The hepatoprotective activity of Fenugreek seeds' extract against carbon tetrachloride induced liver toxicity in rats

Said, A.M. [a], Al-Khashali, D. [**], Al-Khateeb, E. [**] and Al-Wakeel, N [***]
Department of Pharmacotherapeutics, College of Pharmacy, Almustansirya University
****Department of pharmacology and toxicology, College of Pharmacy, University of Baghdad.***
*****Unit of histopathology, Department of laboratories, Baghdad Medical Cit.***

الخلاصة:

تمت دراسة فعالية حماية الكبد ضد التسمم بواسطة رباعي كلوريد الميثان في الجرذان للمحلول المائي لمستخلص الكحول الاثيلي لبذور الحلبة العراقية بعد ازالة الدهون منها بواسطة الهكسان. باستخدام الفحوصات النسيجية المرضية وقياس نشاطات انزيمات وظائف الكبد في الدم ومحتويات الكبد من الشحوم والكلوتاثايون كدلائل مختبرية, اثبتت بذور الحلبة ان لها فعالية عالية ضد التسمم الناتج عن آلية التأكسد بينما لها فعالية محدودة وبنسبة اقل ضد التسمم الناتج عن آلية تراكم الدهون في الكبد نتيجة ربما يعود لخاصية بعض المواد الفعالة في البذور والتي لها قابلية مواجهة المركبات الوسطية المتكونة خلال عملية التأكسد والتي تؤدي الى تضرر الانسجة بينما بنبة اقل في مواجهه المركبات الوسطية الخاصة بعملية تجمع الدهون.

Abstract

The liver subjected to a number of disorders caused by chemicals namely as *hepatotoxicity*. carbon tetrachloride (CCl4) the most wildly used in induction of liver injury as a hepatotoxic model for scientific researches. The role of plants in treatment and prevention of chemical-induced liver damage, was extensively studied. Accordingly, this study was designed to evaluate the hepatoprotective effect of the aqueous solution of the ethanolic extract of Fenugreek seeds (FGS) against CCl4 induced liver damage in rats. From analysis of results, the extract of FGS showed highly hepatoprotective effect against CCl4 induced-necrosis with less effect against fatty changes, this could be due to the antioxidant action of the active constituents in the extract that counteract and scavenge the intermediates free radicals of CCl4 that generating during lipid peroxidation, which is the main cause of necrotic damage, while limitations of these constituents to inhibit the intermediates involved during fatty changes which is unrelated to the oxidative stress. These results make the FGS as a good candidate to be used against drug induced hepatotoxicity.

Introduction:

The liver is considered as the major organ responsible for conducting various metabolic processes and according to it's highly exposed to the toxic effects of different xenobiotics predisposing to different types of diseases and disorders namely as liver injury (hepatotoxicity) [1]. Some of hepatotoxic compounds are used experimentally such carbon tetrachloride (CCl_4) as a model for scientific researches to investigate drug-induced liver toxicity because it is widely studied and has more than one approved mechanism of liver toxicity [2]. Many researches have been oriented to study the effects of plants with antioxidant activity that used traditionally by the herbalists to support liver function and treatment of hepatic diseases [3]. Fenugreek is annual herb that grows natively and cultivated in different parts in the world used both in medicine and with food as spice show antioxidant effect through their used in diabetes mellitus due to the presence of different active constituents such as flavonoids, alkaloids, vitamins and amino acids [4]. Accordingly, this study was designed for the evaluation the hepatoprotective effect of the aqueous solution of the ethanolic extract of fenugreek seeds (FGS) against carbon tetrachloride (CCl_4) induced liver damage in rats.

Materials and methods:

Extraction of active Constitutes from FGS:

One kilogram (1000 gm) of dried seeds of fenugreek (*Trigonella feouam-graceum, F. Legumenosea*) FGS has been crushed and milled, defatted by soxhlet apparatus with 1500 ml of n-hexane until disappearance of the yellowish color which spends about 6 hours. The remaining residue which is oil free was left at room temperature for 24 hr, this residue represent the defatted FGS and was extracted by alcohol through reflex method with 2 liters of 80% ethanol for 6 hr at 40 ^{o}C, then the mixture was left to cool and filtered by filter paper. The filtrate was evaporating by rotary evaporator vacuum at 40 ^{o}C until ethanol free extract was remained that contained the total active constituents of active ingredients in FGS. The total weight yielded of this extract was 173.641 gm (each 1 gm of the seeds powder contains 173.641 mg of the active constituents extract). The extract then dissolved with up to 1000 ml of distilled water (D.W.) in that 1 ml of the present solution contained the extract of 1 gm of the crude FGS powder, the solution is then administered to the animal.

Randomization and treatment of animals:

Thirty adult rats of *Rattus norvegicus* from both sexes weighing 200-250 gm rats divided into three groups, 10 of each, in which groups I & II received 2 ml/kg dose of D.W. while group III received 347.282 mg/kg/day of FGS extract as 2 ml/kg (equivalent to 2 gm/kg/day of FGS powder) and the treatment continued for 30 days [5]. .At 31st day, animals of groups II & III received single

dose 2ml/kg of 1:1 v/v (CCl_4 in corn oil) to induce liver damage [6], while group I received corn oil only and served as control. The animals were feed commercial pellet and tap water in free access *ad libitum*. All animals were all sacrificed after killed by anesthetic ether at 32nd day and their blood and liver samples were obtained for examinations.

Preparation and analysis of liver homogenate:

After killing the animals, Livers are quickly excised, placed in chilled phosphate buffer solution (pH 7.4) at 4 ^oC, blotted with filter paper and weighed. One gram of liver is then taken to prepare 10% tissue homogenate for determination of hepatic constituents using the same buffer solution type utilizing electrical tissue homogenizer at set 3 for 1 minute at 4 ^oC. All preparations were freshly prepared and frozen (-18^oC) unless worked immediately. Determination of lipid peroxidation performed by estimation of malondialdehyde (MDA), the degridative product of peroxidized lipid depending on the method of Buege and Aust [7]. Total thiol groups contents, which can be used as an indicator for reduced glutathione (GSH) in the liver was determined according to the method of Ellman [8], Determination of hepatic triglycerides (TGS) as an indicator of lipid contents was used of fatty liver assessment by an adaptation method of direct determinant of serum TGS according to the enzymatic reactions method of Fossati and Prencipe [9].

Preparation and analysis of Serum Samples:

Post-mortem serum samples prepared, after separation from the blood that collected by cardiac puncture from the animals, and centrifuged at 3000 rpm for 15 minutes. The supernatant layer then analyzed using standard laboratories procedures and ready made kits for estimation of the activities of aspartate transferase (AST), alanin transferase (ALT) [10], and alkaline phophatase (ALP)[11], as liver function test (LFT) enzymes.

Histopathological examination:

One gram of the liver from each animal was cuted into 3 mm pieces, fixed in 10% formadehyde solution, then dehydrated using increasing strengths of ethanol and cleaning the tissues using xylene then impregnated with paraffin wax, heated and blocked by pouring in embeded models. Blockes were cut by microtome into 5 um thick sections, washed in water bath and left in oven for dewaxing. Then stained with haematoxyline and eosin and examined under light microscope. The degree of the injury was evaluating in increasing grade as focal, mild, moderate and sever for each liver injury type [12].

Statistical analysis:

The results were presented as mean ± standard deviation (SD). The statistical analysis includes unpaired *t*-test and the significance level of all tests was taken as P value<0.05.

Results:

The levels of biochemical parameters, AST, ALT and ALP activities in serum with the levels of MDA, GSH and TGS in liver homogenate of all groups are illustrated in figures 1-6. The histopathological examination of liver sections from each animal that done to demonstrate the degree of the each hepatic injury types induced by CCl4 and the effect of FGS in this study are illustrated in figures 8-10. Analysis of data revealed significant amelioration of oxidative stress in group III treated with FGS extract administration as compared during hepatotoxicity induced by CCl_4 in group II by lowering MDA and elevating GSH to the control level in group I after they are changed by CCl_4, while fatty changes decreased from sever in group II to mild in group III as indicated by TGS hepatic levels and histopathological examinations. The levels of LFT activities decrease with FGS extract treatment after they increase during CCl_4 but their levels still higher than control. FGS extract show also attenuation of inflammation that were caused by CCl_4.

Discussion:

The alcoholic method considered the first step in the extraction of many bioactive constituents from plant materials, due to ethanol properties. The uses of aqueous alcohol (alcohol and water mixing) allow the extraction of both polar and non-polar compounds [13]. N-hexane was used for defatted fenugreek oil from the seeds [14], in order to exclude the unwanted fatty components that interfere with both extraction processes and biological effects inside the body.

The effect of CCl_4 on rat liver in group II is well observed. The types of liver injuries caused by CCl_4 are observed from the significant increments of hepatic constituents in tissue homogenates analysis; MDA and TGS in group II which are indicators of oxidative stress and fat accumulation respectively. These results are compatible with the aspects of CCl_4 induced liver toxicity in that two major mechanisms, lipid peroxidation and covalent binding that caused by $CCl_3OO^{\cdot}$ and $CCl^{\cdot}{}_3$ which lead to necrosis and steatosis respectively [15,16].These results are supported by the histopathological examination which showed the presence of necrotic damage and fatty changes in liver sections (figure 8). The hepatic glutathione (GSH) decreased upon CCl_4 induced liver toxicity [17]. LFT enzymes are increase in their activities in serum in all type of liver injuries [1], including CCl_4 induced hepatotoxicity [18].

The antioxidant properties of FGS were observed during the use of FGS as hypoglycemic and hypolipidemic agent in diabetes mellitus (DM), due to the involvement of lipid peroxidation in the pathogenesis of the disease and the effective treatment of antioxidant in combination with antidiabetic drugs, the influence of FGS powder in rats received 2 gm/kg/day dose for 30 days in Alloxan diabetic rats normalizes the alterations of enhanced lipid peroxidation and increase susceptibility to oxidative stress associated with depletion of antioxidants in liver, kidney and pancreas [5]. Administration of FGS powder to

diabetic rats prevents necrosis caused by alloxan through histopathological examination of hepatic tissue after stabilization of free radicals metabolism by the antioxidant properties of FGS [19].

The GSH level was preserved by FGS treatment against CCl_4 toxicity and it could be one of the important factors that prevent lipid peroxidation seen in group II. The administration of FGS powder enhanced the activity of glutathione peroxidase and glutathione reductase, that are found to be decreased during diabetes in the rats' liver with correction of blood glucose level [20]. Other study showed that increase the level of GSH by FGS in livers could be result not due to activation of antioxidant enzymes only but through stimulation of endogenous synthesis of GSH [21], which is an additional explanation for the increase in hepatic GSH in group III compared to group II . Administration of FGS powder in 2gm/kg/day enhances the circulating antioxidants, vitamin E, vitamin C, GSH and GPX in both chemical-induced colon carcinogenic and normal rats with decrease the level of MDA [22]. The aqueous extract of FGS showed protective effect against subchronic administration of alcohol-induced liver and brain damage in rats due to the antioxidant properties of FGS which counteract the lipid peroxidation process caused by ethanol [23].

Flavonoids are groups of polyphenolic compounds which are present in most plants, concentrating in seeds, fruits and flowers. Flavonoids have been shown to be potent antioxidants capable of scavenging lipid peroxyl radicals [24]. The mechanism of antioxidant action of flavonoids was not related to the direct scavenging activity only, but also flavonoids were showed to increase the tissue GSH contents when fed to the experimental animals through stimulation of GSH synthesis related enzymes [25], and GSH antioxidant enzymes GPX and GR [26], which are all lead to increase GSH level and total thiol status inside the tissue. Fenugreek was identified as a plant of highly flavonoids contents in commonly Indian food of 85 dietary stuffs by biochemical analysis [27]. In other work, fenugreek was identified as a plant of higher antioxidant phenolics activity and the ethanolic extract of FGS showed higher antioxidant activity as compared with aqueous extract. That study indicate also a positive and highly statistical relationship between total phenolic with antioxidant activity and the extraction with ethanol was found to be more efficient than water for the polyphenolic constituents [28]. The major flavonoid, quercetine that present in FGS was shown a protective activity against CCl_4 induced hepatotoxicity [29].

The histopathological examination showed a decline of fatty changes from severs in group III to mild-moderate form in group II while hepatic TGS in group III still higher than of group I although it decrease significantly as compared with group II. These results indicate that the severity of fatty changes was decreased by FGS treatment and partial protection was given only in contrast to that seen in prevention of lipid peroxidation. In a study of the hepatoprotective effect of GSH in rats liver exposed to CCl_4-induced lipid

peroxidation and covalent binding that caused by it's free radicals derivatives, $CCl_3OO^{\cdot}$ and $CCl^{\cdot}_3$ respectively, GSH showed protective action against $CCl_3OO^{\cdot}$ but not $CCl^{\cdot}_3$ [30]. This is compatible with the data observed in this study where although GSH preserved upon treatment with FGS during CCl_4 toxicity but partial inhibition of fatty liver observed, which can be considered as one of the explanation.

The occurrence of mild fatty changes in group III explain increase the levels of LFT enzymes; AST, ALT and ALP comparing to group I, although they are significantly below group II. This is may be attributed to the increased lipid accumulation predisposing to the most of the indicated histopathological changes [1].

Conclusion

From data present, the extract of FGS showed highly hepatoprotective effect against CCl_4 induced-lipid peroxidation with less effect against fatty changes, could be due to the antioxidant action of the flavonoids active constituents in the extract that counteract and scavenge the intermediates free radicals of CCl_4 that generate during the peroxidation while limitations of these constituents to inhibit the intermediates involved during fatty changes which is unrelated to the oxidative stress.

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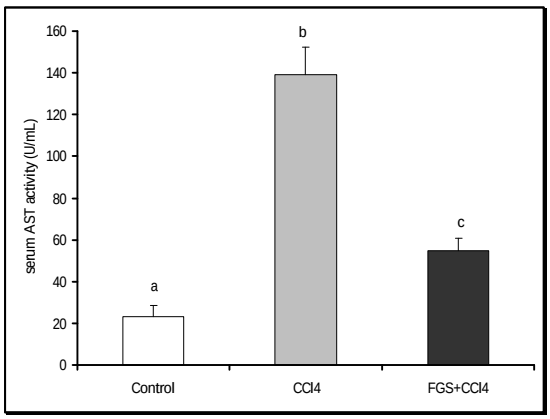

Figure 1: The effect of treatment on serum AST activity

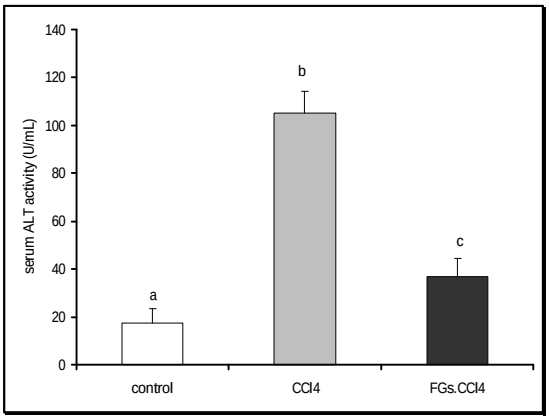

Figure 2: The effect of treatment on serum ALT activity

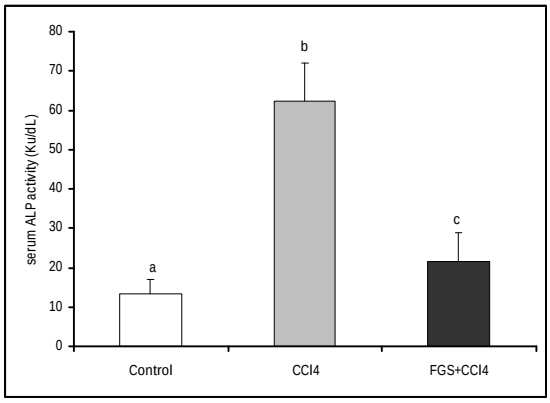

Figure 3: The effect of treatment on serum ALP activity

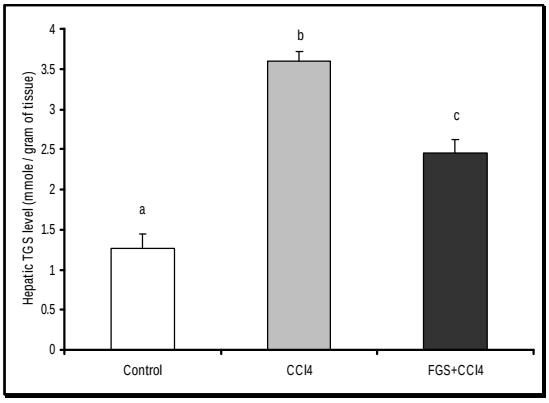

Figure 4: The effect of treatment on hepatic TGS

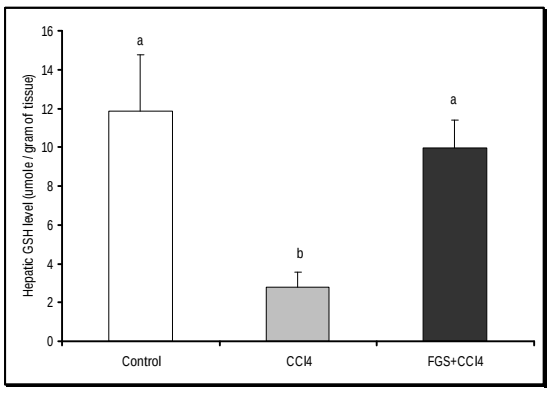

Figure 5: The effect of treatment on hepatic GSH

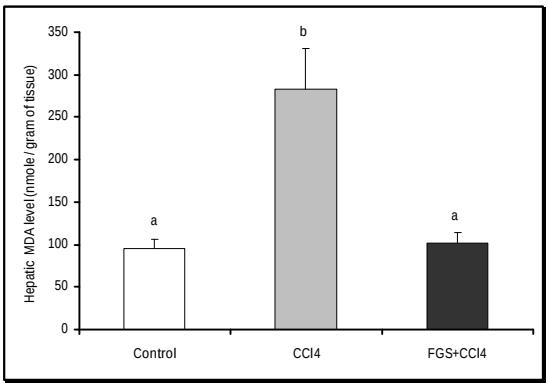

Figure 6: The effect of treatment on hepatic MDA

Figures 1-6: Effects of treatment with Fenugreek seeds extract prior CCl_4 on the level of biochemical parameters. n=10. Values with non-identical superscripts (a,b&c) considered significantly different (P<0.05).

A B

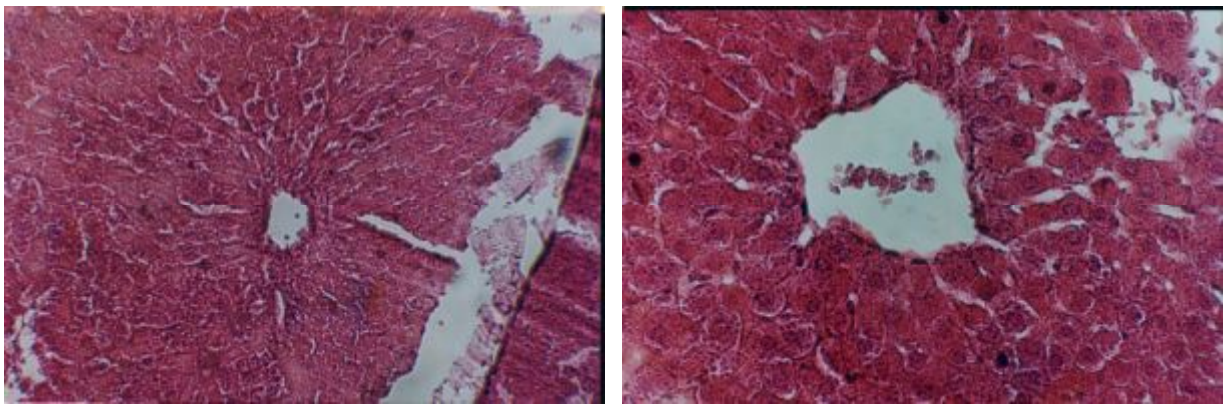

Figure-7: Liver section from control group show normal hepatic architect with branch of central hepatic vein. A: (10*10), B: (10*20). Staining: Haematoxylline & Eosin.

A B

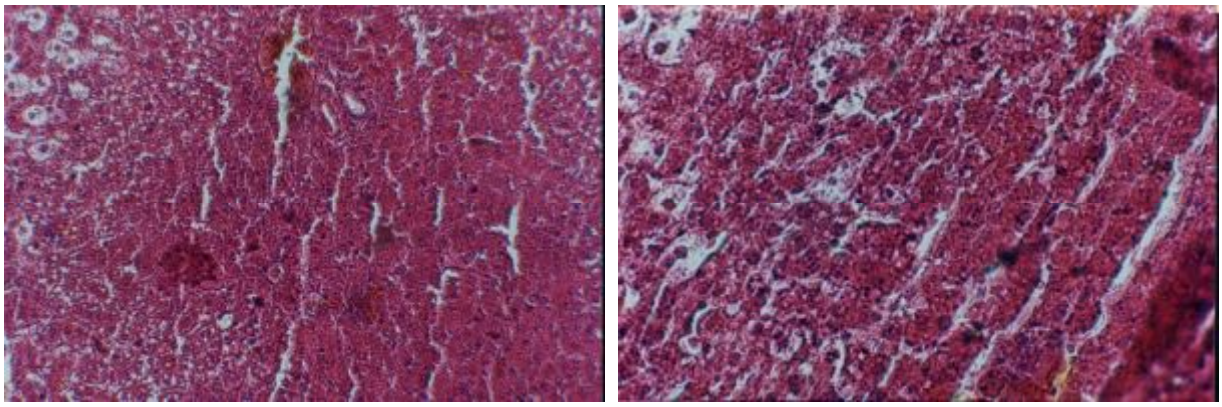

Figure-8: Liver section from CCl4 group show necrotic damage with inflammatory infiltration and .Fatty changes. A: (10*10), B: (10*20). Staining: Haematoxylline & Eosin.

A B

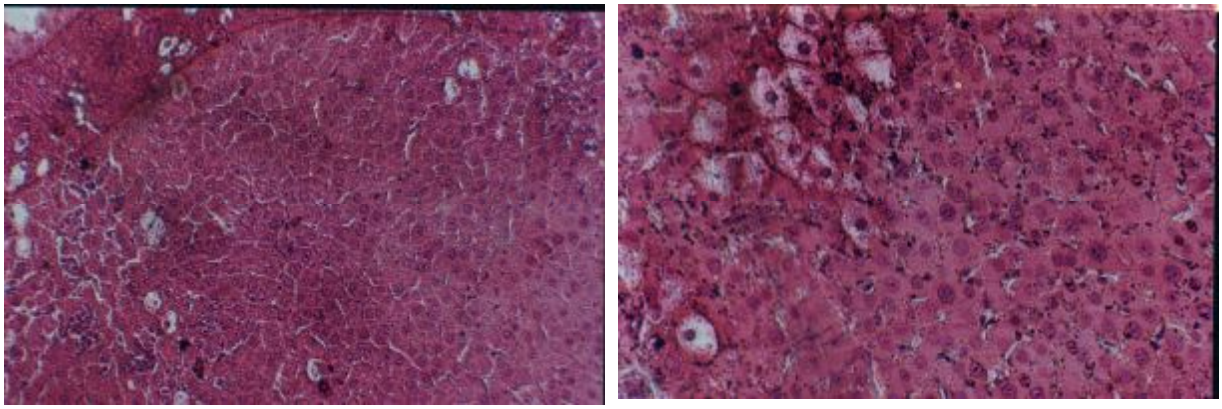

Figure 9: Liver section from FGS + CCL4 group show normal regenerating hepatocytes and Mild fatty changes. A: (10*10), B: (10*20). Staining: Haematoxylline & Eosin