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Favorable effects of *Anethum graveolens* on liver oxidative stress and cholesterol 7 alpha-hydroxylase levels in non-alcoholic fatty liver disease (NAFLD) rat models

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ABSTRACT

Background: High-fat high-cholesterol diet induces a phenotype similar to non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) in humans. In NAFLD and NASH, cholesterol and bile acid metabolisms are impaired to accumulate lipids and toxic bile acids along with cholestatic hepatic damage. Recently, the use of herbal-derived cholesterol lowering products has attracted much attention as possible therapeutic strategies for NAFLD. Hence, the aim of this study was to determine the effects of an Anethum graveolens (dill) on liver cholesterol 7 alpha-hydroxylase and liver fat accumulation in rats.

Method: Thirty-six rats were randomly divided into 6 groups (n = 6) and received normal diet (ND) or a mixture of chow diet+2% cholesterol+0.5% cholic acid + 20% corn oil as high cholesterol/fat (HC–HF) diet (NAFLD model). Animals were also treated daily with dill tablet or dill extract (300 mg/kg). At the end of the 30 days experiments, serum and liver lipid profile and liver total antioxidant capacity were determined. Cholesterol 7 alpha-hydroxylase mRNA and protein expression levels were determined in the liver and histopathological changes in liver tissues were analyzed by microscope.

Results: Lipid profiles significantly decreased in dill treated groups ($p < 0.05$). Liver total antioxidant capacity significantly ($p < 0.05$) increased and MDA levels markedly ($p < 0.05$) reduced both in dill tablet and dill extract treated groups ($p < 0.05$). Both types of treatments caused significant increases in liver cholesterol 7 alpha-hydroxylase gene expression ($p < 0.05$). Histopathological examinations showed that treatment with dill normalized the hypercholesterolemia-induced changes in liver histology.

Conclusion: Administration of dill significantly reduced liver fat, oxidative stress and increased cholesterol 7 alpha-hydroxylase enzyme at the both mRNA and protein levels. Dill extract was found more effective than its commercially available tablet.

1. Introduction

The significant role of a high cholesterol diet has been studied comprehensively in animal models and humans. Experiments in animals have shown that dietary cholesterol is involved in the prevalence of several chronic diseases, including hyperlipidemia, non-alcoholic fatty liver disease (NAFLD) and cardiovascular disease (CVD). These diseases share hepatic homeostasis derangement, which may be driven by differential expression of key genes in lipid metabolism [1,2]. In NAFLD and non-alcoholic steatohepatitis (NASH), lipid and bile acid metabolism are impaired and, consequently lipids and toxic bile acids accumulate in the liver. Cholesterol 7α-hydroxylase (CYP7A1) has a vital role in regulation of cholesterol and bile acid metabolisms. It has been shown that overexpression of this enzyme effectively decreased liver oxidative stress and cholesterol level, and reversed hepatic inflammation and fibrosis in animal models [1,3]. Li et al. showed that transgenic overexpression of cholesterol 7α-hydroxylase in the liver inhibits high-cholesterol/high-fat diet-induced obesity and reduces

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inflammation in the liver [4].

High cholesterol-high fat diet (HC–HF) is accompanied with augmented reactive oxygen species (ROS) and accumulation of lipid peroxides. Hence, ROS participates in the etiology of NAFLD by motivating antioxidant depletion and inducing of inflammation and lipid peroxidation. It has been established that feeding animals a high fat diet induces liver steatosis and liver injury, which are characteristic of NAFLD and consequently offers an appropriate model for the initial stages of the disease [5,6]. Increased triglyceride, cholesterol levels and oxidative stress are chief factors in the prevalence of NAFLD induced by HC-HF diet [7]. In this context, dietary antioxidants have key roles in the prevention of various human disorders, including metabolic syndrome, diabetes, NAFLD, and cardiovascular disease [8].

Previous evidence showed that antioxidant supplements have significant role in the prevention and treatment of NAFLD [5] and Epidemiological studies have reported that fruits, herbs, and spices are associated with lower risks of this disease [8]. The use of herbal medicine as substitute or complementary treatment for health problems have recently been increased worldwide [9]. *Anethum graveolens* (dill) is one of the traditional herbal plant with many useful effects [10,11]. Dill is recognized as a rich source of phenolic, flavonoids, saponin, cardiac glycosides and terepene [12,13]. These components have potential antioxidant and hypolipidemic effects [13,14]. It has been established that administration of essential oil, leaf and seed of dill in animal models considerably normalized lipid profile including TG, TC, LDL-C, VLDL-C, and restored glucose levels and significantly raised high-density lipoprotein (HDL) cholesterol levels [15,16]. Dill has also shown potential antioxidant and antiradical activities. Although there is substantial evidence to support the beneficial effects of dill in the treatment of hypercholesterolemia, the exact mechanism for the hypolipidemic properties of dill has not been explained so far. Therefore, the present study was aimed to investigate the effects of dill tablet and hydroalcoholic extract of dill on liver cholesterol 7 alpha-hydroxylase gene expression in order to reveal a mechanism for the hypolipidemic effects of dill.

2. Methods

2.1. Preparation of hydroalcoholic extract

The leaves of dill were obtained from a local market and botanical identification and authentication was confirmed. The leaves of dill were air-dried at room temperature and crushed and macerated. One hundred gram of powder was defatted with 99% ethanol and water in a 1: 1 proportion for 48 h at room temperature. The samples were filtered 3 times through Whatman No.2 filter paper and evaporated under the vacuum at 40 °C. Extracts were stored in sealed vials at −20 °C until further analysis. Dill tablet was purchased from Iran Darouk Company (Tehran, Iran).

2.2. Animal model

Wistar male rats weighting about 220 g (8 weeks old) were obtained from the Animal House of Hamadan University of Medical Sciences (Hamadan, Iran). Animals were kept under room temperature, 65 ± 5% humidity, and 12-h light/dark cycles. After 7 days, animals were randomly divided into 6 groups ($n = 6$ for each group) based on our previous works [17,18]. Rats received standard chow diet as normal diet (ND) or a mixture of chow diet+2% cholesterol+0.5% cholic acid + 20% corn oil as high cholesterol/fat (HC–HF) diet. Animals were also treated daily with dill tablet (300 mg/kg) or dill extract (300 mg/kg) as following; control group: (ND), group 2: (HC–HF), group 3: (HC–HF + dill tablet), group 4: (HC–HF + dill extract), group 5: (ND + dill tablet), and group 6: (ND + dill extract). Rats were received the dill tablet and dill extract daily via oral tubing. At the end of one month treatment, animals were anesthetized and sacrificed. Blood samples were collected after an overnight fasting from the heart and centrifuged at 4000×*g* for 10 minutes to obtain serum. Liver tissues were dissected and stored in liquid nitrogen for further analysis. All steps of this experiment were approved by the ethical committee of Hamadan University of Medical Sciences and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (1996) was considered in all experiments and animal care procedures. The minimum number of animals was used, safety procedures were compiled in all mandatory experimental works, and minimum suffering of animals was considered in all experiments. The concentration of dill tablet (300 mg/kg) which was used in the present study was based on the previously described optimal concentration [13,18]. Other report indicated that the 300 mg/kg dill hydroalcoholic extract is effective dose [17]. Hence in this experiment we used 300 mg/kg dill and hydroalcoholic extract.

2.3. Determination of biochemical parameters

The levels of fasting blood sugar (FBS), serum total cholesterol, and triglyceride were measured using colorimetric commercial kits. LDL-C and VLDL-C were calculated using following equations, respectively: LDL-C = TC –HDL-C – TG/5, and VLDL = TG/5.

2.4. Liver sampling and antioxidant test

To investigate the antioxidant status, 500 mg of liver tissues were homogenized in lysis buffer (Sigma-Aldrich, USA) at 4 °C. The samples centrifuged at 4000×*g* and 4 °C for 10 min and supernatant were used for protein determination by Bradford method. The amount of Malondialdehyde (MDA) was determined calorimetrically using thiobarbituric acid assay [19] and total antioxidant capacity (TAC) was measured using ferric ion reducing antioxidant potential (FRAP) method with some modifications [20,21].

2.5. Liver lipids levels

To determine the effects of dill tablet and dill extract on liver cholesterol and triglyceride content, 500 mg of liver tissues were homogenized in 10 ml of 1:2 v/v mixtures of methanol and chloroform at 4 °C. The homogenates were centrifuged and then lower layer used to determine the TC and TG levels according to the previously published papers [22,23].

2.6. Histopathological examination of liver

To study possible morphological changes caused by hypercholesterolimia, the liver tissues were fixed in 10% formalin, embedded in paraffin, and sectioned into 5 μm thicknesses using a microtome. Slides were stained by hematoxylin and eosin [24] and analyzed using light microscope (Olympus, Tokyo, Japan). Histopathological scoring system for NAFLD was done according to previous published paper [25].

2.7. Quantitative real-time polymerase chain reaction (qRT-PCR)

The cholesterol 7 alpha-hydroxylase gene expression levels were measured in liver tissues by qRT-PCR. Total RNA from frozen tissue samples were extracted using Trizol reagent (Invitrogen Co. USA). RNA was quantified using NanoDrop™ One^{C} (Thermo Fisher Scientific, USA) and its quality and integrity were confirmed by 1% agarose gel electrophoresis. Specific primers were designed using AlleleID7.6 software as forward: 5′-GGGCAGGCTTGGGAATTTTG-3′ and reverse: 5′-AGTGAGCATTGGTCCCGAAG-3′ primers for cholesterol 7 alpha-hydroxylase and as forward: 5′-GTAACCCGTTGAACCCCATT-3′ and reverse: 5′-CCATCCAATCGGTAGTAGCG-3′ primers for 18S-RNA housekeeping gene. One microgram of total RNA was reverse transcribed into cDNA using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas Co. Lithuania) and a Bio-Rad thermocycler (Bio-Rad Laboratories, USA) [26,

27]. Finally, relative gene expression of cholesterol 7 alpha-hydroxylase was calculated by the $2^{-\Delta\Delta Ct}$ method [17].

2.8. Protein levels of cholesterol 7 alpha-hydroxylase

Cholesterol 7 alpha-hydroxylase protein level was determined in liver tissue samples using ELISA kit according to the manufacturer's instruction (Eastbiopharm Co. China).

2.9. Statistical analysis

Statistical analysis was carried out using the Statistical Package for Social Sciences version 16 (SPSS Inc., Chicago-USA). Values were presented as mean ± SD and a one-way ANOVA followed with post hoc Tukey test was used for comparison between the groups. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Serum lipid profile and FBS

FBS did not differ between groups and all animals had almost a similar level of fasting blood glucose (Table 1). The HC-HF received animals showed significant raises in the serum TC, TG, and LDL-C levels, as well as marked reduction in HDL-C level. Those animals received HC-HF diet and treated with dill extract or dill tablet showed significant ($p < 0.01$) reduction in TC, TG, LDL-C, and VLDL-C levels accompanied with an increase in HDL-C compared with non-treated HC-HF group (Table 1).

3.2. Liver lipids

Measurement of liver lipids in HC-HF group showed significant increases in cholesterol and triglyceride accumulation while, treatment by 300 mg/kg dill extract or dill tablet significantly reduced cholesterol and triglyceride levels (Fig. 1).

3.3. Antioxidant enzymes activity

Administration of dill extract or commercial dill tablet at the dose of 300 mg/kg augmented total antioxidant capacity compared with the control group.

MDA level, as a marker of lipid peroxidation, was higher in HC-HF fed animals compared with chow diet fed animals ($p < 0.05$). Administration of dill extract or dill tablet reduced the MDA level in HC-HF treated animals compared with untreated HC-HF fed rats (Fig. 2).

3.4. Gene expression levels of cholesterol 7- alpha hydroxylase

HC-HF fed rats displayed significant reduction in cholesterol 7-alpha hydroxylase gene expression in comparison with control animals ($p < 0.001$). Whereas, animals treated with dill extract or dill tablet (300 mg/kg) had significant increase in cholesterol 7-alpha hydroxylase gene expression compared with HC-HF group (Fig. 3).

3.5. Protein levels of cholesterol 7- alpha hydroxylase

Similar to the reduced gene expression, HC-HF fed animals showed significant decrease in cholesterol 7 alpha-hydroxylase protein concentration compared with normal animals ($p < 0.001$). Administration of dill extract or dill tablet significantly increased cholesterol 7 alpha-hydroxylase protein levels compared with HC-HF fed animals ($p < 0.001$) (Fig. 4).

3.6. Histopathological analysis

The microscopic analysis of the liver of the HC-HF group showed that (Fig. 5) the euchromatin nuclei has lost their structure, and the regular hepatic structure which is seen in the normal state, has been altered in the HC-HF group. HC-HF induced the incidence of fatty liver, but dill extract or dill tablet reduced the pathogenesis. Compared to the normal group, a mild infiltration of inflammatory cells was seen in the HC-HF group. Infiltration of macrophage, mild fibrosis and steatosis as well as change of sinusoid structure, mild congestion, Kupffer cell hyperplasia, and increased foam cells were observed in HC-HF group compared with the normal group. Furthermore, the HC-HF rats showed hepatic steatosis with ballooning degeneration, lipid droplet accumulation, and inflammatory cell infiltration (Fig. 5). However, treatment with dill extract or dill tablet normalized all of these alterations in HC-HF group. Treatment of normal animals had no harm effects on liver structure (Fig. 5). Following H&E staining, the nonalcoholic fatty liver disease activity score (NAS) was calculated which showed a significantly higher NAS scores in HC-HF rats compared with the other groups (Fig. 6).

4. Discussion

Dietary high cholesterol is involved in the prevalence of non-alcoholic fatty liver disease [1,2] where lipid and bile acid metabolism are impaired and, consequently lipids and toxic bile acids accumulate in the liver. Cholesterol 7α-hydroxylase (CYP7A1) has a vital role in regulation of cholesterol and bile acid metabolisms and it has been shown that overexpression of CYP7A1 effectively decreases liver oxidative stress and cholesterol level, and reverses hepatic inflammation and fibrosis [1,3]. Thus, in the present study we aimed to investigate the effects of dill tablet and hydroalcoholic extract of dill on liver cholesterol 7 alpha-hydroxylase gene expression in order to reveal a mechanism for the hypolipidemic effects of dill.

Rats which received one-month hyperlipidemic and hypercholesterolemic diet [containing corn oil (20%) and cholesterol (2%)] developed hepatic steatosis and dyslipidemia, as confirmed by histopathological examinations. Rats also showed elevated serum TC, TG, LDL-C, HDL-C, or liver lipid profile.

The present study showed marked declines in serum cholesterol, TG and LDL-C levels and a significant increase in HDL-C level in rats when received 300 mg/kg dill tablet or hydroalcoholic extract. It is well recognized that high levels of total cholesterol (TC) and triglyceride are the main risk factors for atherosclerosis, NAFLD, and cardiovascular-related mortality and morbidity worldwide [7,28]. Previous studies

Table 1
Effect of dill (Anethum) tablet and hydroalcoholic extract of dill on glucose levels and lipid profiles.

Groups	FBS (mg/dL)	Triglyceride (mg/dL)	Cholesterol (mg/dL)	LDL-C (mg/dL)	VLDL-C (mg/dL)	HDL-C (mg/dL)
Control	73.14 ± 4.95	100.29 ± 8.48*	69.00 ± 6.73*	6.94 ± 4.55*	20.05 ± 1.69*	6.94 ± 4.55*
HC-HF	76.00 ± 8.72	138.57 ± 12.43#	197.43 ± 10.19#	134.57 ± 10.42#	27.71 ± 2.48#	134.57 ± 10.42#
HC-HF + Dill tablet	74.57 ± 5.09	104.86 ± 7.13*	111.57 ± 4.96*	32.42 ± 5.66*	20.97 ± 1.42*	32.42 ± 5.66*
HC-HF + Dill extract	74.43 ± 6.18	65.43 ± 5.16*	108.00 ± 7.76*	45.75 ± 10.74*	11.14 ± 1.22*	45.75 ± 10.74*
Control + Dill tablet	72.14 ± 11.09	80.14 ± 6.12*	76.71 ± 2.49*	51.71 ± 3.9*	16.03 ± 1.22*	8.97 ± 4.71*
Control + Dill extract	75.57 ± 7.18	89.71 ± 9.60*	80.86 ± 9.42*	19.91 ± 8.32*	17.94 ± 1.92*	19.91 ± 8.32*

HC-HF; high cholesterol-high fat diet, TC; total cholesterol, TG; Triglycerides, HDL; high-density lipoprotein, LDL; low-density lipoprotein, VLDL; very low-density lipoprotein. Data were presented as the mean ± S.D. "*" represents $p < 0.001$ compared with HC-HF group and "#" indicates $p < 0.001$ compared with normal group.

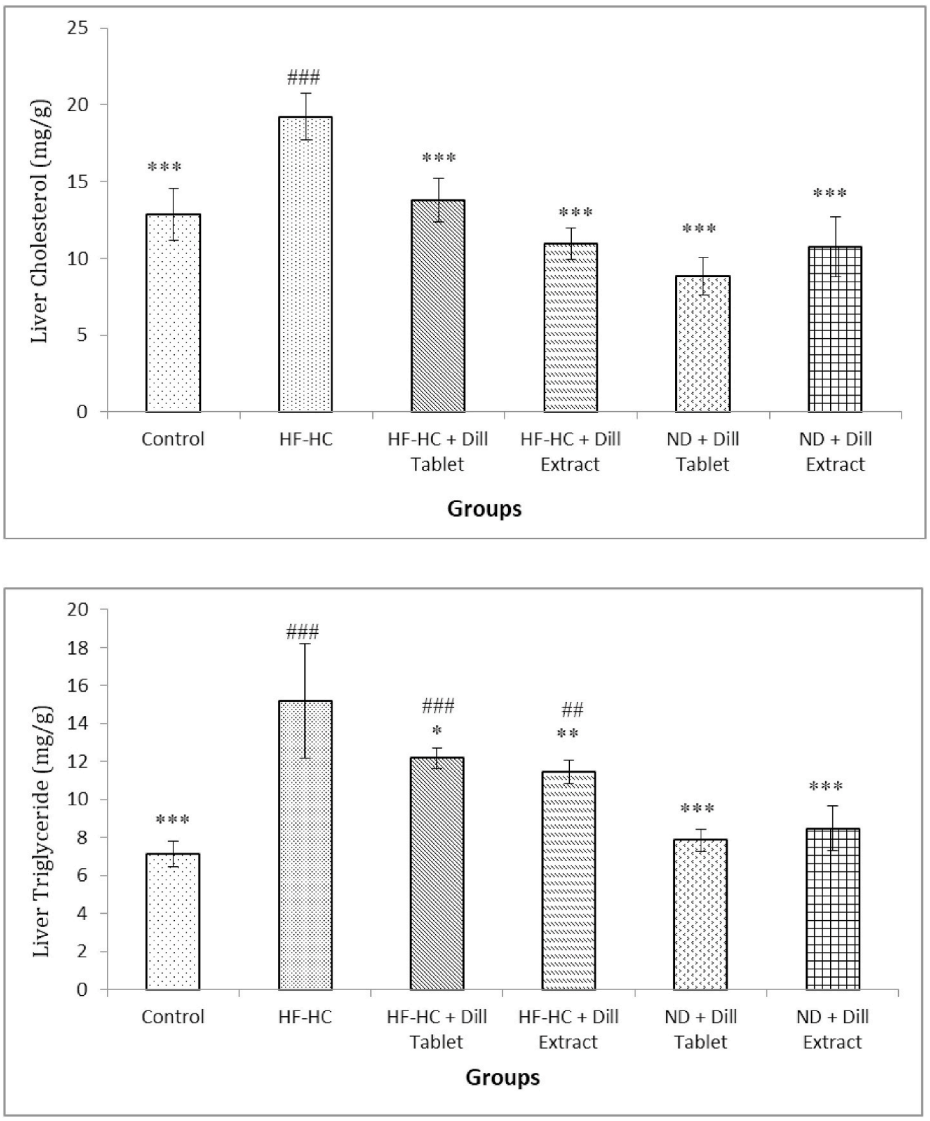

Fig. 1. Effect of *Anethum* extract and dill tablet on liver cholesterol (top) and triglyceride contents (bottom). Dill tablet and hydroalcoholic extract of dill significantly reduced liver triglyceride (TG) and cholesterol in HC-HF group. Data were presented as the mean ± S.D. ***$p < 0.001$, ** $p < 0.01$ and *$p < 0.05$ compared with HC-HF group. $^{###}p < 0.001$ and $^{##}p < 0.01$ compared with normal group. HC-HF; high cholesterol-high fat diet, ND: normal diet.

[11,17] have established that the hydroalcoholic extract is more useful than the extracts obtained other fractions of plant. In agreement with the previous literature [29,30], our results showed no significant differences in FBS levels.

The present experiment investigated the effects of dill on CYP7A1 mRNA and protein expression levels in high cholesterol fed rats and the result showed that dill significantly augmented this enzyme level. Cholesterol 7a-hydroxylase is known as the initial and rate-limiting step in bile acid synthesis pathway, as the main root for cholesterol catabolism and excretion [31]. The rate of cholesterol excretion is a vital factor in disorders such as atherosclerosis and fatty liver [31]. Here for the first time, we reported beneficial effects of dill in lowering liver cholesterol and triglyceride, at least in part by conversion of cholesterol to bile acid via increasing 7α-hydroxylase gene expression and protein levels. Increase of CYP7A1 indicated that dill raises the conversion of cellular cholesterol to bile acids, which may explain its cholesterol-lowering properties.

Some studies have shown that flavonoides including carvon, limonene, α-phellandrene probably are responsible for hypolipidemic and antioxidant effects of dill by inhibition of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) or Acetyl-CoA carboxylase (ACC), the key enzyme in metabolism of cholesterol [32]. It has been proposed that dill components may also stimulate LDL clearance by increasing of hepatic LDL receptors [33]. Other researchers suggested declining of intestinal cholesterol absorption or decreasing the synthesis of fatty acid [33], and improvement of lipoprotein metabolism [15]. Some others, have suggested that antioxidant effects of dill are responsible for hypolipidemic effect [34]. Studies reported that dill has hepatoprotective effect probably due to polyphenol contents [35]. We have previously shown that this plant have significant antiradical, antioxidant and antiglycation effects [13,19,23,36].Chemical compounds of dill may also be accountable for hypoglycemic effects of dill [37].

Various herbal medicines have been known to exhibit hepatoprotective properties by increasing cellular antioxidant capacity [9,37]. In this study, we examined the antioxidant capacity in the liver of dill treated animals, and showed potential rise in total antioxidant levels and reduction of malondialdehyde (MDA), compared with those of untreated HC-HF fed rats. Total antioxidant capacity significantly reduced in HC-HF treated animals while dill administration augmented this marker. On the other hand, high cholesterol diet exhibited its toxic properties by increasing lipid peroxidation (as determined by MDA level) probably through enhancement in generation of ROS [31].

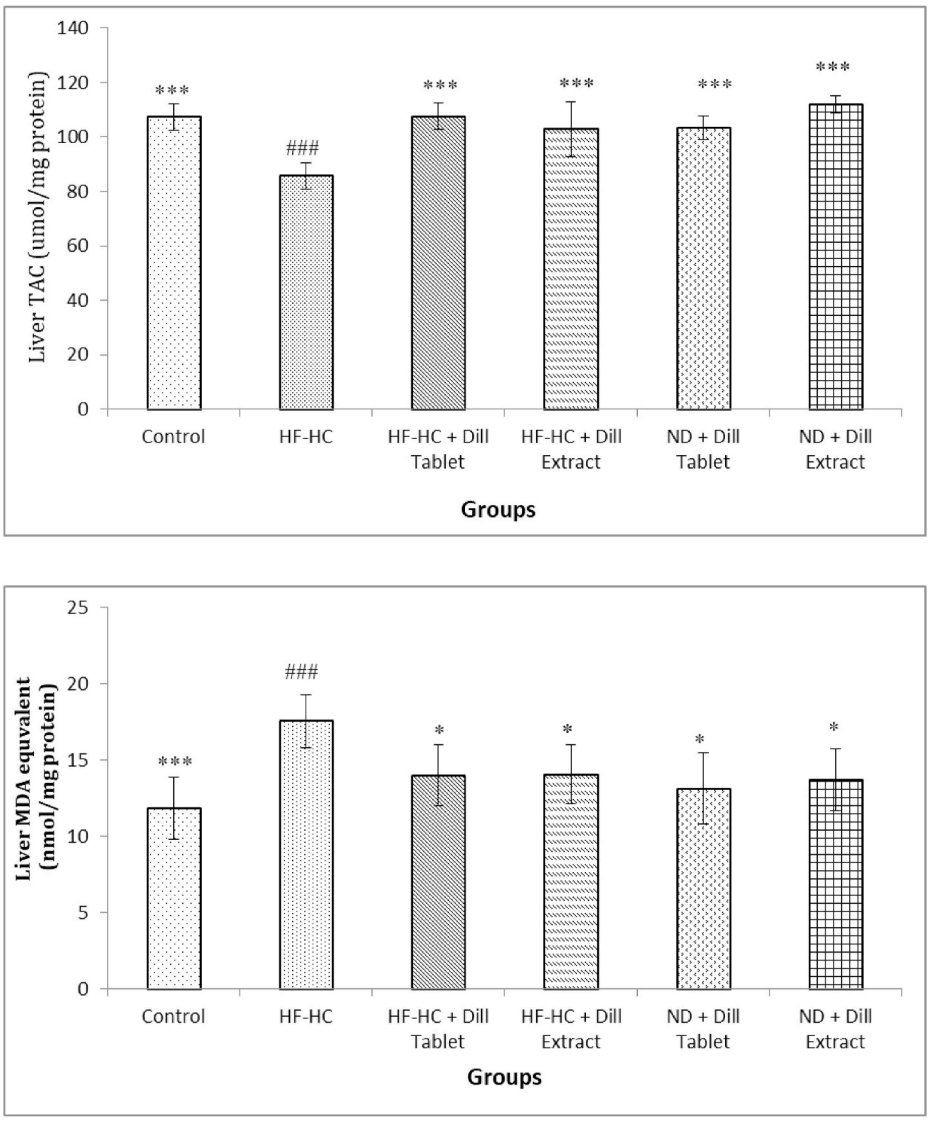

Fig. 2. Effect of *Anethum* extract and dill tablet on liver total antioxidant capacity (top) and malondialdehyde levels (bottom). Dill tablet and hydroalcoholic extract of dill significantly increased TAC and decreased MDA levels in HC-HF group. All values are presented as mg/gram tissue. Data were presented as the mean ± S.D. ***$p < 0.001$ and *$p < 0.05$ compared with HC-HF group. $^{###}p < 0.001$ compared with normal group. HC-HF; high cholesterol-high fat diet, ND: normal diet.

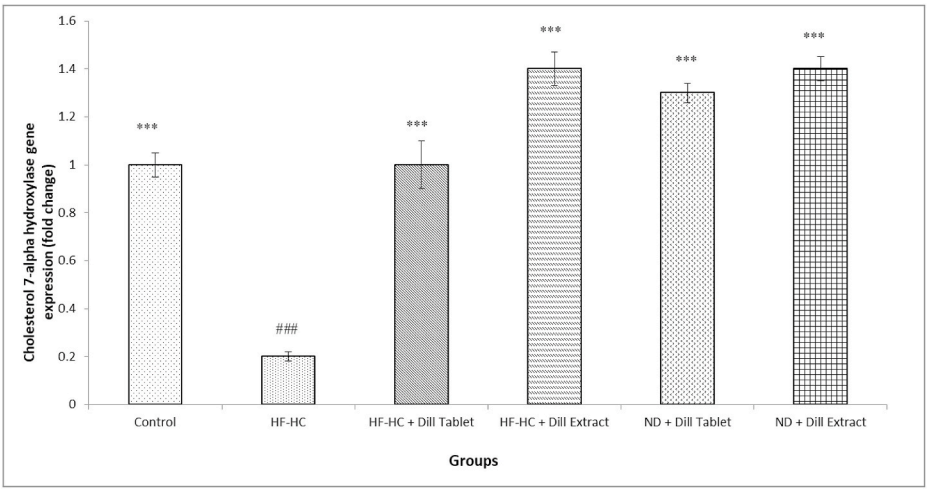

Fig. 3. Effect of *Anethum* extract and dill tablet on cholesterol 7- alpha hydroxylase gene expression in different group. Data were presented as the mean ± S.D. ***$p < 0.001$ compared with HC-HF group and $^{###}p < 0.001$ compared with normal group. HC-HF; high cholesterol-high fat diet, ND: normal diet.

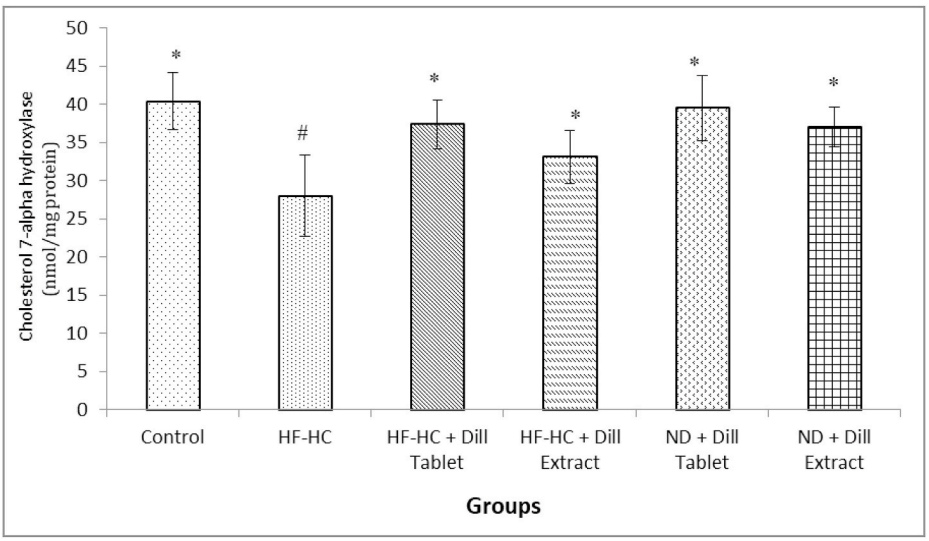

Fig. 4. Effect of *Anethum* extract and dill tablet on cholesterol 7 alpha-hydroxylase protein level. Dill tablet and hydroalcoholic extract of dill significantly increased this protein concentration in HC-HF group compared with untreated animals. Data were presented as the mean ± S.D. ***p < 0.001, ** p < 0.01 and *p < 0.05 compared with HC-HF group. #p < 0.05 compared with normal group. HC-HF; high cholesterol-high fat diet, ND: normal diet.

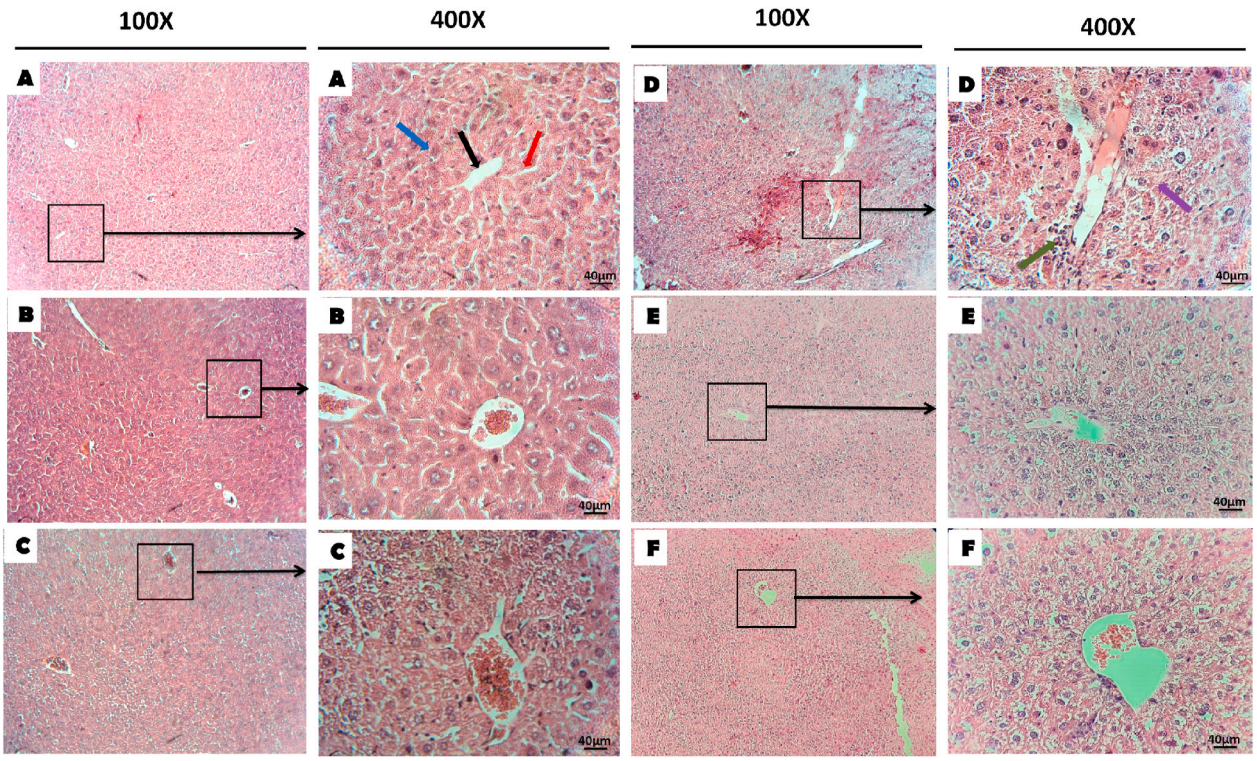

Fig. 5. Effect of *Anethum* extract and dill tablet on liver morphology based on H&E staining. histopathological examination of liver showed that treatment of HC-HF treated animal with dill (Anethum) extract and tablet alleviated structural changes compared with untreated rats (magnification 100 × and 400 ×). Black arrow: central vein, red arrow: sinusoidal space, blue arrow: hepatocyte, purple arrow: foam cell, green arrow: necrotic cell. A: Control, B: Control + dill tablet, C: Control + dill extract, D: HC-HF, C: HC-HF + dill tablet, F: HC-HF + dill extract. HC-HF; high cholesterol-high fat diet. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Numerous studies have also established high MDA concentration in HC-HF fed animals [38]. Increased MDA level could be responsible for some of the pathological properties in the hypercholesterolemia. In this experiment, we have shown a significant rise in lipid peroxidation in liver of HF-HC fed rats [39]. In contrast, dill administration markedly reduced the level of MDA in HF-HC fed animals. It was reported that dill, which has D-limonene, alpha-pinene, isoliquertigenin and quercetin has potential antioxidant activity [11]. Moreover, the authors of this paper previously showed that dill significantly augmented antioxidant capacity in the diabetic animals [11,18,36].

Analysis of histopathological changes of liver showed hepatic steatosis and oxidative damage in a HC-HF fed animals. HC-HF fed animals showed numerous histological changes including congested central veins, foam cell formation, vacuolated hepatocytes and necrosis.

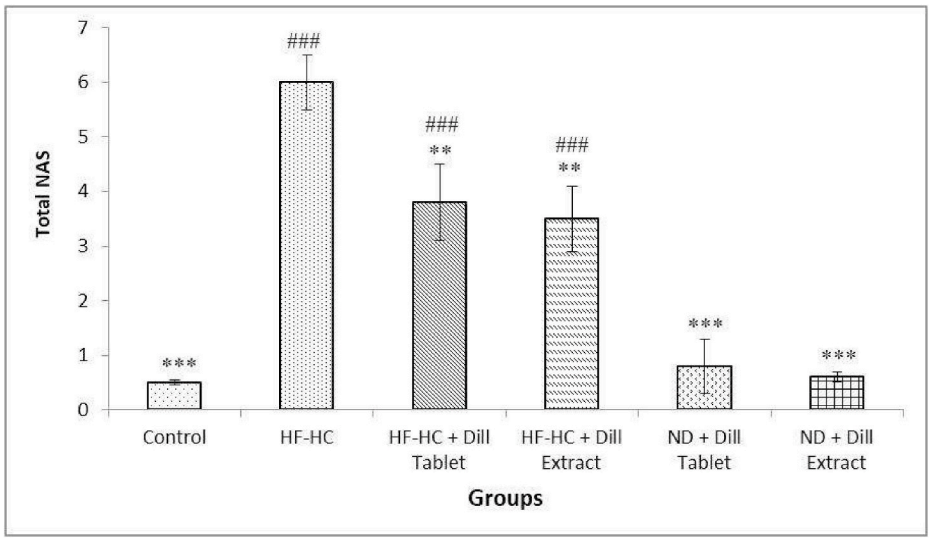

Fig. 6. Effect of *Anethum* extract and dill tablet on liver morphology and nonalcoholic fatty liver disease activity score (NAS).

However, treatment of HC-HF fed animal with dill tablet or hydro-alcoholic extract remarkably normalized morphological changes in liver. Therefore, it can be assumed that dill administration can provide hepatoprotection against high cholesterol diet-induced harmful damages to the liver tissue.

In conclusion, this experiment showed that dill is a valuable edible vegetable that ameliorated fatty liver and reduced cholesterol levels, with no detrimental effects on rats. Serum cholesterol and LDL-C reductions were associated by rises of CYP7A1 levels.

Availability of data and material

Data are available upon reasonable request.

CRediT authorship contribution statement

Ebrahim Abbasi: Conceptualization, Data curation, Methodology, Writing – original draft. **Mohammad Taghi Goodarzi:** Project administration, Supervision, Data curation. **Heidar Tayebinia:** Project administration, Supervision, Data curation. **Massoud Saidijam:** Project administration, Supervision, Data curation. **Iraj Khodadadi:** Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

The authors report no conflict of interest.

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