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The Effects of Fenugreek Seed Powder on Oxidant and Antioxidant Factors in Male Rats with Acetaminophen-induced Liver Toxicity

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ABSTRACT

BACKGROUND AND OBJECTIVE: Acetaminophen can induce liver damage via oxidative stress. This study aimed to evaluate the protective effects of fenugreek seed powder on oxidant and antioxidant factors in acetaminophen-induced liver toxicity.

.**METHODS:** In this experimental study, 24 male Wistar rats, weighing 250-280 g, were divided into four groups: control, normal saline+fenugreek seed powder, acetaminophen and acetaminophen+fenugreek seed powder. After 24 h of fasting, groups Normal and Normal+ Fenugreek received normal saline and groups Acetaminophen and Acetaminophen + Fenugreek received 1000 mg/kg acetaminophen by gavage. After six hours, the normal saline and acetaminophen groups received normal saline, whereas the groups receiving normal saline+fenugreek powder and acetaminophen+fenugreek powder were administered 1000 mg/kg of fenugreek seed powder via gavage. Twelve hours after the second gavage, the rats were sacrificed and liver tissues were frozen and assessed.

FINDINGS A decline in malondialdehyde and hydrogen peroxide levels was observed in group acetaminophen+fenugreek, compared to the acetaminophen group (0.2608±0.03 and 1.56±0.153 vs. 6.7±0.344 and 2.38±0.104 in acetaminophen groups; $p<0.001$ and $p<0.05$, respectively). Also, the activities of peroxidase and catalase increased in these groups, compared to the acetaminophen groups (19.07± 3.366 and 1.174±0.242 vs. 8.108 ± 2.46 and 0.614±0.089 in acetaminophen groups, respectively).

CONCLUSION: The results of this study showed that fenugreek seeds could lead to reduced oxidative stress in acetaminophen-induced liver toxicity

KEY WORDS: Acetaminophen, Fenugreek seeds, Liver, Oxidative stress, Rat.

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Introduction

Drug-induced liver injury (DILI) is one of the most common causes of discontinued use of medications. Moreover, DILI is often the main reason for denial of drug approval and withdrawal of drugs from the market. This issue is a major challenge for clinicians, health authorities and pharmaceutical companies (1). Use of over 800 medications has been associated with liver toxicity. So far, acetaminophen overdose has been known as the most common cause of DILI (2). Acetaminophen (N-acetyl-p-amino-phenol or

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paracetamol) is used as an analgesic and antipyretic agent. This medication was widely available in the United States in 1955. Until the late 1990s, acetaminophen was not recognized as a cause of acute liver failure in the United States. However, in a study by Larson et al. (2005), acetaminophen accounted for almost 42% of all cases of acute liver failure (3). Approximately 90% of acetaminophen is transformed into non-toxic metabolites through the second phase of metabolism upon use and a small percentage (<5-10%) is metabolized by cytochrome P450 (CYP) isomers, leading to the production of the toxic metabolite of acetaminophen, i.e., N-acetyl-p-benzoquinone imine (NAPQI). Under normal conditions, NAPQI is detoxified by conjugation with glutathione.In acetaminophen-induced toxicity, following the cellular elimination of glutathione, NAPQI induces hepatotoxicity and cellular death by binding to cellular macromolecules. Consequently, mitochondrial respiration becomes impaired, hepatic adenosine triphosphate level decreases, mitochondrial permeability transition pore is unlocked and cytochrome C is released from the mitochondria. Multiple P450 (CYP) enzymes can convert acetaminophen to NAPQI, among which CYP2E1, CYP1A2 and CYP3A4 play major roles in this process (4).Since prehistoric times, various medicinal plants have been used in traditional medicine. For thousands of years, these plants as natural medicines with therapeutic effects and pharmacological characteristics have been used around the world (5). Medicinal herbs contain high levels of antioxidants, which can delay or inhibit the oxidation of lipids or other materials (6). Antioxidant characteristics of herbal plants are related to phenolic antioxidants such as phenolic acids, flavonoids, terpenes, tocopherols, vitamin C and a group of carotenoids (7). Fenugreek is an annual leguminous plant, the seeds of which contain 6-10% lipid, 44-59% carbohydrate and 20-30% protein (8, 9). In comparison with other legumes, fenugreek seeds contain higher ratios of minerals (i.e., Ca, P, Fe, Zn and Mn) (10). The seeds also contain some aromatic components such as n-alkanes, terpenes, nonalactone and saponins (11). Also, these seeds are known to be rich in polyphenolic flavonoids, which have antioxidant activities and protect the cellular structures from oxidative damage (12, 13). The present study aimed to determine the protective effects of fenugreek seed powder on oxidant and antioxidant factors in acetaminophen-induced liver toxicity.

Methods

In this experimental study, 24 male Wistar rats, weighing 250-280 g, were obtained from the animal house of Shahid Bahonar University of Kerman and were stored in the same center. The rats were stored under standard light, temperature and humidity and were given sufficient food and water a few days before the experiment. Fenugreek seeds were purchased from an apothecary shop and approved by a botanist. About 1000 mg/kg of fenugreek seed powder was dissolved in normal saline and administered in rats via gavage. Pure acetaminophen powder, which was provided by Darou Pakhsh Co. (Tehran, Iran), was dissolved in normal saline and administered via gavage. The rats were randomly divided into four groups of six rats:

Control group (C): The rats received normal saline via gavage after 24 hours of fasting. Six hours later, normal saline was administered via gavage.

Fenugreek seed powder group (T): After 24 hours of fasting, the rats received normal saline. Six hours later, fenugreek seed powder (1000 mg/kg) was administered via gavage.

Acetaminophen group (A): After 24 hours of fasting, the rats received 1000 mg/kg of acetaminophen via gavage. Six hours later, normal saline was administered.

Fenugreek seed powder+acetaminophen group (A+T): The rats received acetaminophen after 24 hours of fasting via gavage. Six hours later, fenugreek seed powder was administered via gavage. Twelve hours after the second gavage, the rats were anesthetized and sacrificed. A refrigerated centrifuge (Centrifuge 5804R, Eppendorf Company, Germany), a centrifuge device (BioChem Co., USA), a digital scale (HR-200)

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for weighing acetaminophen and fenugreek seed powder (with the accuracy of 0.0001 g), a digital scale (EK1200, Japan) for weighing the rats (with the accuracy of 0.01 g), a -75 °C freezer for storing the tissues and a spectrophotometer (Varian Cary® 50, Australia) were used in this study.

Evaluation of antioxidant factors: In this study, Bradford assay was applied to prepare the tissue extracts in order to measure the activities of catalase, peroxidase and proteins (14). In this assay, 0.5 g of the tissue was removed and homogenized in 3 ml of 50 mM phosphate-buffered saline (PBS), containing 1mM EDTA. Then, the obtained extract was centrifuged for 10 min at 1000 g in the refrigerated centrifuge.

Measurement of catalase activity: Catalase activity was measured by calculating H_2O_2 reduced absorption (H_2O_2 reduction) at 240 nm, using the Dhindsa and Motowe method (15). The reaction mixture contained 50 mM PBS (pH=7) and 15 mM hydrogen peroxide. By adding 100 mL of the tissue extract to the mixture, the reaction started. The absorbance changes were calculated after the onset of the reaction. The amount of hydrogen peroxide was calculated by using the extinction coefficient (€= 40 mM^{-1} cm^{-1}).

Evaluation of peroxidase activity: Peroxidase activity was measured by using guaiacol and measuring the absorbance rate of tetra-guaiacol, composed of guaiacol (produced by peroxidase activity) at 470 nm. The reaction mixture contained 50 mM PBS (pH=7), 0.3% hydrogen peroxide and 1% guaiacol. The reaction started by the addition of 20 mL of the enzyme extract to 2.5 ml of the reaction extract at 25 °C. The amount of produced tetra-guaiacol was calculated, based on the absorbance changes and the extinction coefficient of tetra-guaiacol (€= 25.5 mM^{-1} cm^{-1}) (16). The enzyme activity was reported according to the number of enzymes per mg of total protein in an appropriate volume of the obtained extract via Bradford method (14).

Measurement of protein concentration: Bradford method was applied to prepare the tissue extracts in order to measure catalase, peroxidase and protein levels (14). In this method, 0.5 g of the tissue was homogenized in 3 ml of PBS (50 mM). Then, the obtained extract was centrifuged for 10 min at 10,000 g in the refrigerated centrifuge. About 0.1 ml of the tissue extract and 5 ml of the biuret reagent were added to the test tube and were immediately vortexed to measure the protein concentration. After 25 minutes, the absorbance was determined at 595 nm. To prepare the biuret, 0.1 g of Coomassie Brilliant Blue G-250 was dissolved in 50 ml of ethanol 95% for one hour, and then, 100 ml of phosphoric acid 85% was added drop by drop. Finally, the total volume of the solution was increased to one liter by using distilled water.

The protein standard curve: To plot the protein standard curve, 1.4 g of standard bovine serum albumin was dissolved in one liter of distilled water and different concentrations (i.e., 50, 100, 200, 400, 700, 1400 and 2800 mg/L) were obtained. All processes were repeated by using biuret reagent, as previously explained for the unknown samples. The absorbance rate was determined at 595 nm by the spectrophotometer. To obtain different protein concentrations, the standard curve was plotted (fig 1).

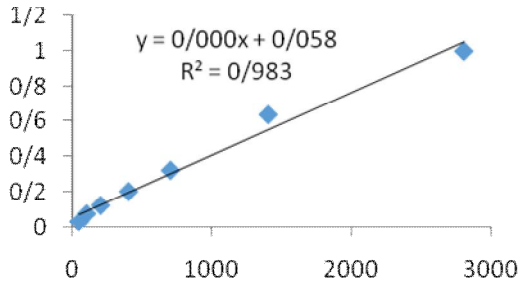

Figure 1. The protein standard curve

Measurement of hydrogen peroxide (H_2O_2): Measurement of H_2O_2 was performed by using the method proposed by Velikova and colleagues (17). About 0.1 g of the tissue was homogenized in 1 ml of trichloroacetic acid 0.1% (TCA) and the obtained extract was centrifuged for 10 min at 1000 g in the refrigerated centrifuge. Then, 0.5 ml of the centrifuged solution was added to 0.5 ml of PBS (10 ml, pH=7) and 1 ml of potassium iodide (1M). The absorbance rate was determined at 390 nm. The amount of

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hydrogen peroxide was calculated by the extinction coefficient (0.28mM-^{1}cm^{-1})and reported in micromoles per gram of tissue weight.

Measurement of malondialdehyde (MDA): The measurement of MDA concentration was performed by using Heath and Packer method (18). According to this method, 0.2 g of the tissue was homogenized in 5 ml of TCA 0.1% and the obtained extract was centrifuged for 10 min at 10,000 g. Four ml of 20% TCA solution, composed of 0.5% thiobarbituric acid (TBA), was added to 1 ml of the tissue extract and the obtained mixture was heated in a warm water bath for 30 min at 95°C. Then, it was cooled immediately in ice and centrifuged at 10,000 g for 10 min. The absorbance rate was determined by the spectrophotometer at 532 nm. The specified compound for the absorption of this wavelength is red complex (MDA-TBA). The absorption of non-specific pigments was determined at 600 nm and was subtracted from this amount. Finally, the extinction coefficient (155 mM-^{1}cm^{-1}) was used to calculate MDA concentration.

Data analysis: For data analysis, one-way ANOVA and Tukey's post-hoc test were performed, using SPSS. $p<0.05$ was considered statistically significant.

Results

Peroxidase: The peroxidase activity showed a significant reduction in group A (8.108±2.468), compared to group C (22.404±3.462) ($P<0.05$). Also, peroxidase activity in the A+T group increased, compared to group A, although the difference was not statistically significant (fig 2).

Catalase: Catalase activity showed a significant reduction in groups A(0.614±0.089)and T(0.346±0.0872), compared to group C(1.71±0.239) ($p<0.01$ and $p<0.001$, respectively). Also, catalase activity increased in the A+T group, compared to group A, although the difference was not significant (fig 3).

Hydrogen peroxide (H2O2): H_2O_2 concentration showed a significant increase in group T, compared to groups C, A and A+T($p<0.001$). Furthermore, there was a significant reduction in the A+T group (1.56±0.153), compared to group A(2.38±0.1048) (fig4).

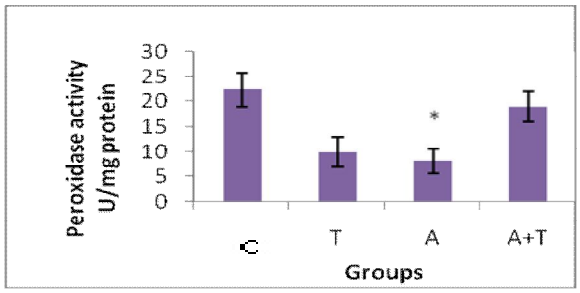

Figure 2. Assessment of changes in peroxidase activity in the groups

(Each column represents Mean±SEM, n=6) *A significant difference compared to group C ($p<0.05$) (C= control, T= fenugreek seed powder, A= acetaminophen, A+T= acetaminophen+fenugreek seed powder)

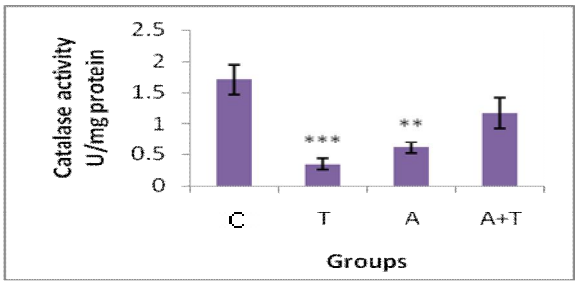

Figure 3. Assessment of changes in catalase activity in the groups

(each column represents Mean±SEM, n=6) **A significant difference compared to group C ($p<0.01$), ***A significant difference compared to group C ($p<0.001$) (C= control, T= fenugreek seed powder, A=acetaminophen, A+T= acetaminophen+fenugreek seed powder)

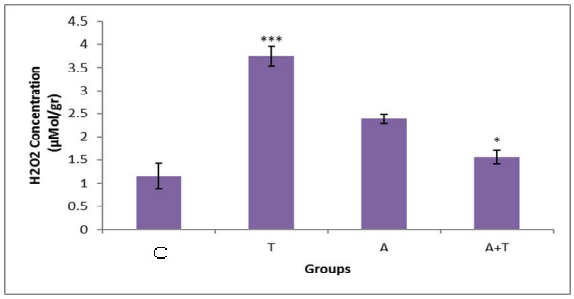

Figure 4. Comparison of changes in the concentration of H_2O_2 in all groups

(each column represents Mean±SEM, n=6) ***A significant difference compared to groups C, A and A+T ($p<0.001$), ***A significant difference compared to group A ($p<0.05$) (C= control, T=fenugreek seed powder, A=acetaminophen, A+T= acetaminophen+fenugreek seed powder)

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MDA: MDA concentration showed a significant increase in group A (0.2608±0.0301), compared to groups C (0.61±0.2017), A+T (0.2608±0.0301) and T (0.341±0.103) ($p<0.001$) (fig 5).

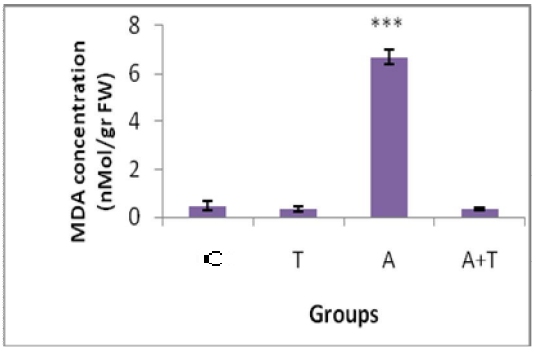

Figure 5. Comparison of changes in the concentration of MDA in the groups

(each column represents Mean±SEM, n=6) ***A significant difference compared to groups C, T and A+T ($p<0.001$) (C= control, T= fenugreek seed powder, A= acetaminophen, A+T= acetaminophen+fenugreek seed powder)

Discussion

This study showed that fenugreek seeds could significantly reduce changes in antioxidant factors (catalase and peroxidase), MDA level and hydrogen peroxide. Overall, acetaminophen can induce liver necrosis if used at high doses via over-production of NAPQI. NAPQI can form covalent bonds with liver membrane proteins, leading to the production of reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxides and hydroxyl radicals (19).In our study, similar to the results reported by Cheng et al. and Chen et al., an increase was observed in the level of H_2O_2 in rats receiving acetaminophen (20, 21). ROS can profoundly affect cell membranes and induce lipid peroxidation.

Lipid peroxidation is a marker of oxidative stress, which arises in the interaction between ROS and unsaturated fatty acids and leads to the formation of MDA and 4-hydroxynonenal, causing damage to cell membranes, cell necrosis and inflammation (22). In this study, a significant elevation was observed in lipid peroxidation in the liver of acetaminophen-treated rats (assessed by measuring MDA level), which was in agreement with the results reported by Saxena et al. (23). High doses of acetaminophen cause a significant reduction in the activity of antioxidants. Catalase and peroxidase are two enzymes responsible for the elimination of ROS, which catalyze the conversion of hydrogen peroxides to water. The decreased activity of these antioxidants may lead to increased use of superoxides and hydrogen peroxides, which in fact leads to the production of hydroxyl radicals (24). In the present study, a decline was observed in the activity of catalase and peroxidase in the liver of acetaminophen-treated rats.

The reduction in the activities of catalase and peroxidase in the liver of these rats was also confirmed in a study by Gupta et al. (25). Administration of 250 and 600 mm/kg of acetaminophen in rats leads to a decrease in cytosolic glutathione peroxidase (26). According to our study, 1000 mg/kg of fenugreek seed powder in acetaminophen-treated rats prevented the increase in the amount of hydrogen peroxide and MDA and the decrease in catalase and peroxidase activities in the liver of rats.

These results were in line with reduced oxidative stress reported in other studies and showed that fenugreek seed powder could significantly improve antioxidant activities in thioacetamide-induced hepatotoxicity and prevent the increased lipid peroxidation in rats (27).

In the present study, use of fenugreek seed powder in rats led to an increase in hydrogen peroxide, a slight decrease in MDA level and a decline in catalase and peroxidase activities in the N+T group, compared to the control group. These findings were in accordance with the results reported in a study by Sakr and colleagues. In their study, use of fenugreek seed powder led to decreased catalase activity and MDA level and an increase in the activity of superoxide dismutase, leading to H_2O_2 production (28). Mitchell et al. showed that fenugreek seeds are rich in polyphenols, which promote the antioxidant capacity (12). Polyphenols, especially flavonoids, are among the most powerful herbal antioxidants. Polyphenols can form complexes with reactive metals such as iron, zinc and copper and reduce their absorption (29). High

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levels of these elements (metal cations) in the body can lead to the production of free radicals and cause oxidative damage in cell membranes and DNA (30). Furthermore, polyphenols function as free radical scavengers and neutralize these elements before they damage the cells (31).

Other plants such as turmeric (32), ginger (32) and soy milk (33) are rich in flavonoids. The efficacy of soy milk in liver damage was assessed in a study by Yakubu and colleagues. The results revealed that this plant can prevent liver damage caused by oxidative stress (33). Also, flavonoids can exert pro-oxidant effects including the formation of free radicals instead of scavenging them (34). In a previous study, flavonoids such as apigenin and naringenin with a phenol-type substitution pattern in B-Ring led to an increased risk of the formation of reactive oxygens (30-50 times), when incubated in the presence of glutathione, thymus and bone marrow enzymes such as peroxide (35). It has been observed that these flavonoids can act as pro-oxidants and cause lipid peroxidation at concentrations which other flavonoids are still active as antioxidants and obstruct lipid peroxidation.

Chemical and enzymatic oxidation of flavonoids leads to the production of toxic flavonoid quinone radicals, which may be scavenged by glutathione; therefore, flavonoids are reconstructed and thiyl radical are formed from glutathione. Thiyl radicals may react with glutathione to produce a disulfide radical anion, which rapidly reduces molecular oxygen to superoxide anion radicals. Another mechanism of pro-oxidant activity of flavonoids is related to the formation of oxidation products (quinine type). This mechanism occurs especially when three or four subunits of catechol are present in flavonoid molecules. Oxidation of catechols to quinones can produce powerful electrophiles, leading to DNA alkylation (36).The obtained results showed that fenugreek seeds can lead to a reduction in oxidative stress in liver damage induced by acetaminophen. It is possible that the efficacy of fenugreek seed powder in the increased antioxidant defense be related to flavonoid compounds.

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