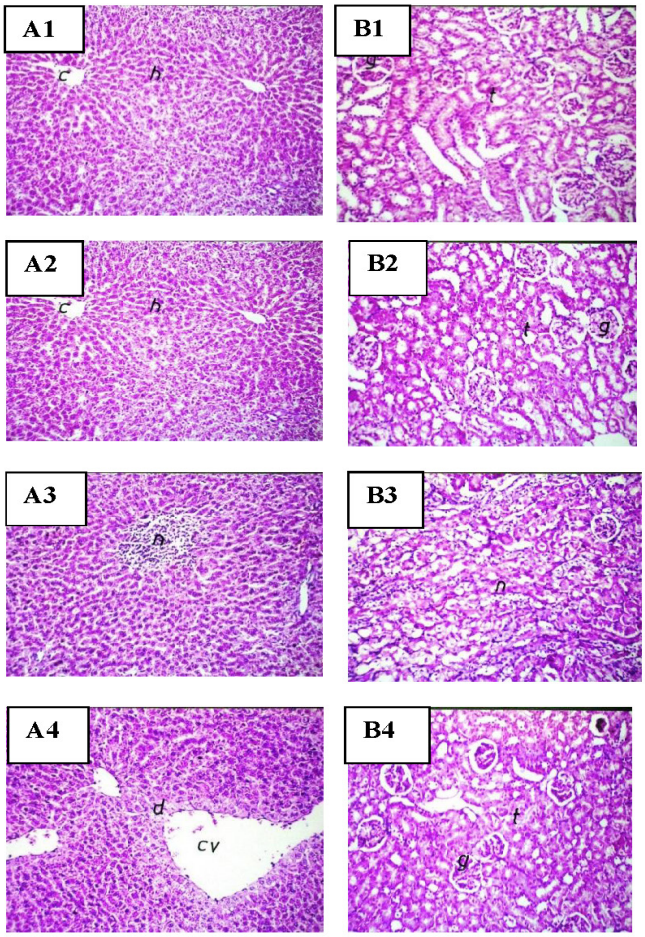

Figure 1. Effects of FG on CP -induced histopathological changes in rat (A) liver and (B) kidney. Liver and kidney sections were stained with hematoxylin and eosin (H&E) and examined with a light microscope by 400× magnification. (1): Placebo group, (2): FG group, (3): CP group; (4): FG+ CP groups. C- Central vein, h- hepatocyte, g- glomeruli, t- tubules, n- necrosis.

ment of sinusoids. In kidneys of rats treated with FG, the morphology revealed a nearly regular appearance of glomeruli and tubuli. Similarly, FG treatment partially preserved the liver parenchyma, where the appearance of the hepatocytes, sinusoids and Kupffer cells was near to normal morphology (Fig. 1).

Effect of FG on the CP-enhanced gene expression of apoptotic marker p38 MAPK and pro-inflammatory marker TNFα in liver and renal tissue homogenates

Cisplatin significantly increased the expression level of p38 MAPK in liver ($p=0.000$, $p< 0.001$) and kidney ($p= 0.000$, $p< 0.001$) tissues of CP group indicating the apoptosis of hepatic and renal cells (Fig. 2). The expression level of the p38 MAPK was significantly ($p= 0.000$, $p< 0.001$) lowered in the FG+CP group compared to the CP group. The differences in p38 MAPK levels were insignificant ($p=0.067$, $p > 0.05$; $p=0.98$, $p > 0.05$ in kidney and liver, respectively) between placebo and FG group.

The expression level of pro-inflammatory cytokine**,** TNFα was elevated significantly in CP treated group in comparison with the placebo ($p= 0.000$, $p< 0.001$) (Fig. 2). Pretreatment with FG has significantly ($p= 0.000$, $p< 0.001$ in both in kidney and liver) decreased the TNFα expression level. There is no significance difference in the expression level of TNFα between placebo and FG group ($p= 0.069$, $p > 0.05$; $p= 0.95$, $p > 0.05$ in kidney and liver, respectively).

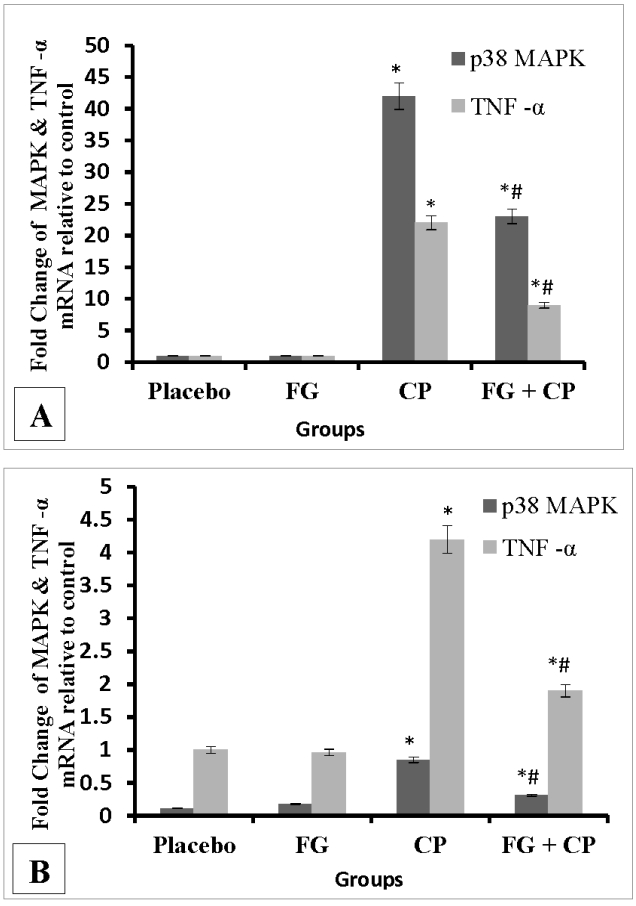

Figure 2. Effects of FG on CP -induced increase of p38MAPK & TNF-α mRNA expression levels in (A) liver and (B) kidney in rats. Values are expressed as means ± standard error of 10 rat**s** in each group. *P < 0.05, compared to the placebo group (N). *P < 0.05, compared to the cisplatin group (CP).

Discussion

Anticancer agents are used as common therapy against different kinds of cancer. Those agents usually demolish the physiological homoeostasis in various organs during treatment of cancer. Physiological side effects in non-tumor cells induced mostly by radical formation and oxidant injury. Effective anticancer therapy with cisplatin is limited owing to its induced toxicity to various organs including the heart, kidneys and liver (22, 23). So we aimed to demonstrate the effect of fenugreek seeds extract on CP induced renal and hepatic injuries in rats.

Herbs and spices are considered to be essential in medical therapies for delaying aging and biological tissue deterioration. Fenugreek (*Trigonella foenum-graecum Linn*) is a leguminous plant cultivated in several Asian and African countries. The seeds of fenugreek are commonly used in Egypt, India, and in oriental countries as a spice in food preparations due to their strong flavor and aroma (11). Fenugreek seeds are rich source of many active phytochemicals such as saponins, coumarin, fenugreekine, nicotinic acid, sapogenins, phytic acid, scopoletin, and trigonelline, which are thought to account for many of its presumed therapeutic effects (24).

Cisplatin treatment resulted in a significant reduction ($p < 0.05$) in body weight, compared to placebo group. Administration of fenugreek alone did not cause any significant change in body weight. However, prior treatment with FG resulted in a significant protection against the cisplatin-induced reduction of body weight. The decreased body weight due to cisplatin treatment observed in the present study have been previously reported (25, 26). The weight loss of animals treated with cisplatin can be at least partially due to the drug toxicity which accelerates the water elimination in urine. Also, cisplatin-induced weight loss might be due to gastrointestinal toxicity and thereby reduced ingestion of food (27).

Transaminases are the most sensitive biomarkers directly implicated in the extent of cellular damage and toxicity, because they are cytoplasmic in location and are released into the circulation after cellular damage (28). Alterations in AST and ALT are reported in hepatic diseases and in myocardial infarction (22, 29). Treatment with FG alone did not cause any significant change in AST and ALT activities, but treatment with cisplatin significantly increased serum AST, and ALT activities compared to placebo. The presence of FG with cisplatin minimized its toxic effect on serum liver enzymes to approach the control levels and markedly improved the histopathology of the liver. This is consistent with prior report demonstrating that Trigonella foenum graecum seed extract administration could blunt Thioacetamide (TA) induced increase in activities of marker enzymes of hepatocellular injury, viz. alkaline phosphatase (ALP), gamma glutamyl transferase (GGT) (30). The present study revealed that the significantly decreased level of AST, ALT and total bilirubin and the significantly increased level of total protein compared to CP group in serum is due to FG antioxidant effect and its ability to act as a free radical scavenger, thereby protecting membrane permeability after treatment with extract indicate hepatoprotective and curative effect.

The significant disturbance in the activities of AST and ALT by treatment with cisplatin has been previously reported (9, 31). The ability of cisplatin to cause alterations in the activity of these enzymes could be a secondary event following cisplatin-induced liver damage with the consequent leakage from hepatocytes (31). Actually cisplatin contributed various mechanisms to liver dysfunction consist of cellular toxicity, vasoconstriction in the renal microvasculature, and proinflammatory effects, by producing free radicals oxidative stress which participated for decline antioxidant enzyme level and lead to cell injury (32), although apoptotic lesions seem to characterize the damaged liver parenchyma (9). Hepatotoxicity is not considered as a dose limiting toxicity for cisplatin (9). It is known that cisplatin is significantly taken up in human liver and there is a suggestion that the drug accumulates in significant amounts in hepatic tissue particularly, when it is injected in high-doses (32). Generally, liver toxicity of cisplatin is characterized by mild to moderate elevation of serum transaminases (9). In the present study, elevation in the plasma levels of transaminases are indicators of impaired liver functions.

Kidneys represent the major control system maintaining body homeostasis. The plasma concentrations of urea and creatinine determine renal function and are thus biomarkers for kidney diseases (33). Rats treated with FG alone showed no significant change in the levels of urea and creatinine compared to placebo. Meanwhile, cisplatin treatment caused significant increasing in levels of both parameters. Rats pre-exposed to FG before cisplatin exhibited a significant reduction in the levels of urea and creatinine compared to cisplatin alone, this was consistence with others (34) that demonstrated that FG improves renal function and ameliorate renal histopathological alterations. The suggested mechanism may be related to reducing oxidative stress.

The increase in both urea and creatinine levels due to cisplatin treatment has been previously reported (9, 26, 35). Nephrotoxic damage by cisplatin is indicated by increase in blood urea nitrogen (BUN) and creatinine levels. Excretion of cisplatin is predominantly renal and the kidney is considered to be the primary target organ for cisplatin toxicity. Consequently, the impairment of kidney function by cisplatin is recognized as the main side effect and the dose limiting factor associated with its use, occurring either acutely or after repeated treatment (36).

Oxidative stress significantly contributes to CP-associated cytotoxicity, and the use of antioxidants could counteract such cytotoxic effects of CP. Administration of CP resulted in oxidative stress as indicated by a marked increase in MDA levels, a lipid peroxidation marker, while FG decreased those levels. On the other hand, total antioxidant activities are depleted in CP-treated group in a corroboration with a previous study (29), while FG obviously ameliorated the pattern of TAA and corrected this depletion. These results are consistent with a previous report demonstrating considerable antioxidant activities for FG, as represented by significant free radical scavenging activity and inhibitory effects on lipid peroxidation (37).

The major cytotoxicity of cisplatin is inhibition of DNA synthesis and the production of reactive oxygen species. Consequently, the mechanism underlying FG nephroprotection and hepatoprotection may be due to the marked radical scavenging ability of FG (24). Hydroxyl radicals, either directly or indirectly, activate p38 MAPK (mitogen activated protein kinase) which plays an important role in mediating cisplatin-induced renal and liver injury and inflammation, through the production of TNF-α (38).

The present study revealed that p38 MAPK and TNF-α expression were abrogated substantially by the treatment of FG, thus reducing inflammatory response implicated in the acute renal toxicity caused by the cisplatin.

It has been observed that the reactive oxygen and nitrogen species can induce an apoptotic signal cascade by inducing MAPK signaling through p38 MAPK (39) as well as in inflammation (40).

The protection offered by the *Trigonella foenum-graecum* extract could have been due to the presence of any of the active principles contained in the FG extract. Phytochemical screening has shown FG to contain high concentration of polyphenolic flavonoids. Taking into consideration that flavonoids, mainly quercetin extracted from other medicinal plants with nephroprotective and hepatoprotective effects, have been described to inhibit nephrotoxicity and hepatotoxicity induced by xenobiotics (41, 42).

Flavonoids are capable of modulating the activity of enzymes and affect the behavior of many cell systems and exerting beneficial effects on body. Flavonoids generally have been shown to protect against various forms of disorders such as coronary heart diseases, liver and kidney disorders. This is thought to be as a results of induction of detoxifying enzymes such as epoxide hydroxylase, glutathione-s-transferase, UDP-glucuronosyl transferases. Although the molecular mechanism through which the induction of these detoxification enzymes is not well known, it has been suggested that these phytochemicals interact with various intracellular signaling cascades (43). It can be conclude from the present study that the inhibitory effect of FG extract on inflammatory and apoptotic pathway induced by CP administration. So the observed hepato and nephroprotective activity of FG may be due to the presence of flavonoids.

In conclusion, the findings of the present study reinforce the significant role of ROS, inflammatory pathway in pathogenesis of the cisplatin-induced nephrotoxicity and hepatotoxicity. It further demonstrates that abrogation of inflammatory response and apoptotic signal induction by natural antioxidants could be an effective strategy for prophylaxis of cisplatin induced renal and liver damage. FG has a powerful antioxidant effect that can alleviate the CP-induced nephrotoxicity and hepatotoxicity through inhibition of TNF-α mediated inflammation, P38 MAPK mediated- cell apoptosis, as well as by restoration of biochemical and histopathological changes against cisplatin administration resulting in improvement of kidney and liver functions and might be clinically useful. However, there is a need for further studies on this issue before clinical application can be recommended.

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