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Olive Leaf Extract Improves the Atherogenic Lipid Profile in Rats Fed a High Cholesterol Diet

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Coronary heart disease because of atherosclerosis is still the most common cause of mortality. Elevated levels of low-density lipoprotein and total cholesterol are major risk factors for atherosclerotic cardiovascular disease. The aim of this study was to evaluate the effects of the olive leaf extract on serum lipid profile, early changes of atherosclerosis and endothelium-dependent relaxations in cholesterol-fed rats. For this purpose, rats were fed by 2% cholesterol-enriched or standard chow for 8 weeks. Some rats in each group were also fed orally by olive leaf extract at doses of 50 or 100 mg/kg/day. Atorvastatin at dose of 20 mg/kg of body weight daily was also given as positive control. After 8 weeks, lipid profiles of rat serums were analyzed. Antioxidant enzyme activities (superoxide dismutase and glutathione peroxidase) and degree of lipid peroxidation (malondialdehyde levels) were also measured in the hearts isolated from rats. In addition, expression of adhesion molecules and endothelium-dependent relaxations of isolated thoracic aortas of rats were evaluated. Total cholesterol and LDL-cholesterol levels were found to be increased in cholesterol-fed rats, and both doses of olive leaf extract and atorvastatin significantly decreased those levels. In conclusion, because the olive leaf extract attenuates the increased cholesterol levels, it may have beneficial effects on atherosclerosis. Copyright © 2015 John Wiley & Sons, Ltd.

Keywords: olive leaf; cholesterol; statin; atherosclerosis; rat.

INTRODUCTION

Coroner heart disease is a serious health threat in the developed world and responsible for about 20% of all deaths (Cassar *et al.*, 2009). Atherosclerosis, the cause of coronary heart disease, is a chronic condition that thickens the walls of medium and large arteries. The atherosclerotic process is very complicated and not fully understood. However, low-density lipoprotein (LDL) -oxidation has been considered to play a vital role in the pathogenesis of atherosclerosis (Mitra *et al.*, 2011). In addition, it has been recognized that elevated total cholesterol and LDL levels are major risk factors for atherosclerotic cardiovascular disease (Levine *et al.*, 1995; Ballantyne, 1998). Clinical trials have demonstrated that cholesterol-lowering interventions through diet and drugs reduce major cardiovascular events in patients (Kwiterovich, 1997; Stamler *et al.*, 2000).

Different dietary plants have been found to have significant cholesterol-lowering effects in various experimental models and patients (Ghasi *et al.*, 2000; Choudhary *et al.*, 2005). Olive from *Olea europae* is a major component of the Mediterranean diet. There is considerable evidence that this low-calorie diet reduces cardiovascular mortality (Trichopoulou *et al.*, 2003). Olive oil, the olive fruit and its leaves contain the phenolic compound oleuropein, which is known to be responsible for most of the characteristic therapeutic effects. Therapeutic uses of the olive leaves date back centuries. As extracts, herbal teas and powders, they have long been used to treat various conditions such as internal infections, cardiovascular diseases and chronic and degenerative disorders (El and Karakaya, 2009).

This study was planned primarily to investigate the effects of olive leaf extract (OLE) on lipid parameter and atherogenic index in high-cholesterol diet fed rats. Vascular activity and histopathological studies were also conducted in the thoracic aortas of rats. Additionally, antioxidant enzyme levels were measured in the heart tissues to evaluate whether OLE administration might exert an effect on lipid peroxidation and antioxidant status in experimental rats.

MATERIALS AND METHODS

Preparation of olive leaf extract. The plant material was dried at room temperature and reduced to coarse powder and soaked in 75% aqueous ethanol at room temperature under stirring for two days and filtered (Plant:solvent ratio was 1:20). The soaking and filtration were repeated twice, and combined filltrate was evaporated *in vacuo* with 20.8% yield of OLE. Total

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phenolic substances, total flavonoid content and antioxidant activity of OLE were determined by Folin–Ciocalteu method, $AlCl_3$ complex formation and DPPH radical scavenging method respectively. Each analysis was carried out in triplicate.

Determination of total phenolic compounds. Briefly, the OLE (1 mg/0.1 mL aqueous methanol) was diluted with 2.8-mL distilled water. Then 0.1 mL of Folin–Ciocalteu (%50 in water) was added, and the content was mixed throughly. After 3 min 2 mL of 2% sodium carbonate (Na_2CO_3) was added, and the mixture was allowed to stand for 1 h with intermittent shaking. The absorbance was measured at 750 nm in a spectrophotometer. Gallic acid was used as a standard phenolic compound.

Determination of total flavonoids. The determination of total flavonoids in OLE was determined as follows: 1 mg/0.5 mL OLE was diluted with 1.5 mL of 95% ethanol, and 0.1 mL of 10% aluminum chloride hexahydrate ethanolic solution and 2.8 mL of distilled water were added, and after incubation for 40 min at room temperature the absorbance was determined by spectrophotometrically at 415 nm. The total flavonoid concentration was expressed as quercetin equivalents.

DPPH free radical scavenging activity. Antioxidant activity was evaluated by free radical scavenging activity and compared to α-tocopherol by using the DPPH superoxide anion radical. Four milliliters of DPPH methanolic solution (0.004%) was added to 0.25 and 0.5 mg/mL OLE. The solution was vortexed thorougly and incubated in dark for 30 min. The absorbance was measured at 517 nm against blank sample lacking scavenger. The DPPH inhibition percentage was calculated.

Animals and experimental model. Male Wistar rats (n = 40), 250–300 g in weight, were housed under laboratory conditions, a 12-h light/12-h dark cycle, at temperature 21 ± 2 °C. They were allowed free access to tap water and diets. The experimental procedures are approved by the Animal Care and Use Commitee of Celal Bayar University.

The hypercholesterolemic rat model was performed using a high-cholesterol diet (HCD) consisting 2% cholesterol and 0.5% cholic acid, and control rats were given standard chow diet (SCD) without cholesterol supplementation. The control and hypercholesterolemic rats were divided into eight groups:

Group 1: Control rats group (SCD, rats treated with saline)

Group 2: Control atorvastatin treated group (SCD + AV, control rats treated with atorvastatin 20 mg/kg)

Group 3: Control olive leaf extract 50 (SCD + OLE50, control rats treated with olive leaf extract 50 mg/kg)

Group 4: Control olive leaf extract 100 (SCD + OLE100, control rats treated with olive leaf extract 100 mg/kg)

Group 5: Hypercholesterolemic group (HCD, hypercholesterolemic rats treated with saline)

Group 6: Hypercholesterolemic atorvastatin treated group (HCD + AV, hypercholesterolemic rats treated with atorvastatin 20 mg/kg)

Group 7: Hypercholesterolemic olive leaf extract 50 (HCD + OLE50, hypercholesterolemic rats treated with olive leaf extract 50 mg/kg)

Group 8: Hypercholesterolemic olive leaf extract 100 (HCD + OLE100, hypercholesterolemic rats treated with olive leaf extract 100 mg/kg)

Olive leaf extract was dissolved in saline and administered via oral gavage daily (8 weeks). All animals were fed their respective diets until the end of study.

At fasting status, the rats were anesthetized by using an intraperitoneal injection urethane (1000 mg/kg, 20%). Thoracic aorta was removed, placed in cold Krebs– Henseleit solution, cleaned gently of adherent connective tissue and cut into rings (approximately 3-mm length). A part of the aortic tissue was used for immunohistochemical studies. Blood samples from abdominal aorta and heart tissue were collected, when rats were killed, for the measurement of biochemical parameters and antioxidant enzyme levels: superoxide dismutase (SOD), glutathione peroxidase (GPx) and malondialdehyde (MDA).

Determination of lipid levels. Blood samples were separated by centrifugation (10 min, 3000 rpm) and kept at −80 °C until the day of measurement. Plasma total cholesterol (TC), triglyceride (TG) and high-density lipoprotein (HDL) were measured by a commercially avaible enzyme kit in an autoanalyzer system (Beckman Coulter, USA). Plasma LDL was determined using the Friedewald equation: LDL cholesterol = TC-HDL-TG/5 (Friedewald *et al.*, 1972). Atherogenic index was calculated as the ratio of non-HDL (TC-HDL) to HDL.

Vasocontractile responses. The aortic rings were suspended under a resting tension 1 g in 20-mL organ chambers containing oxygenated (5% $C0_2$, 95% O_2) and warmed (37 °C) Krebs solution (pH: 7.4) with the following composition (mM): NaCl 118. 0, KCl 4. 7, $NaHCO_3$ 25. 0, $CaCl_2$ 1.8, NaH_2PO_4 1.2, $mgSO_4$ 1.2 and dextrose 11.0. All preparations were fixed with two stainless steel wires, one was connected to a force displacement transducer for the measurement of isometric contractions and for record on computer using an transducer data acquisition system (MP 150 Biopac Systems, Ankara, Turkey). After 1-h washing and equilibration period, contractile responses to phenylephrine were taken in rings to determine an precontractile tone in vessels. Relaxations induced by acetylcholine (10^{-9}–10^{-5} M) and sodium

nitroprusside (10^{-9}–10^{-5} M) were obtained from aortic rings precontracted with about EC_{80} concentration of phenylephrine.

Enzyme assays. Tissues were homogenized in Tris–HCl buffer (50 mM, pH 7.4) for 2 min at 5000 rpm. The homogenate was then centrifuged at 5000 ×g for 60 min to remove debris. Protein assays were made using the Lowry method (Lowry *et al.*, 1951). SOD activity was measured according to Sun *et al.* (1988) by reducing nitroblue tetrazolium (NBT) by the xanthine–xanthine oxidase system, which is a superoxide generator. GPx activity was measured according to the method as described previously (Paglia and Valentine, 1967). The enzymatic reaction was initiated by adding H_2O_2 and the change in absorbance at 340 nm was monitored by a spectrophotometer. The level of MDA was determined in heart tissue samples of the rats by by measuring the formation of thiobarbituric acid-reactive substances (Wasowicz *et al.*, 1993). The fluorescence was measured as arbitrary unit by a spectrofluorometer (Hitachi model F-4010, Japan) excitation at 525 nm and emission at 547 nm. The results were expressed as nmol of MDA/mg protein.

Histopathological studies. Hematoxylin–eosin (H&E) and immunohistochemical staining for VCAM-1, P-selectin, E-selectin and TNF-alfa were accomplished on formalin (%10) fixed and paraffin embedded tissue sections of thoracic aorta with monoclonal mouse antibodies. All incubation procedures were performed at room temperature. Staining was graded as weak (0), patchy (1) and strong diffuse (2).

Statistical analysis. Data were presented as mean ± S.E.M. Statistical significance was determined using one-way ANOVA followed by multiple comparison test (Tukey's test). The Kruskal Wallis H and Mann–Whitney U-tests were used to compare the histopathological data. Differences were considered to be statistically significant for $p < 0.05$.

RESULTS

Properties of olive leaf extract

The amount of total phenolic contents in OLE was determined as 0.393 ± 0.004% gallic acid equivalent, and the flavonoid content in OLE was calculated as 0.558 ± 0.003 mg QE/100 mg. The free radical scavenging capacity was found as 68.66 ± 0.601% and 94.90 ± 0.540% for 0.25 mg/mL and 0.5 mg/mL OLE, respectively.

Effect of olive leaf extract on lipid profile, lipid peroxidation and antioxidant status

Table 1 shows effect of OLE on serum lipid parameters in rats. There was about three-fold increase in serum total cholesterol and LDL-cholesterol levels of rats fed hypercholesterolemic diet ($p < 0.05$). These levels decreased significantly in OLE supplemented group in comparison with HCD group. No significant difference was found in HDL-cholesterol levels between groups. Serum triglyseride levels slightly increased in hypercholesterolemic group compared to controls, but this difference also has no statistical significance. Rats receiving hypercholesterolemic diet showed a remarkable increase in the atherogenic index ($p < 0.05$). Treatment with OLE (50 mg/kg and 100 mg/kg) significantly reduced the atherogenic index. The results of lipid peroxidation and antioxidant enzyme levels in heart tissue of rats showed no significant difference among the groups (Table 2).

Vascular activity and histopathological studies on aortic tissue

As shown in Fig. 1, phenylephrine-induced contractile responses in aortic strips were similar in all groups. In addition, endothelium-dependent relaxation induced by acetylcholine showed no difference among control and hypercholesterolemic rats (Fig. 2). No significant difference was determined between the groups with H&E staining. The expressions of adhesion molecules, VCAM-1, P-selectin, E-selectin and TNF-alpha, interpreted as the activation of endothelium, were

Table 1. Lipid profile and atherogenic index in various groups

Group	HDL cholesterol (mg/dL)	Triglyseride (mg/dL)	Total cholesterol (mg/dL)	LDL cholesterol (mg/dL)	Atherogenic index
SCD	29.20 ± 4.55	56.40 ± 4.00	47.40 ± 7.10*	7.00 ± 5.83*	0.68 ± 0.25*
SCD + AV	24.60 ± 4.52	63.20 ± 20.09	42.60 ± 3.32*	5.60 ± 3.58*	0.95 ± 0.36*
SCD + OLE50	35.60 ± 7.08	73.80 ± 14.94	52.80 ± 7.03*	2.40 ± 1.50*	0.61 ± 0.17*
SCD + OLE100	26.20 ± 6.12	77.60 ± 11.53	44.20 ± 5.54*	2.40 ± 1.40*	0.96 ± 0.45*
HCD	24.80 ± 1.90	81.80 ± 10.55	144.60 ± 16.03	103.60 ± 15.86	4.86 ± 0.58
HCD + AV	30.00 ± 3.88	50.00 ± 10.34	100.00 ± 16.92*	60.20 ± 18.07*	2.60 ± 0.70*
HCD + OLE50	27.20 ± 1.11	70.60 ± 12.58	82.60 ± 10.97*	41.40 ± 10.68*	2.10 ± 0.45*
HCD + OLE100	22.00 ± 2.38	62.20 ± 8.35	95.00 ± 17.56*	62.60 ± 18.77*	3.29 ± 1.35*

SCD, rats treated with saline; SCD + AV, control rats treated with atorvastatin 20 mg/kg; SCD + OLE50, control rats treated with olive leaf extract 50 mg/kg; SCD + OLE100, control rats treated with olive leaf extract 100 mg/kg; HCD, hypercholesterolemic rats treated with saline; HCD + AV, hypercholesterolemic rats treated with atorvastatin 20 mg/kg; HCD + OLE50, hypercholesterolemic rats treated with olive leaf extract 50 mg/kg; HCD + OLE100, hypercholesterolemic rats treated with olive leaf extract 100 mg/kg.

*Different from HCD group, $p < 0.05$. Results are given as ±SEM, n = 5 for each group.

Table 2. Lipid peroxidation and antioxidant enzyme levels in heart tissue

Group	SOD (u/mg prot)	GPx (u/mg prot)	MDA (nmol/mg prot)
SCD	46.73 ± 6.26	0.61 ± 0.05	0.12 ± 0.01
SCD + AV	41.42 ± 2.67	0.56 ± 0.13	0.14 ± 0.01
SCD + OLE50	40.61 ± 8.75	0.63 ± 0.10	0.15 ± 0.02
SCD + OLE100	40.74 ± 5.77	0.61 ± 0.05	0.13 ± 0.01
HCD	42.88 ± 13.48	0.53 ± 0.10	0.13 ± 0.02
HCD + AV	42.52 ± 5.69	0.58 ± 0.05	0.09 ± 0.01
HCD + OLE50	44.62 ± 5.35	0.61 ± 0.16	0.18 ± 0.06
HCD + OLE100	42.78 ± 4.98	0.75 ± 0.05	0.13 ± 0.01

SCD, rats treated with saline; SCD + AV, control rats treated with atorvastatin 20 mg/kg; SCD + OLE50, control rats treated with olive leaf extract 50 mg/kg; SCD + OLE100, control rats treated with olive leaf extract 100 mg/kg; HCD, hypercholesterolemic rats treated with saline; HCD + AV, hypercholesterolemic rats treated with atorvastatin 20 mg/kg; HCD + OLE50, hypercholesterolemic rats treated with olive leaf extract 50 mg/kg; HCD + OLE100, hypercholesterolemic rats treated with olive leaf extract 100 mg/kg. Results are given as ±SEM, n = 5 for each group.

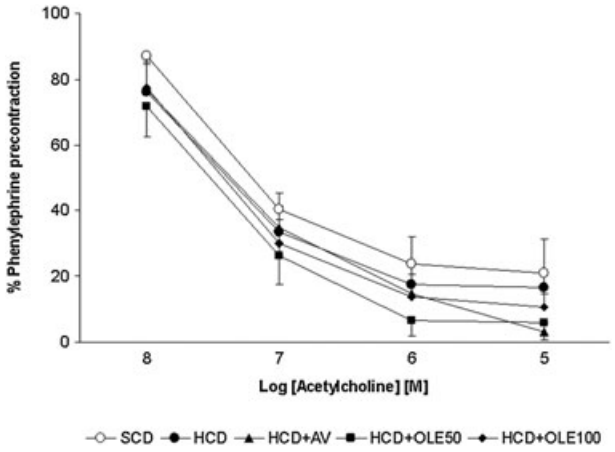

Figure 2. Acetylcholine-induced endothelium-dependent relaxation in rat thoracic aorta. Relaxation was presented as the percentage of phenylephrine precontraction in endothelium intact thoracic aortic rings. Data are expressed as means ± SEM. (n = 5 for each group). SCD, rats treated with saline; HCD, hypercholesterolemic rats treated with saline; HCD + AV, hypercholesterolemic rats treated with atorvastatin 20 mg/kg; HCD + OLE50, hypercholesterolemic rats treated with olive leaf extract 50 mg/kg; HCD + OLE100, hypercholesterolemic rats treated with olive leaf extract 100 mg/kg.

classified based on the staining patterns: No staining, intermittent patchy staining and diffuse staining patterns which were scored as 0, 1 and 2, respectively. Although a high incidence was detected for intermittent patchy staining pattern, no statistically significant difference was found between the patterns or scores of the groups (data not shown).

DISCUSSION

This study was designed to evaluate the effects of OLE on serum lipid profile, some indicators of atherosclerosis and endothelium-dependent relaxations in HCD fed rats.

Drug therapy or diet treatment can regulate cholesterol levels and decrease mortality in people with cardiovascular disease. Scientific evidence has shown that

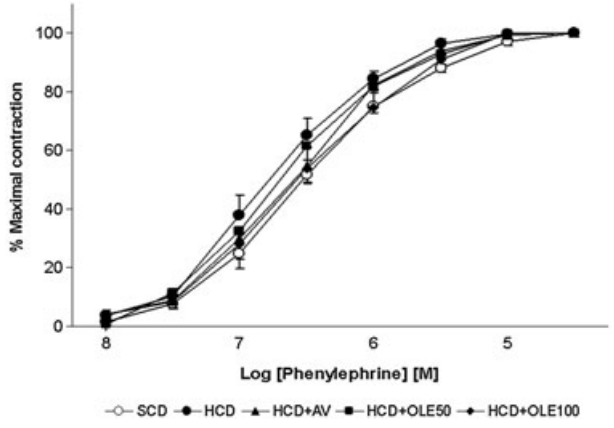

Figure 1. Phenylephrine induced contractile responses in rat thoracic aorta. Data are expressed as means ± SEM. (n = 5 for each group). SCD, rats treated with saline; HCD, hypercholesterolemic rats treated with saline; HCD + AV, hypercholesterolemic rats treated with atorvastatin 20 mg/kg; HCD + OLE50, hypercholesterolemic rats treated with olive leaf extract 50 mg/kg; HCD + OLE100, hypercholesterolemic rats treated with olive leaf extract 100 mg/kg.

OLE have a remarkable impact on blood pressure, and olive oil helps prevent the oxidation of LDL-cholesterol, which is one of the earliest events in developing atherosclerosis (Masella *et al.*, 2004). The active component of olive leaf is a phenolic compound known as oleuropein. Oleuropein has been found to be antioxidant, antiinflammatory, anti-atherogenic, anti-cancer, antimicrobial and antiviral (Omar, 2010). Therefore, it is often used as dietary supplement in Mediterranean countries. Oleuropein has been shown to decrease plasma lipid levels in rabbits and plasma glucose levels in streptozotocin-induced diabetic rats (Andreadou *et al.*, 2006; Onderoglu *et al.*, 1999). In our study, we found that HCD increased total cholesterol, LDL-cholesterol and atherogenic index, and OLE administration as well as atorvastatin restored lipid profile in HCD fed rats. Atorvastatin is a well known drug which is used primarily for lowering blood cholesterol. Our results also showed that there was no significant difference in cholesterol levels between the groups treated with OLE or atorvastatin.

Endothelial dysfunction is considered a potential early indicator of atherosclerosis. It has been reported that vascular reactivity was impaired in HCD fed rats (Slowing *et al.*, 2001). However, we did not found any difference in vascular responses between the experimental groups. Several studies have also reported that the expression of vascular adhesion molecules was elevated in the aorta of HCD fed rats (Lee *et al.*, 1999; Sandhya and Rajamohan, 2006). In contrast, our results showed no statistically difference in staining pattern between any of groups. Additionally, antioxidant enzyme levels and lipid peroxidation products did not change in heart tissues of experimental groups compared to controls. Our results are concordant with the findings of previous studies emphasizing that OLE has a significant effect on antioxidant enzyme activity in rats (Jemai *et al.*, 2008; Fki *et al.*, 2007). On the other hand, in our study, there were no signs of oxidative stress and endothelial damage in hypercholesterolemic rats that did not receive treatment. These findings also significantly differ from some published earlier experimental studies

that have found links between high fat/high cholesterol diet, endothelial dysfunction and oxidative stress (Oztürk *et al.*, 2015; Kobayasi *et al.*, 2010). A possible explanation for these contradictory results is that the duration of administration may not long be enough to observe the influence of dietary fat on oxidative stress and the antioxidant effects of OLE.

Because we found that treatment with OLE in HCD fed rats improves the lipid profile because of lowering the cholesterol levels, the hypocholesterolemic effect of OLE was compared to atorvastatin, which is the most used of the statins. Statins are a class of drugs used to reduce cholesterol levels by inhibiting the production of cholesterol in the liver. Because hypercholesterolemia is one of the major causes of cardiovascular morbitity and mortality, these agents are effective in preventing heart disease. On the other hand, rare but serious side effects associated with statins including liver injury and muscle damage have been reported (Golomb and Evans, 2008). Although statins are proven to be well tolerated drugs, they should be used with care and knowledge of their adverse effects. Lifestyle changes, such as exercise and diet, can help reduce blood cholesterol levels and may be preferred before starting medication treatment.

The Mediterranean diet has been shown to reduce the risk of cardiovascular mortality. Olive foods such as olive oil and olive leaf are major components of that diet, and oleuropein is responsible for most of their beneficial effects. Olive leaf also contains siginificant amounts of oleuropein which is traditionally removed from olives because of its biting taste. Our findings demonstrated a potential and beneficial effect of OLE in reducing of the atherogenic index. In this study, atherogenic index, defined as the ratio of non-HDL to HDL was significantly decreased in HCD fed rats treated with OLE. Non-HDL to HDL ratio was found to be a better marker of risk factor for cardiovascular disease in metabolic syndrome, diabetes mellitus and dyslipidemia (Kim *et al.*, 2013; Pandeya *et al.*, 2012). LDL-cholesterol is also strong predictor of cardiovascular risk. Additionally, we found that LDL-cholesterolC and TC levels were raised in HCD fed rats, and OLE administration significantly reduced these high levels.

In conclusion, the results of this study indicated that OLE supplementation decreased serum total cholesterol and LDL-cholesterol in HCD fed rats while HDL-cholesterol and TG levels remained unchanged. As alternative to statins and other drugs, OLE supplementation may help in improving the lipid profile in hypercholesterolemia.

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The authors report no conflicts of interest.

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