Thymol decreases apoptosis and carotid inflammation induced by hypercholesterolemia through a discount in oxidative stress

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Abstract
Objective: Atherosclerosis sclerosis is a chronic inflammatory disease that can lead to cardiovascular and cerebrovascular disorders that are generally along with hypercholesterolemia and oxidative stress. Various surveys have shown that thymol is a polyphenolic compound with anti-inflammatory and antioxidant properties. This study aimed to investigate the anti-inflammatory and antiapoptotic effects of thymol on carotid tissue of hypercholesterolemic rats.
Materials and Methods: Forty male Wistar rats were randomly divided into 4 groups with 10 members each (n = 10): a control group with a normal diet (ND), a group with a high-cholesterol (2%) diet (HD), a group with a high-cholesterol diet combined with thymol (24 mg/kg HD + T), and a group with a thymol diet (T). After preparing serum from peripheral blood of rats, lipid measurements were obtained, including total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglycerides (TG), by using a colorimetric method; the levels of oxidized LDL (OxLDL) were obtained through enzyme-linked immunosorbent assay (ELISA). The activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) antioxidant enzymes, as well as the concentrations of malondialdehyde (MDA) and serum total antioxidant capacity (TAC), were determined with the use of colorimetric methods. The protein expressions of Bcl2 and cleaved caspase 3 and the phosphorylation of p38 mitogen-activated protein kinase (MAPK) in rat carotid tissue were determined by an immunoblotting method.
Results: The rats fed with a high-cholesterol diet for 8 weeks increased TC and OxLDL in HD group compared with the ND group ($P<0.01$; OxLDL HD vs ND (214.42 ± 17.46 vs 69.13 ± 9.92; $P<0.01$); (229.39 ± 13.26 vs 67.89 ± 5-14 (215.58 ± 12.46 vs 229.35 ± 13.26; $P<0.05$, OxLDL HD vs HD + T 105.53 ± 10.44; $P<0.01$). Both of them were decreased with the intervention of thymol in the HD + T group compared with the HD group.
The amount of phosphorylation of p38 (p-p38) and the protein expression of cleaved caspase 3 showed a significant increase in the HD group compared with the ND group ($P<0.01$). In contrast, the expression of Bcl-2 in the high-cholesterol diet group decreased compared with the control group ($P<0.01$). A comparison of the p-p38 and the protein expression of cleaved caspase 3 between the T + HD and the HD groups showed that in both cases, thymol caused a decrease (p<0.01), whereas Bcl-2 effected an increase ($P<0.05$). Regarding the oxidant and antioxidant indices, thymol significantly decreased the MDA level and increased the total antioxidant content ($P<0.01$).
Conclusion: The results of this study indicate that thymol significantly decreases the expression of inflammatory and apoptotic proteins in carotid tissue. However, this decrease is probably not mediated by an effect on lipid metabolism because thymol decreases the total level of cholesterol but has no significant effect on the LDL-C level as the atherogenic index. In addition, thymol possibly exerts an antioxidant effect without the direct involvement of antioxidant enzymes.
Keywords: Atherosclerosis, Bcl2 protein, Cholesterol, Cleaved caspase 3, p38 mitogen-activated protein kinase

Introduction

Oxygen molecules exposed to different radiation and stress by taking an electron from other molecules, cause damage other molecules, cells and DNA. This activity can result in various diseases, including cancer, brain damage, and heart disease, such as atherosclerosis sclerosis, as well as weakness of the immune system. The production of reactive oxygen species (ROS), even in normal conditions, is inevitable in metabolic pathways; however, the antioxidants available in the body and obtained in the diet neutralize the effects of ROS (1).

Hypercholesterolemia is directly and closely related to atherosclerosis (2,3) and is characterized by increasing plasma concentrations of low-density lipoprotein (LDL), and triglycerides (TG). The accumulation of LDL in the intima and their oxidation lead to the formation of atherosclerotic plaques, which is one the most common causes of clogged arteries, heart attacks and strokes, and cardiovascular diseases (CVDs) (4-7). In fact, the damage caused by atherosclerosis represents a wide range of particular cellular and molecular responses that, as a whole, can be regarded as inflammatory diseases (5). The

oxidation of LDL by ROS leads to production of oxidized LDL (OxLDL), releasing phospholipids, peroxidation of lipids and proteins, and production of malondialdehyde (MDA).

Activation of endothelial cells is more in areas of vessel that are under the hemodynamic pressure. OxLDL is the simulator of inflammatory reactions (5,8), which attracts blood monocytes, and calls and reproduces macrophages (5,9,10). This oxidative stress leads to the consumption of the support recovery substance of the antioxidant system, decreasing the level of glutathione and the activity of antioxidant enzymes, such as glutathione peroxidase (GPx), catalase, and SOD. Increasing lipid peroxidation and/or decreasing antioxidant protection have been observed in metabolic diseases and unhealthy lifestyles and can cause endothelial dysfunction and atherosclerosis. Clinical trials have shown that increasing ROS decreases antioxidant formation, especially in patients with coronary artery disease (CAD) (11,12). The oxidative stress stimulates inflammatory reactions and plays an important role in the progression of atherosclerosis by launching the signaling (13,14).

Protein of p38 mitogen-activated protein kinase (MAPK) as an important pro-inflammatory marker controls cellular responses to stress and inflammation. Modified LDL, cytokines, and stressful events cause the phosphorylation and activation of p38 in endothelial cells and macrophages (15).

The family of B-cell leukemia/lymphoma-2 proteins includes 2 types of antiapoptotic members, such as Bcl-2 and Bcl-xL, as well as proapoptotic members, such as the multi-Bcl-2 homology- (BH-) and domain proteins Bax and Bak, and is the mediating factor of the mitochondria-dependent apoptosis pathway (16). The Bcl-2 proteins act as major regulators of basic and non-basic signaling pathways of apoptosis. These proteins can be used as therapeutic targets in the treatment of atherosclerosis (17). The mitochondrial pathway is triggered by proteins of the Bcl2 family or ions of Bak and Bax, whereas the extrinsic pathway is triggered by the tumor necrosis factor (TNF) family or the activation of caspase 8, followed by the activation of caspase 3 (18-20).

Thymol and carvacrol are 2 important natural terpenoids that have antibacterial, antioxidant, and anti-inflammatory effects. These compounds have been reported to cause inhibition of inflammatory edema and migration of leukocytes in an animal model, as well as a decrease in key mediators of inflammation, such as cyclooxygenase (COX)-2, and stimulation of the enzyme of nitric oxide synthase (iNOS) and inflammatory cytokines, such as TNF, TNFα, and interleukin (IL)-1β (21). Thymol is a compound with an antioxidant effect against oxidative stress. The leaves and essential oil of thymol are used in the food industry as flavor enhancers and preservatives, as well as in herbal medicine. In 2011, El-Nekeety et al studied the antioxidant properties of *Thymus vulgaris* oil against oxidative stress in male rats and reported a powerful antioxidant effect of thymol

(22). Thymol directly refines free radicals and activates physiologic defense mechanisms to exert its antioxidant activities (23,24). As a powerful antioxidant with the ability to increase the serum total antioxidant capacity and inhibit peroxidation, thymol can prevent the progression of atherosclerosis (25).

Given the above-mentioned discussion, this study aimed (*a*) to investigate the effect of thymol on antioxidant, apoptotic, and inflammatory markers; and (*b*) to investigate the possible mechanism of this effect by considering changes in antioxidant responses or peripheral blood lipid levels.

Materials and Methods
Animals
Male Wistar rats were purchased from the animal laboratory of the Pasteur Institute in Karaj, Iran. The number of rats placed in each group was determined by using the following formula (26):

$$N=(Z1-a+Z1-\beta)2(SD12+SD22)/(M1M2)$$

The criteria for inclusion in the study were a normal weight of about 270 ± 30 g, young age (8 weeks), good health, male gender, and a normal lipid profile. The exclusion criteria were presence of hyperlipidemia and behavioral abnormalities. For 1 week, the animals were kept at a temperature of 21°C, humidity of 55%, and a 12-hour light/dark cycle for them to get used to the environment (27), with access to sufficient food and water.

Study Design
The rats were randomly assigned to 4 groups. The first was a control group with a normal diet (ND). The second group had a high-cholesterol diet (HD); 2% cholesterol was given through regular food and water for 2 months. Cholesterol with > 99.9% purity was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The third group had a diet of combined high cholesterol and thymol (HD+T); the animals received thymol with 2% cholesterol (t0501; Sigma, St. Louis, MO, USA) at a dose of 24 mg/kg for 2 months. The fourth group was the thymol group (T).

Sampling
Twenty-four hours after the last intervention and after 12 hours of fasting, the mice were deeply anesthetized by an intraperitoneal injection of 90 mg/kg ketamine and 9 mg/kg xylazine. Blood samples were collected from the heart before the removal of the carotid. Then, a sample of 6 to 7 mm was removed from each carotid (28,29) and frozen in nitrogen liquid for western blot analysis. Whole blood was poured into tubes without an anticoagulant; this was centrifuged at 3000 rpm for 5 minutes after 1-hour incubation at 37°C and complete coagulation. Then, supernatant (serum) was collected and stored at -20°C for biochemical analyses (30).

Lipid Profile
The amount of plasma lipid indicators, including TG, total cholesterol (TC), and high-density lipoprotein cholesterol

(HDL-C), was measured by using biochemistry laboratory kits (Tehran, Iran), applying a photometric method with the use of a VITROS 5600 autoanalyser (Ortho-Clinical Diagnostics, Inc., USA). To measure LDL-C, the Friedewald formula of LDL-C = TC-TG/5-HDL-C was used (31). The concentrations of all variables were expressed in mg/dL. The serum levels of OxLDL were measured by sandwich enzyme-linked immunosorbent assay (ELISA) with the use of an ELISA kit (MBS729489; My Bio Source Ltd, USA).

Plasma Antioxidant Profile
To measure the activity of the GPx enzyme in hemolyzed blood, a Ransel kit (Randox Laboratories Ltd, Admore, Northern Ireland) was used, the design of which was based on the analysis of H_2O_2 in the presence of GSH by GSH-Px and a decrease in GSH. The activity of the SOD enzyme in hemolyzed blood was measured with a Ransod kit (Randox Laboratories Ltd, Admore, Northern Ireland); this was based on the inhibitory effect of superoxide dismutase (SOD) on superoxide anions (O^{-2}) caused by the enzymes of the xanthine/xanthine oxidase system, which ultimately produces nitrite. The activities of the 2 above-mentioned enzymes were expressed in U/g Hb.

Total Antioxidant Capacity Level
The total antioxidant capacity (TAC) was measured with the use of a Randox kit (Randox Laboratories Ltd, Admore, Northern Ireland).

Malondialdehyde Level
The thiobarbituric acid (TBA) method was used to determine the amount of MDA based on the reaction between MDA and TBA and the formation of TBA-reactive substances (TBARS). The measured levels were expressed in nmol/mL of serum.

Western Blotting
Western blotting was used to measure the protein expression of phospho-p38, Bcl-2, and cleaved caspase 3 based on the protocol of Santa Cruz online, according to a previously used method (32). First, carotid tissue proteins were extracted by using RIPA lysis buffer (Sigma) plus anti-protease cocktails (Sigma Chemical Co., MO, USA). The protein concentrations were determined by the Bradford assay method by using a commercially available kit (Sigma Chemical Co., MO, USA). Then the proteins were separated by electrophoresis on 10% acrylamide gel; after the separation, the bands were transferred onto nitrocellulose membrane Hybond ECL nitrocellulose membranes (Sigma Chemical Co., MO, USA). The membranes were blocked with phosphate-buffered saline containing 5% nonfat milk powder and 1.0% Tween 20 and were subsequently incubated for 1 hour at a temperature of 4°C overnight with anti-rabbit polyclonal primary antibodies of Bcl-2, p-p38, cleaved caspase 3, and beta-actin as a control (Santa Cruz, USA) and HRP-conjugated anti-rabbit secondary antibodies (1:5000; Santa Cruz Biotechnology); this was followed by detection through a chemiluminescence method by using X-ray film. The band densities were measured with the densitometry software ImageJ, version 62.1 (National Institutes of Health, Bethesda, Maryland, USA), and then normalized against the beta-actin band. After the normalization of the bands, the relative density curve was plotted to quantify the density.

Statistical Analysis
The average relative densities obtained were entered into the SPSS software, version 16, and the data distribution was analyzed by applying the Kolmogorov-Smirnov test. Comparison of the averages between groups was done by using analysis of variance (ANOVA) and Tukey post hoc test. A *P* value of less than 0.05 was considered as significant.

Results
Thymol Decreases TC in the Blood of Hypercholesterolemic Rats
As shown in Tables 1 and 2, feeding rats with a high-cholesterol diet for 8 weeks increased the levels of TC, LDL-C, and TG in the HD compared with the ND group. The amounts of TC and LDL-C showed a significant increase in both groups; however, the increase in TC was almost three fold that of LDL-C in the HD group.

No significant difference in the mean HDL-C level was found between the ND and the HD group. In the HD+T group, the consumption of thymol significantly decreased the TC levels; however, no significant changes were observed in the other indicators.

Table 1. Comparison of Lipid Profile Values Between the Studied Groups (n=10)

Group	TC (mg/dL)	LDL Cholesterol (mg/dL)	HDL Cholesterol (mg/dL)	TG (mg/dL)
ND	67.89±5.14	15.20±2.34	33.66±2.90	50.12±7.16
P value	<0.001***	<0.001***	0.988	0.001***
HD	229.35±13.26	177.39±10.38	35.27±4.69	65.41±10.66
P value	<0.05*	1	0.942	0.179
HD+T	215.58±12.46	177.43±18.41	37.58±5.79	73.48±7.36
T	64.67±3.74	14.13±2.04	34.42±3.24	48.52±6.17

Abbreviations: LDL, low-density lipoprotein; HDL, high-density lipoprotein; ND, normal diet; HD, hypercholesterolemic diet; HD+T, hypercholesterolemic diet + thymol; T, thymol.
* *P* <0.05, ** *P* < 0.01. ****P* < 0.001.

Table 2. Comparison of Antioxidant Profile Values Between the Studied Groups

Group	TAC (mmol/L)	GPx (U/g Hb)	SOD (U/g Hb)	MDA (umol/l)
ND	1.74±0.53	85.29±2.62	829.14±65.90	2.95±0.68
P value	0.05*	0. 01**	0.001***	0.001***
HD	1.178±0.24	71.08±9.32	681.68±73.65	6.37±0.85
P value	0.001**	0.624	0.335	0.001
HD+T	1.99±0.33	76.72±14.37	627.40±63.34	2.37±0.49
T	2.59±0.34	97.12±5.76	758±35.66	2.06±0.47

Abbreviations: GPx, glutathione peroxidase; SOD, superoxide dismutase; MDA, malondialdehyde; TAC, total antioxidant capacity.
* $P <0.05$, ** $P < 0.01$. *** $P < 0.001$.

Thymol Decreases Serum Levels of OxLDL in Hypercholesterolemic Rats

As shown in Figure 1, the serum levels of OxLDL in the HD group were significantly increased compared with the ND group. The consumption of thymol decreased the serum levels of OxLDL in the HD + T group.

Thymol decreases the phosphorylation of p38 (P-P38) and the protein expression of cleaved caspase 3 and increases the expression of Bcl-2

As shown in Figure 2, the p-p38 and the protein expression of cleaved caspase 3 were significantly increased in the HD group compared with the ND group, whereas the expression levels of Bcl-2 were significantly decreased. In the HD+T group, thymol consumption significantly decreased the p-p38 and the protein expression of cleaved caspase 3 but significantly increased the expression of Bcl-2.

Discussion

This study aimed to investigate the effect of thymol on oxidative indexes and atherogenic lipids in carotid tissue of Wistar rats. Thymol and carvacrol are 2 important natural terpenoids that are the main components of aromatic plants. These compounds also have antibacterial, antioxidant, and anti-inflammatory effects (21). In this study, a high-cholesterol diet (2%) was used to create the atherogenic rat model. Although previous studies have

Figure 1. Serum OxLDL Levels in the Studied Groups (n = 10). Abbreviations: ND, normal diet; HD, hypercholesterolemic diet (2%); HD + T, hypercholesterolemic diet+thymol (24 mg/kg); T, thymol. *** $P<0.001$ versus ND, ### $P<0.001$ versus HD.

reported the creation of such model with 1% cholesterol per cholesterol (2% cholesterol) (33-35), the lipid profile results in this work indicate the successful induction of hypercholesterolemia in rats. After creating the hypercholesterolemic model, treatment with thymol (24 mg/kg) was carried out.

The most important findings of this study were as follows: First, thymol decreased the inflammation and apoptosis caused by a high-cholesterol diet. Second, thymol showed a direct impact on the clearance of oxidants but had no significant impact on the activity of antioxidant enzymes. Third, although thymol decreased the TC plasma level in the hypercholesterolemic model, it had no effect on the atherogenic lipid (LDL-C). Fourth, thymol decreased the level of OxLDL induced in the hypercholesterolemic model to half.

Hypercholesterolemia, especially the LDL type, is often associated with atherosclerosis, which is a chronic inflammatory disease of the arteries. The accumulation of lipids in the arterial wall to higher than the normal level and their oxidation as OxLDL are considered as the main causes of inflammation and apoptosis. In the current study, the level of OxLDL in animal models was found to be increased by approximately four fold, which was in accordance with the study of Kunitomo (36) and Khorrami et al (37), who showed that the level of serum OxLDL was significantly increased in mice fed with a high-cholesterol diet. However, our results also showed that the high-cholesterol diet (2%) for 14 weeks significantly increased the levels of TC, TG, LDL, VLDL (very LDL), and Ox-LDL and decreased the concentrations of HDL/LDL and HDL. The increase in OxLDL may be due to an increase in LDL particles or oxidation factors. van der Zwan et al found a direct relationship between LDL-C and OxLDL (38). In addition, evidence has shown that a high-cholesterol diet increases the level of ROS in peripheral blood and carotid (39-41). After treatment with thymol, it was found that elevation of OxLDL serum level in the presence of thymol has been reduced to half amount, whereas the LDL-C concentration did not change.

These results are in accordance with a previous review of the literature. In another research, Yu et al reported that hyperlipidemic and atherosclerotic New Zealand white rabbits showed a decreasing effect of thymol on OxLDL (25). Some studies have shown a decreasing effect of thymol on the lipid profile. For example, Meeran et
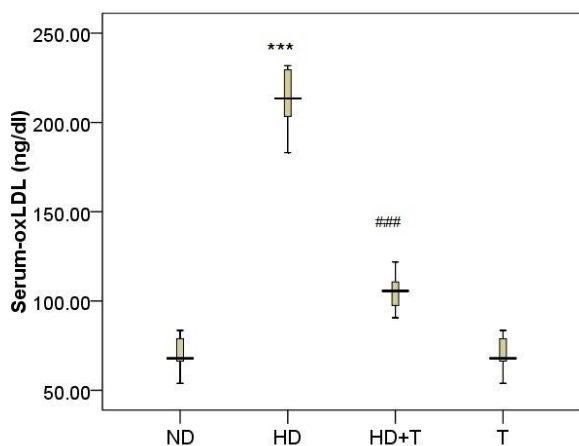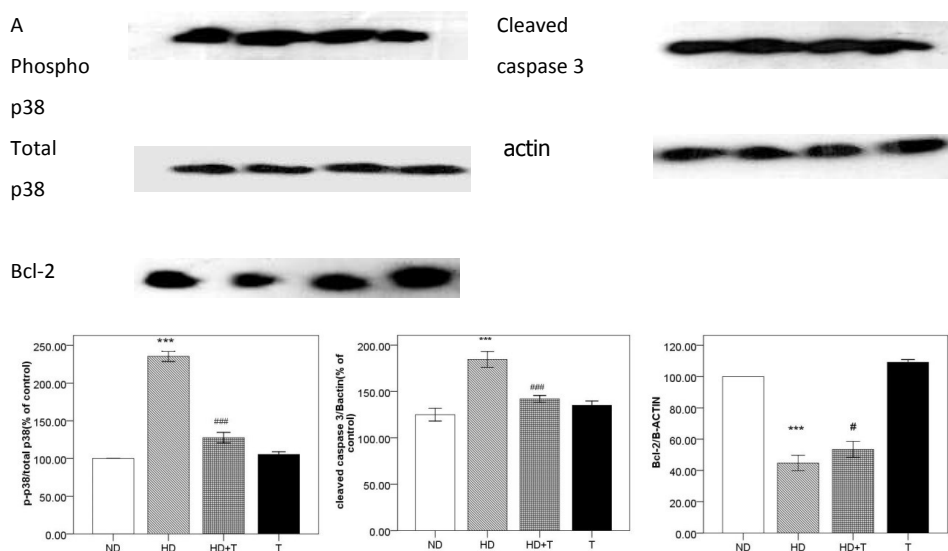
Figure 2. Downregulation of Cleaved Caspase 3 and p-p38 MAPK and Upregulation of Bcl2 in the Carotid Artery of an Atherosclerotic Rat Model After Thymol Administration.
A, immunoblotting of p-p38 MAPK, p38 MAPK, cleaved caspase 3, Bcl2, and B-actin in the ND, HD, HD+T, and T groups. B, Quantitation of immunoblotting of p-p38 MAPK/p38 MAPK. C, Quantitation of immunoblotting of cleaved caspase 3. D, Quantitation of immunoblotting of Bcl2. The values are the means ± SD of 6 animals in each group. *** $P < 0.001$ versus ND, ## $P < 0.01$ versus HD, # $P < 0.05$ versus HD, ### $P < 0.001$ versus HD

al determined the ability of thymol to decrease changes in lipid metabolism, tachycardia, ECG, apoptosis, and cardiac hypertrophy caused by myocardial infarction (MI) induced by isoproterenol in rats. Their results showed that thymol decreased lipid metabolism changes through its anti-tachycardia, anti-hypertrophic, antiapoptotic, and antihyperlipidemic effects (42); however, that study used a different model from the one in the present work.

The oxidation of LDL particles occurs in the sub-intima and in areas with high oxidative stress, such as the accumulation of polymorphonuclear cells, after entering into the area under the intima, they attach to proteoglycan of that area through apo B100 ligand, and they were trapped and oxidized by ROS resulted from peroxidation of arachidonic acid in platelets and macrophages respiratory explosion, in the different degrees in various lipid and protein areas such as apo B100 (43) and create the minimally modified LDL (MMLDL) oxidation with poor oxidation of LDL or OxLDL (LDL with complete oxidation). By increasing the degree of LDL oxidation, these particles away more from the normal metabolism of LDL (44). Thus, after being converted to OxLDL, they are unable to use classic clathrin receivers and become exclusively connected to the recipient of the hyper-family of TLR and LOX-1 on macrophages under the intima and thereafter are phagocytized by macrophages (45). By ingesting large quantities of OxLDL, these macrophages are converted into foamy cells, and different routes of apoptosis and inflammation are activated in them. The cells are eventually lysed, and only one core lipid with a number of macrophage carcasses remains in the atheroma area such that some OxLDLs reenter the general blood circulation (46).

Treatment with a high-cholesterol diet following by increasing the lipids and oxidative stress was along with increasing blood vessel inflammation and programmed apoptosis of carotid cells. The present study on arterial inflammation indicators, such as p-p38 and apoptotic indices, including activation (breaking) of caspase 3 and expression of Bcl2, has shown that the p-p38 and cleaved caspase 3 in the rat model increased, whereas the antiapoptotic Bcl2 protein level decreased. In addition, our results indicated that after 8 weeks of treatment with thymol, the inflammatory and apoptotic effects induced by a high-cholesterol diet were decreased (33-35). Thus, there was a significant decrease in p-p38 and cleaved caspase 3 and an increase in the level of antiapoptotic Bcl2 protein. Taken together, these results suggest that the consumption of thymol decreased the inflammation and cell apoptosis caused by a high-cholesterol diet.

The increase in inflammation and cell apoptosis induced by a high-cholesterol diet has been proven in numerous studies (47,48). The induction of hyperlipidemia in New Zealand white rabbits resulted in an increase in intima thickness, serum lipid variables, and different markers of inflammation and pro-inflammatory cytokines, a finding that is consistent with the results of our study (25). In addition, Liang et al showed an inhibitory effect of thymol on the inflammatory response induced by lipopolysaccharides by decreasing the signaling pathway of NF-κB and MAPK in mouse mammary epithelial cells (mMECs). Studies have also confirmed that thymol inhibits the p-p38MAPK, IκBα, NF-Kb, p65, ERK1/2, and c-JNK in mMECs (49). OxLDL particles cause the movement of Ras from the cytoplasm to the cell membrane and thereby activates at least three types of

Thr/Thykinase, (MKK3, MKK4/SEK1, and MKK6), which cause the phosphorylation and activation of P38 in the downstream (50). The anti-inflammatory effect of thymol has also been observed in the inhibition of inflammatory edema and leukocyte migration in male rats (51). Another study on a rat model of infarction induced by isoproterenol showed a simultaneous decrease in the expression of Bcl-2 and an increase in the proapoptotic bax gene in myocardial tissue; a significant protective effect of thymol was reported to mitigate the changes (42). Thymol has also been found to prevent cardiac toxicity induced by doxorubicin (DOX) by decreasing oxidative stress, inflammation, and apoptosis. DOX causes a sharp decrease in the activity of catalase, SOD, and glutathione and an increase in the quantity of heart MDA products; these compounds participate in the generation of oxidative stress and increases the production of ROS. In the present study, the pretreatment and preventive roles of thymol on lactate dehydrogenase (LDH), aspartate aminotransferase (AST), creatine phosphokinase (CPK), and TNF-α serum, as well as the decrease of caspase-3 in heart tissue, were clarified. Moreover, this effect occurred along with a sharp decrease in lipid peroxidation, as determined by the amount of MDA, an increase in glutathione, and an increase in catalase and SOD activity in cardiac tissue (52). Although these results are apparently not in accordance with the effect of thymol on HL-60 (acute promyelocytic leukemia) cancer cells, which showed a decrease in Bcl2 expression and an increase in Bax, in DNA fragmentation, in the activity of caspases 3 and 9, and in the clearance of ROS in dose-dependent conditions (53). However, it is important to note that in both models, the ROS levels were decreased by thymol. It seems that thymol, by adjusting the ROS levels in physiologic amounts, plays a protective role against oxidative conditions.

Similarly to our study, the results obtained from the antioxidant and antioxidant profiles showed that thymol had no significant effect on the activity of the antioxidant enzymes GPx and SOD. However, it caused an increase in the TAC in peripheral blood and a decrease in the serum levels of MDA. Confirming the results of the current study, an anti-study of the oxidation effects of T. vulgaris (which is rich in thymol) on oxidative stress induced by aflatoxin in male rats showed that T. vulgaris extract increased the TAC and thereby prevented aflatoxin effects (22). Evaluating oxidative stress in ova albumin-induced asthma in mice and estimating the antioxidant effect of thyme and thymol confirmed that thyme and thymol have increased the amount of GPx and SOD in the body and have reduced MDA (54). The protective effect of thymol isolated from Thymus quinquecostatus Celak against oxidative injuries induced by tert-butyl hydroperoxide (t-BHP) in liver cells showed that thymol inhibited cell injury by preventing oxidative stress induced by decreasing ROS and MDA and increasing glutathione (55).

Oxidative stress is associated with the number of free radicals in the body, which inhibit the activity and safety level of antioxidants, or is defined by an increased

amount of MDA, decreased activity of SOD, catalase, and GSH-PX, and decreased TAC. The total antioxidant capacity includes the enzymes of antioxidants and other substances, such as vitamins C and E, uric acid, and any other materials, such as carvacrol, thymol, and other polyphenols, with the ability for ROS clearance. An increase in total antioxidants, along with a decrease in MDA and no change in antioxidant activity, reflects the fact that thymol contains substances that directly impact the clearance of oxidative agents and that this effect is independent of the activity of antioxidant enzymes. Previous studies have also shown the direct effect of thymol on the clearance of ROS.

It is necessary to note that in all the tests carried out, thymol, at the specific dose given, had no particular effect on the control group, thus confirming to some extent the safety of this compound for normal cells. In general, the results of this study indicate that the anti-inflammatory and antiapoptotic effects of thymol can be somewhat correlated with a decrease in OxLDL, which is followed by a decrease in oxidative stress. Considering that thymol did not significantly decrease the levels of LDL-C, it thus seems more reasonable to attribute the inflammatory and antiapoptotic effects of thymol to its antioxidant property.

Conclusion
The results of the present study show that thymol significantly decreases the apoptotic and inflammatory effects induced by hypercholesterolemia in rat carotid tissue and that this decrease is possibly associated with a direct antioxidant effect of thymol. Therefore, because of its antioxidant, anti-inflammatory, and antiapoptotic effects, thymol as a food supplement could be considered as a modulator of vascular damage resulting from hypercholesterolemia. However, further studies are needed to clarify the other effects of this compound.

Conflict of Interest
None to declare.

Ethical Issues
This research was approved by the ethics committee of Tabriz University of Medical Sciences (A125345). All procedures applied in the maintenance, treatment, and sampling of animals were done at the Neurology Research Center of Tabriz University of Medical Sciences.

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References
1. Hayrapetyan S, Vardanyan L, Vardanyan R. Antioxidant Activity of Creeping Thyme (Thymus serpyllum L.) in Cumene Oxidation Reaction. Chem Biol. 2013;2:23-31.
2. Salvayre R, Negre-Salvayre A, Camaré C. Oxidative theory of atherosclerosis and antioxidants. Biochimie. 2016;125:281-296. doi:10.1016/j.biochi.2015.12.014.
3. Han SN, Leka LS, Lichtenstein AH, Ausman LM, Schaefer EJ, Meydani SN. Effect of hydrogenated and saturated, relative

to polyunsaturated, fat on immune and inflammatory responses of adults with moderate hypercholesterolemia. J Lipid Res. 2002;43(3):445-452.

4. Baskaran G, Salvamani S, Azlan A, Ahmad SA, Yeap SK, Shukor MY. hypocholesterolemic and antiatherosclerotic potential of *Basella alba* leaf extract in hypercholesterolemia-induced rabbits. Evid Based Complement Alternat Med. 2015;2015:751714. doi:10.1155/2015/751714.

5. Epstein FH, Ross R. Atherosclerosis—an inflammatory disease. N Engl J Med. 1999;340(2):115-126.

6. Willems S, van der Velden D, Quax PH, et al. Circulating immunoglobulins are not associated with intraplaque mast cell number and other vulnerable plaque characteristics in patients with carotid artery stenosis. PLoS One. 2014;9(2):e88984. doi:10.1371/journal.pone.0088984

7. Liu X, Zhu RX, Li L, He ZY. Association of *LOX-1* gene polymorphisms with cerebral infarction in northern Chinese Han population. Lipids Health Dis. 2014;13:55. doi:10.1186/1476-511x-13-55.

8. Yin Y, Liu W, Ji G, Dai Y. The essential role of p38 MAPK in mediating the interplay of oxLDL and IL-10 in regulating endothelial cell apoptosis. Eur J Cell Biol. 2013;92(4–5):150-159. doi:10.1016/j.ejcb.2013.01.001.

9. Libby P. Inflammation in atherosclerosis. Arterioscler Thromb Vasc Biol. 2012;32(9):2045-2051.

10. Pirro M, Schillaci G, Paltriccia R, et al. Increased ratio of CD31+/CD42− microparticles to endothelial progenitors as a novel marker of atherosclerosis in hypercholesterolemia. Arterioscler Thromb Vasc Biol. 2006;26(11):2530-2535.

11. Conti P, Shaik-Dasthagirisaeb Y. Atherosclerosis: a chronic inflammatory disease mediated by mast cells. Cent Eur J Immunol. 2015;40(3):380-386. doi:10.5114/ceji.2015.54603.

12. Winarsi H, Yuniaty A, Nuraeni I. Improvement of antioxidant and immune status of atherosclerotic rats adrenaline and egg-yolks -induced using cardamom-rhizome-ethanolic-extract: an initial study of functional food. Agriculture and Agricultural Science Procedia. 2016;9:264-270. doi:10.1016/j.aaspro.2016.02.147.

13. Alavinezhad A, Boskabady MH. Antiinflammatory, antioxidant, and immunological effects of *Carum copticum* L. and some of its constituents. Phytother Res. 2014;28(12):1739-1748.

14. Lubrano V, Balzan S. Enzymatic antioxidant system in vascular inflammation and coronary artery disease. World J Exp Med. 2015;5(4):218-224. doi:10.5493/wjem.v5.i4.218.

15. Kardakaris R, Gareus R, Xanthoulea S, Pasparakis M. Endothelial and macrophage-specific deficiency of P38alpha MAPK does not affect the pathogenesis of atherosclerosis in ApoE-/- mice. PLoS One. 2011;6(6):e21055. doi:10.1371/journal.pone.0021055

16. Su J, Zhou L, Xia MH, Xu Y, Xiang XY, Sun LK. Bcl-2 family proteins are involved in the signal crosstalk between endoplasmic reticulum stress and mitochondrial dysfunction in tumor chemotherapy resistance. Biomed Res Int. 2014;2014:234370. doi:10.1155/2014/234370.

17. Kutuk O, Basaga H. Bcl-2 protein family: implications in vascular apoptosis and atherosclerosis. Apoptosis. 2006;11(10):1661-1675. doi:10.1007/s10495-006-9402-7.

18. Fadeel B, Orrenius S. Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease. J Intern Med. 2005;258(6):479-517.

19. Karami-Tehrani F. Caspase dependent apoptosis induced by cladribine in the estrogen receptor negative breast cancer cell line, MDA-MB468. Journal of Sciences, Islamic Republic of Iran. 2003;14(4):303-310.

20. Blanco-Colio LM, Villa A, Ortego M, et al. 3-Hydroxy-3-methyl-glutaryl coenzyme A reductase inhib-itors, atorvastatin and simvastatin, induce apoptosis of vascular smooth muscle cells by down-regulation of Bcl-2 expression and Rho A prenylation. Atherosclerosis. 2002;161(1):17-26.

21. Gholijani N, Gharagozloo M, Kalantar F, Ramezani A, Amirghofran Z. Modulation of cytokine production and transcription factors activities in human Jurkat T cells by thymol and carvacrol. Adv Pharm Bull. 2015;5(suppl 1):653-660. doi:10.15171/apb.2015.089.

22. El-Nekeety AA, Mohamed SR, Hathout AS, Hassan NS, Aly SE, Abdel-Wahhab MA. Antioxidant properties of *Thymus vulgaris* oil against aflatoxin-induce oxidative stress in male rats. Toxicon. 2011;57(7):984-991.

23. Raskovic A, Pavlovic N, Kvrgic M, et al. Effects of pharmaceutical formulations containing thyme on carbon tetrachloride-induced liver injury in rats. BMC Complement Altern Med. 2015;15:442. doi:10.1186/s12906-015-0966-z.

24. Dehkordi MJ, Dehkordi HS, Mobarakeh HI, Khamesipour F, Hamedi B. Assessing the effect of the thyme (*Thyme vulgaris* L.) essence on some biochemical factors in rat's blood serum. Int J Life Sci. 2014;8(3):15-18.

25. Yu Y-M, Chao T-Y, Chang W-C, Chang MJ, Lee M-F. Thymol reduces oxidative stress, aortic intimal thickening, and inflammation-related gene expression in hyperlipidemic rabbits. J Food Drug Anal. 2016;24(3): 556–563.

26. Valentinuzzi ME. Friedman LM, Furberg CD, DeMets DL. Fundamentals of Clinical Trials. 3rd ed. New York: Springer-Verlag; 2004.

27. Oschry Y, Eisenberg S. Rat plasma lipoproteins: re-evaluation of a lipoprotein system in an animal devoid of cholesteryl ester transfer activity. J Lipid Res. 1982;23(8):1099-1106.

28. Xu Y, Zhou S, Fang Z, et al. Inhibition of neointimal hyperplasia in rats treated with atorvastatin after carotid artery injury may be mainly associated with down-regulation of survivin and Fas expression. Pharm Biol. 2014;52(9):1196-1203. doi: 10.3109/13880209.2014.884605.

29. Case GL, He L, Mo H, Elson CE. Induction of geranyl pyrophosphate pyrophosphatase activity by cho-lesterol-suppressive isoprenoids. Lipids. 1995;30(4):357-359.

30. Hashemipour H, Kermanshahi H, Golian A, Khaksar V. Effects of carboxy methyl cellulose and thymol + carvacrol on performance, digesta viscosity and some blood metabolites of broilers. J Anim Physiol Anim Nutr (Berl). 2014;98(4):672-9. doi: 10.1111/jpn.12121.

31. Abo El-Khair DM, El-Safti Fel-N, Nooh HZ, El-Mehi AE. A comparative study on the effect of high cholester-ol diet on the hippocampal CA1 area of adult and aged rats. Anat Cell Biol. 2014;47(2):117-126. doi: 10.5115/acb.2014.47.2.117.

32. Faramoushi M, Amir Sasan R, Sari Sarraf V, Karimi P. Cardiac fibrosis and down regulation of GLUT4 in exper-imental diabetic cardiomyopathy are ameliorated by chronic exposures to intermittent altitude. J Cardiovasc Thorac Res. 2016; 8(1): 26–33.

33. Samout N, Bouzenna H, Ettaya A, Elfeki A, Hfaiedh N. Antihypercholesterolemic effect of *Cleome arabica* L. on high cholesterol diet induced damage in rats. EXCLI J. 2015;14:791-800. doi:10.17179/excli2015-169

34. Busnelli M, Manzini S, Froio A, et al. Diet induced

mild hypercholesterolemia in pigs: local and systemic inflammation, effects on vascular injury - rescue by high-dose statin treatment. PLoS One. 2013;8(11):e80588. doi:10.1371/journal.pone.0080588.

35. Beason DP, Hsu JE, Marshall SM, et al. Hypercholesterolemia in-creases supraspinatus tendon stiffness and elastic modulus across multiple species. J Shoulder Elbow Surg. 2013;22(5):681-6. doi: 10.1016/j.jse.2012.07.008.

36. Kunitomo M. [Oxidative stress and atherosclerosis]. Yakugaku Zasshi. 2007;127(12):1997-2014.

37. Khorrami A, Ghanbarzadeh S, Ziaee M, Arami S, Vajdi R, Garjani A. Dietary cholesterol and oxidised cholesterol: effects on sperm characteristics, antioxidant status and hormonal profile in rats. Andrologia. 2015;47(3):310-317. doi: 10.1111/and.12262.

38. van der Zwan LP, Teerlink T, Dekker JM, et al. Circulating oxidized LDL: determinants and association with brachial flow-mediated dilation. J Lipid Res. 2009;50(2):342-349.

39. Kotani K, Koibuchi H, Miyamoto M, Yamada T, Taniguchi N. Relationship between reactive oxygen metabolites and carotid intima-media thickness in subjects with hypercholesterolemia. Medical Principles and Practice. 2010;19(6):496-498.

40. Yagi H, Sumino H, Yoshida K, et al. Biological antioxidant potential negatively correlates with carotid artery intima-media thickness. Int Heart J. 2016;57(2):220-225.

41. Oyinloye BE, Adenowo AF, Kappo AP. Reactive oxygen species, apoptosis, antimicrobial peptides and human inflammatory diseases. Pharmaceuticals (Basel). 2015;8(2):151-175. doi:10.3390/ph8020151.

42. Meeran MFN, Jagadeesh GS, Selvaraj P. Thymol attenuates altered lipid metabolism in β-adrenergic agonist induced myocardial infarcted rats by inhibiting tachycardia, altered electrocardiogram, apoptosis and cardiac hypertrophy. J Funct Foods. 2015;14:51-62.

43. Liu ML, Ylitalo K, Salonen R, Salonen JT, Taskinen MR. Circulating oxidized low-density lipoprotein and its association with carotid intima-media thickness in asymptomatic members of familial combined hyperlipidemia families. Arterioscler Thromb Vasc Biol. 2004;24(8):1492-1497. doi:10.1161/01.atv.0000135982.60383.48

44. Pedruzzi E, Guichard C, Ollivier V, et al. NAD(P)H oxidase Nox-4 mediates 7-ketocholesterol-induced endoplasmic reticulum stress and apoptosis in human aortic smooth muscle cells. Mol Cell Biol. 2004;24(24):10703-10717. doi:10.1128/mcb.24.24.10703-10717.2004

45. Saulnier-Blache JS. Lysophosphatidic acid: a "bioactive" phospholipid (French). Med Sci (Paris). 2004;20(8-9):799-803.

46. Hulten LM, Ullstrom C, Krettek A, et al. Human macrophages limit oxidation products in low density lipoprotein. Lipids Health Dis. 2005;4:6. doi:10.1186/1476-511x-4-6.

47. Zhang O, Zhang J. Atorvastatin promotes human monocyte differentiation toward alternative M2 macrophages through p38 mitogen-activated protein kinase-dependent peroxisome proliferator-activated receptor γ activation. Int Immunopharmacol. 2015;26(1):58-64.

48. Nissen SE, Tuzcu EM, Schoenhagen P, et al. Statin therapy, LDL cholesterol, C-reactive protein, and coronary artery disease. N Engl J Med. 2005;352(1):29-38.

49. Liang D, Li F, Fu Y, et al. Thymol inhibits LPS-stimulated inflammatory response via down-regulation of NF-kappaB and MAPK signaling pathways in mouse mammary epithelial cells. Inflammation. 2014;37(1):214-222. doi:10.1007/s10753-013-9732-x.

50. Zhu Y, Liao H, Wang N, et al. LDL-activated p38 in endothelial cells is mediated by Ras. Arterioscler Thromb Vasc Biol. 2001;21(7):1159-1164.

51. Fachini-Queiroz FC, Kummer R, Estevao-Silva CF, et al. Effects of thymol and carvacrol, constituents of Thymus vulgaris L. essential oil, on the inflammatory response. Evid Based Complement Alternat Med. 2012;2012:657026. doi:10.1155/2012/657026.

52. El-Sayed ESM, Mansour AM, Abdul-Hameed MS. Thymol and carvacrol prevent doxorubicin-induced cardiotoxicity by abrogation of oxidative stress, inflammation, and apoptosis in rats. J Biochemical Mol Toxicol. 2016;30(1):37-44.

53. Deb DD, Parimala G, Saravana Devi S, Chakraborty T. Effect of thymol on peripheral blood mono-nuclear cell PBMC and acute promyelotic cancer cell line HL-60. Chem Biol Interact. 2011;193(1):97-106. doi:10.1016/j.cbi.2011.05.009.

54. Al-Khalaf MI. Thyme and thymol effects on induced bronchial asthma in mice. Life Sci J. 2013;10:693-699.

55. Kim Y-S, Hwang J-W, Kang S-H, et al. Thymol from Thymus quinquecostatus Celak protects against tert-butyl hydroperoxide-induced oxidative stress in Chang cells. J Nat Med. 2014;68(1):154-162.