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Effect of Fenugreek Seed and Leaves on Some Hematological and Biochemical Parameters in CCl4-induced Liver Injury

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A B S T R A C T

This study was carried out to evaluate the effect of fenugreek plant on CCL4 –induced liver injury by following the hematological and biochemical parameters. To achieve this purpose forty male albino rats were used and divided to four groups. The first group represented control group which received normal diet and intraperitoneal injection with oil (0.5ml/kg). The second group represented the CCL (1ml/kg) model. The third group received 200 mg/kg fenugreek leaves extract by gavage. The forth group received 500 mg/kg fenugreek seed extract by gavage. The fenugreek seed and leave extracts treated group showed significant differences in AST, ALT, ALP, direct bilirubin, MDA, GSH, liver SOD, WBC, LYM and PLT when compared to CCl_4 treated rats. These results indicate that these plants can be used as a good source of antioxidant and hepatic protective activities as well as a good antibiotic agent against some pathogenic bacteria. The methanolic extract of fenugreek seeds with different concentrations in ml inhibited the growth of the pathogenic *E. coli*, *Staphylococcus aureus* and *Bacillus subtilis* bacteria more than the aqueous extract for the fenugreek leaves and seeds.

Introduction

Medicinal plants are important part of health care. Large varieties of plants (more than 1200) are available with known therapeutic effects (Kipkore *et al*., 2014). Approximately 70–80% people worldwide depend on medicinal plants to cure various human ailments including viral diseases (Wang and Liu, 2013). Moreover, herbal drugs have gained much importance due to their easily adaptability, low cost and fewer side reactions on patients (Edziri *et al*., 2011).

Natural antioxidants can protect the body against the adverse effects of CCl4 and some other toxins (Kader *et al*., 2014, Amini *et al*., 2012). Medicinal plants have been used to treat various disorders throughout the history of human life, but the use of synthetic drugs was highly prevalent since the middle of last century (Sewell and Rafieian-Kopaei, 2014). With the rapid detection of their adverse side effects of synthetic drugs on public health, the trend is increasing for application of medicinal plants as alternatives to synthetic ones (Bahmani *et al*., 2014a,b).

Fenugreek (*Trigonella foenum graecum Linn)* is an annual herb that belongs to the family Leguminosae. The seeds of fenugreek are commonly used in the Middle East and South Asia as a spice in food preparation and used as traditional medicines in diabetes, high

cholesterol, inflammations and gastrointestinal ailments (Basu *et al*., 2010; Belguith-Hadriche *et al*., 2010).

Liver diseases are one of the major causes of mortality and morbidity worldwide, drug-induced liver toxicity is a major cause of hepatic dysfunction (Abboud and Kaplowitz, 2007). Oxidative stress is considered as a mechanism in contributing to the initiation and progression of hepatic damage in a variety of liver disorders. Cell damage occurs when there is an excess of reactive species derived from oxygen and nitrogen or deficiency of antioxidants (Girish and Pradhan, 2008a). Oxidative stress, involving enhanced generation of reactive oxygen species (ROS), has been implicated in the etiology of many human diseases. Antioxidants capable of neutralizing ROS and their actions are considered beneficial. In this context, natural dietary components with antioxidant activities could be important (Bandyopadhyay *et al*., 1999; Yamamoto, 2000).

Among environmental toxins, carbon tetrachloride (CCl4) dedicated most of conducted studies to itself (Olagunju *et al*., 2009).

Fenugreek has a good antimicrobial property because. It contains certain bioactive components such as volatile oils, alkaloids, mucilage. All these components in Fenugreek adds on to its antibacterial activity. They contain multiple constituents with antimicrobial activity including phenols, quinones, flavones, tannins, terpenoids, and alkaloids (24).

Aim of this study was to study the antioxidant activity of fenugreek plant and its hepatic protective activity and to determine the oxidative stress and antioxidant markers and some hematological parameters in CCL4 treated rat groups. Also the aim of this study is to evaluate the effect of ethanolic and aqueous extracts of the seeds and leaves of fenugreek against various pathogenic bacteria growth.

Materials and Methods

Materials

Plant preparation

A Fenugreek (*Trigonella foenum graecum*) seeds and leaves sample were collected from the local market of Baghdad. Dry fenugreek seed and leaves were cleaned and ground into small pieces by a blender and 70 % ethanol was used extraction by soxhelt extraction method for six hours.

The extracts were combined, and evaporated to dryness under reduced pressure at 60 Co by a rotary evaporator. Extracts were placed in dark bottle, and stored at -4 C° until further analysis. The extract was suspended in distilled water for hepato protective studies (Bukhari *et al*., 2008).

Experimental animals

Forty male albino rats (*Rattus norvegicus*), weighing about 250 – 350gm were used.

The animals were given standard rat diet chow and housed in plastic cages bedded with wooden chips in a room with controlled temperature of 24±3ºC, 12/12 hours light/dark schedule in an animal house belong to Biology department, College of Science, Salahaddin University-Erbil.

Standard chaw ingredients included (wheat 66.6%,soya 25.6%, oil sun flower 4.4%, lime stone 1.5%, salt 0.63%, methionine 0.158%, Lysine 0.24%, choline chloride 0.062% and trace elements 0.05%)

Experimental Design

The experimental rats were divided randomly to 4 groups. This experiment was carried out for four weeks as explained below:

Group 1: Control rats (n=10)

The rats of this group were given olive oil intraperitoneally (0.5 ml/kg body weight) for four weeks.

Group 2: CCl4 treated rats (n=10)

The rats of this group were given CCl4 intraperitoneally 1ml/kg b.w. (1:1 in olive oil) for four weeks

Group 3: Fenugreek (n=10)

The rats of this group were given CCl4 intraperitoneally 1ml/kg b.w. (1:1 in olive oil) and fenugreek seeds extract 500 mg/kg dissolved in distilled water and given to rats by gavage daily for four weeks.

Group 4: Fenugreek leaves (n=10)

The rats of this group were given CCl4 intraperitoneally 1ml/kg b.w. (1:1 in olive oil) and Fenugreek leaves extract 200 mg/kg dissolved in water and given to rats by gavage daily for four weeks

Methods

Tissue preparation

Anesthesia, dissecting, liver and kidney removing

All animals were anesthetized with Ketamine hydrochloride 80mg/Kg (Trittau, Germany) and Xylazin 12mg/Kg (Interchem, Holland). The liver was removed then divided into two equal parts, one part cut into small pieces (less than 0.5cm 3 thicknesses) then kept in formalin, while the other part stored at refrigerators until homogenized for estimation of SOD, HYP and GSH.

Tissue homogenate

Liver washed with cold saline. Pieces of each tissue used for homogenization by 20 mM cold phosphate buffer saline (pH 7.4).The liver tissues homogenized (10%w/v) using handheld glass homogenizer (Chowdhury *et al*., 2013). Homogenates were centrifuged at 6000 rpm for10 minutes. The supernatants were collected and stored at -80Co until assayed.

Estimation of glutathione in liver tissue

The procedure of (Moron *et al*., 1979) was followed with some modification. Weighting 1 gm of liver tissue and homogenate by using handled homogenizer with 10 ml of cold tris buffer solution. One ml of tissue homogenate was added to 0.25ml of 25% trichloroacetic acid. After centrifugation for 5 minutes at 3000rpm 0.2 ml of supernatant was taken in a test tube, adding one ml o.15mole imidazole solution then adding 1.7ml distilled water and o.1ml 5.5(DTNB) solution finally absorbance was read at 412nm after 3minutes of adding DTNB.

The concentration of GSH was calculated according to the absorbance of blank (B), test (T) and standard (S) solutions by the following equation:

GSH conc. (µmol/mg of tissue) = *conc. Standard * 100 (3.1)

Determination of liver tissue superoxide dismutase

Liver samples were washed with 0.9% NaCl to remove red blood cells. The tissue was then

blotted dry and weighed followed by homogenization in 200 µl buffer (0.05 M potassium phosphate and 0.1 mM EDTA, pH 7.8) and centrifuged at 15,000xg for 30 min at 4˚C. The supernatant was used for determination of SOD. Superoxide dismutase was measured using the Superoxide Dismutase assay kit provided by Elabscience (Elabscience, WuHan P.R.C).

The concentration of SOD was determined by competitive-ELISA method.

The concentration of SOD in the samples is then determined by comparing the OD of the samples to the standard curve (Figure 1).

Blood collection

At the end of the treatment period, blood samples were collected from anesthetized rats through cardiac puncture. The collected blood samples were immediately placed into test tube and centrifuged and the sera were stored at -80Co (Sanyo – Ultra – Low Temperature, Japan) until assayed. While, for hematological analysis blood were collected in EDTA tube.

Hematological analysis

White blood cell (WBC) count, LYM and PLT count were determined automatically by using automated hematology analyzer (Sysmex model: K-1000, Japan).

Determination of Liver Function Paramet

Alkaline Phosphatase, Aspartate Aminotransferase, Alanine Aminotransferase and bilirubin were achieved automatically by using full automated (COBAS Integra 400plus-roche, Germany).

Statistical analysis

One way analysis of variance followed by Newman-Keuls post hoc test comparison procedures were used to compare between means of different groups. Data are represented as the mean±standard error (M±SE). Graphpad prism program, version 6.01, computer program was used for statistical analysis. $P<0.05$ was considered statistically significant. Citations and references were managed by Endnote X 7 (Endnote software, Thomson Reutter, Canada)

The Antibacterial Effects of Leave and Seed Watery and Alcoholic Extracts:-

The inhibitory of many concentrations of leave and seeds was carried out to determine the lowest concentration needed to inhibit visible bacterial growth by fixed concentration of experimental isolates of bacteria after an overnight incubation. The inhibition value of was confirmed based on the inhibition and growth observed on the agar plate which had been carried out as follow:

Leave and seeds in different weights (0.01, 0.02, 0.1, 0.2 and 0.5) gm were added to freshly prepared growth media in 250 ml Erlenmeyer flasks containing 100 ml sterile Nutrient agar, these media poured in sterilized petri dish and inoculated with 1ml of suitable dilution incubated at 37C for 24hr. The test was carried out in triplicate and the mean value was calculated (AL-Bayaty *et al.*, 2011).

Antibacterial Activity of Leave and Seed Watery and Alcoholic Extracts by Well Diffusion Agar Leave and seeds 0.01 g, 0.02 g, was analyzed for inhibition activities against tested bacteria by agar –well diffusion Muller-Hinton agar seeded with bacterial isolates. The inoculums were prepared by adding (5) isolated colonies grown on Nutrient agar plate to (5) ml of nutrient broth and incubated at $37C^0$ for 18 hrs. and compared with (0.5) Mcfarland tube. A sterile

swabs was used to obtain an inoculums was streaked on Muller-Hinton agar plate and left to dry. Wells (5) mm were hollowed out in agar using a sterile cork borer, a volume of (50) µl of tested extracts compounds were dropped separately in each well, and incubated at 37 ^{0}C for 24 hrs.; inhibition zone around the wells were measured and recorded in millimeter after subtraction 5 mm (well diameter).

Results and Discussion

Effect of fenugreek leaves and seed on liver function tests in carbon tetrachloride treated rats

Table (1) shows the effects of fenugreek leaves and seeds on the liver function tests in CCl4 treated rats. The results of this study showed variations in the level of liver function tests in CCl4 treated rats. The ALP level was significantly decreased in control ($P<0.05$) and fenugreek group ($P<0.01$), modified Harvard style but there were no statistical difference of ALP level in fenugreek leave group when compared to the CCl4 treated rats,. Also, AST levels were significantly decreased ($P<0.001$) in control, both of fenugreek groups when compared to the CCl4 treated rats.

Moreover, it revealed that in all treated groups, serum ALT levels were decreased significantly ($P\leq 0.001$) compared with CCl4 treated rats. With respect to direct bilirubin level, control, also both of fenugreek treated groups were significantly decreased ($P\leq 0.001$) compared to CCl4 treated rats.

Results of the current data showed the increase in ALP, AST, ALT and bilirubin levels in CCl4 treated groups are in agreement with (Girish and Pradhan, 2012). The mechanism of hepatic damage by CCl4 is well documented by Buege and Aust (1978) they were reported that CCl4 is metabolized by Cytochrome P450 enzyme to (CCl3). This in turn reacts with molecular oxygen and gets converted to trichloromethyl peroxy radical. This radical forms covalent bonds with sulfhydryl groups of several membrane molecules like GSH leading to their depletion and causes lipid peroxidation. The lipid peroxidation initiates a cascade of reactions leading to liver necrosis. Liver damage is detected by measuring the activities of liver function marker enzymes like AST, ALT and ALP, which are released into the blood from damaged cells. They are also indicators of liver damage (Meera *et al*., 2009).

Our results showed that extract of fenugreek can prevent the CCl4 induced toxicity in the liver by significantly reduction of AST, ALT, ALP and direct bilirubin levels, these results are in agreement with (Meera *et al*., 2009) they achieved that the normalization of the above enzyme levels in rat liver with the plant drugs estabilishes the hepato protective effect of *T. foenum-graecum* which may be able to induce accelerated regeneration of liver cells reducing the leakage of these enzymes into the blood. The results indicated that fenugreek significantly prevented the increased liver function marker enzyme activity induced by CCl4, indicating an improvement of the functional status of the liver by the fenugreek.

Effect of Fenugreek seed and leave extracts on the some hematological parameters in carbon tetrachloride treated rats

The results showed (Table 2) that WBC count significantly decreased in fenugreek seeds ($P\leq 0.001$), but there were no statistical differences in control, fenugreek leaves when compared with CCl4 treated rats. Moreover, number of LYM significantly decreased in fenugreek seeds ($P\leq 0.05$), while there were no significant differences in control,, fenugreek leaves when compared with CCl4

treated group. Furthermore, the PLT count significantly decreased in control, fenugreek (P≤ 0.01), and, fenugreek leaves (P≤ 0.05) when compared with CCl4 treated rats.

The present study showed that the rats treated with fenugreek significantly decreased WBC, LYM and PLT when compared with CCl4 treated rats.

Effect of fenugreek seed and leave extracts on the liver super oxide dismutase and liver glutathione levels in carbon tetrachloride treated rats

As shown in table (3), the level of liver GSH in fenugreek groups significantly increased (P≤ 0.001), but there was no statistical difference of liver GSH level in control when compared to CCl4 treated group. Also, liver SOD significantly increased in control (P≤ 0.001), fenugreek seeds and leaves (P≤ 0.05)

Glutathion (GSH) is the most important of the sulfur-containing non-enzymatic antioxidant molecules. GSH can also conjugate with free radicals directly, earmarking them for renal excretion, which is especially important for dealing with the products of hepatic cytochrome P450 enzyme activity. The sulfhydryl (–SH) portion of the GSH can be used to reduce a variety of free radicals in a reaction catalyzed by the antioxidant enzyme, glutathione peroxidase (Webb and Twedt, 2008).

In this study, the GSH level was significantly increased in fenugreek treatment. This is in agreement with (Sushma and Devasena, 2010), they showed that administration of fenugreek seed extract minimized the effects of ethanol in tissues. The beneficial effects of fenugreek seeds are well demonstrated by their ability to improve antioxidant status thereby lowering lipid peroxidation. *In vitro* investigations revealed that the aqueous extract of fenugreek seeds effectively inhibited the production of TBARS in the presence of promoters of lipid peroxidation. In this manner, the effect of fenugreek aqueous extract was comparable with α-tocopherol (Thirunavukkarasu *et al*., 2003).

Table.1 Effect of fenugreek seed and leaves treatments on liver function test in CCI4-liver injury rats

Groups	S. ALP (U/L)	S. AST(U/L)	S. ALT(U/L)	S.D. Bilirubin (mg/dL)
CCl4	326±25.59	812.3±91.03	763.8±98.49	0.09625±0.006
Control	243.4±27	196.4±35.68	53.4±6.47	0.026±0.002
Fenugreek leave extract	280.4±10.41	131.3±15.31	45.33±1.55	0.02733±0.004
Fenugreek seed extract	230±17.45	146.4±19.84	41.88±2.6	0.0295±0.005

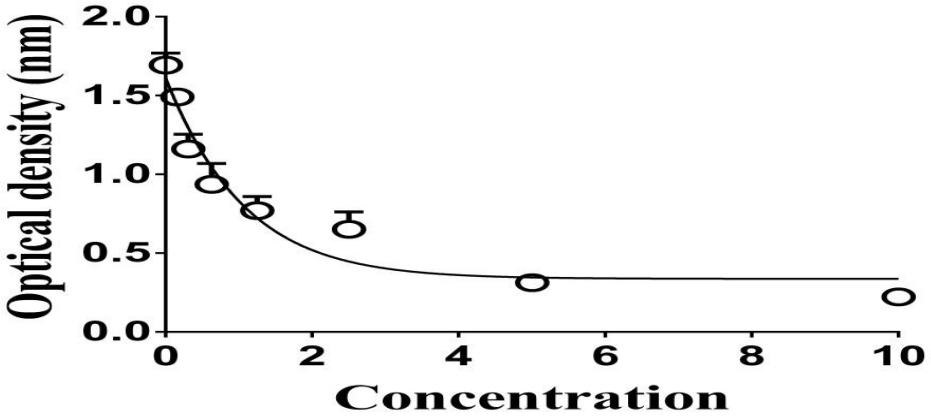
Table.2 Effect of fenugreek seed and leave extracts on the some hematological parametersinCCI4-in liver in jury rats

Groups	WBC *103/µL	LYM *103/µL	PLT*103/µL
CCl4	9.623±0.34	6.033±0.12	915.4±16.91
Control	8.3±0.7	4.65±0.15	522±117.5
Fenugreek leaves	7.2±0.55	4.533±0.27	582.8±47.78
Fenugreek seeds	4.75±0.95	3.75±0.55	536.6±122.1

Table.3 Effect of fenugreek seed and leave extracts on GSH and SOD in CCI4- liver injury rats

Groups	GSH (µmol)	SOD
CCl4	13.33±0.7	0.03576±0.0112
Control	25.19±1.33	0.2804±0.03531
Fenugreek leaves	108.2±4.33	0.2358±0.04062
Fenugreek seeds	130.2±8.71	0.1802±0.05225

Fig.1 Standard curve of superoxide dismutase (SOD)



The antibacterial Activity of Fenugreek Leave and Seed watery and alcoholic extracts, inhibition zone measured in millimeter and percentage of inhibition

Types of bacteria	Concentration	Leaves watery extracts	Leaves alcoholic extracts	Seeds watery extracts	Seeds alcoholic extracts
E. coli	0.01	7(44%)	14(86%)	7(44%)	13.5(86.5%)
	0.02	6(43%)	14(86%)	7(43%)	13(87%)
	0.1	50%	100%	45%	100%
	0.2	50%	100%	46%	100%
	0.5	60%	100%	50%	100%
Staphylococcusaureus	0.01	8(46%)	15(85%)	8(46%)	14(86%)
	0.02	11(66%)	14(86%)	10(65%)	13(87%)
	0.1	68%	100%	60%	100%
	0.2	72%	100%	60%	100%
	0.5	80%	100%	62%	100%
Bacillus subtillus	0.01	6(44%)	12(88%)	6(42%)	11(89%)
	0.02	6 (44%)	11.5(88.5%)	6(42%)	13(90%)
	0.1	50%	100%	42%	100%
	0.2	50%	100%	44%	100%
	0.5	60%	100%	45%	100%

The present study demonstrated that the activity of liver SOD was significantly enhanced by the presence of fenugreek seeds extracts. The mechanism of enhancement was observed by Joshi *et al*., (2014). They conclude that the depleted enzymatic and non-enzymatic anti-oxidants of diabetic rats were restored significantly with the treatment of fenugreek. Such effects may be mediated through the active phytoconstituents present in fenugreek, like 4-hydroxy isoleucine, diosgenin, orientin, quercetin. These active constituents can scavenge, or neutralize the free radicals or other ROS components (Baig *et al*., 2012; Punitha *et al*., 2005).

From this study we support the use of alcoholic fenugreek seeds and leaves extract was more active against the pathogenic bacteria than the watery fenugreek leaves extract and it may have a role in the treatment of some infectious diseases. This is in agreement with R. Chalghoumi *et al*., (2016) they conclude that antibacterial effect was demonstrated by the aqueous extract of fenugreek seeds; however, Iyer *et al*., (2004) they concluded that the organic extracts prepared with chloroform, acetone or methanol showed low to moderately high growth inhibitory effect (8.33 mm ≤ IZ ≤ 20 mm) when tested at a concentration equal to or above 5 mg/ml (24)140p.

In conclusion, from the present study, the following results can be concluded:

From the biochemical and physiological points of view, the model of CCl4 caused several changes in the level of the oxidative parameters, decreasing of GSH but fenugreek seed and leaves were succeeded in attenuating these changes when added to the CCl4 treated group and have shown hepatic protective effect by increasing the liver SOD levels

The model produced oxidative stress and rising in the levels of AST, ALT, ALP, direct bilirubin, but the current seeds and leaves lowered these levels.

Fenugreek seeds and leaves ameliorated inflammation caused by CCl4 treatment via decreasing of WBC and LYM count. Moreover, it decreased thrombogenic activity of CCl4 through decreasing of PLT count

From this study we support the use of alcoholic fenugreek seeds and leaves extract was more active against the pathogenic bacteria than the watery fenugreek leaves extract and it may have a role in the treatment of some infectious diseases.

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