Effects of olive leaves extract on LDL oxidation induced-$CUSO_4$ *in vitro*

Hassan Ahmadvand[1,2], Shahrokh Bagheri[2], Ali Khosrobeigi[2], Maryam Boshtam[3] and Foad abdolahpour[2]

[1]Razi Herbal Medicine Research Center, Faculty of Medicine, Lorestan University of Medical Sciences, Khoramabad, Iran
[2]Department of Biochemistry, Faculty of Medicine, Lorestan University of Medical Sciences, Khoramabad, Iran
[3]Isfahan Cardiovascular Research Center, Isfahan University of Medical Sciences, Isfahan, Iran

Abstract: Oxidation of low-density lipoprotein (LDL) has been strongly implicated in the pathogenesis of atherosclerosis. The use of some natural antioxidant and herbal medicine may lead to the inhibition of production of oxidized LDL and may decrease both the development and the progression of atherosclerosis. The aim of this study was to investigate the effects of Olive leaves ethanol extract (OLE) on LDL oxidation induced-$CuSO_4$ quantitatively *in vitro*. Low-density lipoprotein was incubated with $CuSO_4$ and the formation of conjugated dienes and thiobarbituric acid reactive substances (TBARS). Inhibition of this Cu-induced oxidation was studied in the presence of vitamin E and various concentration of OLE. It was demonstrated that OLE reduced the formation of conjugated dienes and TBARS of LDL against oxidation *in vitro* ($P<0.05$). The inhibitory effects of the OLE on LDL oxidation were dose-dependent at concentrations ranging from (2µg/ml) to (200µg/ml). Moreover, we compared effects of OLE on LDL oxidation with vitamin E as positive control. This study showed that OLE is a source of potent antioxidants and prevented the oxidation of LDL in vitro and it may be suitable for use in food and pharmaceutical applications.

Keywords: Olive leaves extract and Low-density lipoprotein oxidation.

INTRODUCTION

Cardiovascular disease is one of the most reasons of mortality in world. Although an increased concentration of plasma low density lipoprotein (LDL) is believed to be a major risk factor in this regard the underlying mechanisms remain unclear and needs more investigations (Willcox *et al*., 2008 and Lobbes *et al*., 2006). Several reports have indicated the role of oxidized LDL (Ox-LDL) in the pathogenesis of atherosclerosis (Steinberg, 1997; Yekini *et al*., 2009). The uptake of Ox-LDL by macrophages results in the formation of foam cells and Ox-LDL accumulated in vascular endothelial cells, and promotes the development of the characteristic fatty streaks found in atherosclerotic lesions (Ani *et al*., 2007; Witztum and Steinberg, 1991). Also Ox-LDL has been detected in human and animal atherosclerotic lesions. Oxidized LDL has also been reported to compromise endothelial integrity, a silent feature of atherosclerosis (Leopold and Loscalzo, 2009).

Recently, natural foods and food-derived antioxidants such as phenolic phytochemicals have received considerable attention, because they are as chemopreventive agents against oxidative damages (Lee *et al*., 2009). Olive leaf is commonly used to treatment of viral infections, yeast infections, fight colds and flu (Nariman *et al*., 2004). Olive leaf has shown to reduce LDL, or bad cholesterol (Singh *et al*., 2008). In addition, olive leaf increases blood flow by relaxing the arteries and lowers blood pressure (Sudjana *et al*., 2009; Pieroni *et al*., 1996). The major active components in olive leaf are known to be oleuropein and other compound such as hydroxytyrosol, tyrosol, vanillin, luteolin, *p*-coumaric acid, caffeic acid, vanillic acid, rutin and diosmetin, diosmetin-7-glucoside, apigenin-7-glucoside and luteolin-7-glucoside (Lee *et al*., 2009; Soni *et al*., 2006). Several reports have indicated that olive leaf has antioxidant properties and has also antimicrobial properties against mycoplasma, bacteria and fungi (Bianco *et al*., 2006). Recently, high request of whole olive leaf and olive leaf extract has increased for use in foodstuff, functional food materials and food additives (Soni *et al*., 2006; Bianc *et al*., 2006; Fernández-Cabanás *et al*., 2008).

There isn't study about the inhibitory effects of olive leaves extract (OLE) on LDL Oxidation. Therefore, search for LDL oxidation inhibitors, especially in plant give rise to a reliable, safe medicine and cheap in the management and control of cardiovascular and other diseases. Thus in this study, we investigated the effects of LDL incubation with OLE on the modification of LDL induced by $CuSO_4$ in vitro.

MATERIAL AND METHODS

Chemicals

Disodium ethylene diamine tetraacetate (Na_2EDTA), Potassium bromide (KBr), sodium chloride (NaCl), disodium hydrogen phosphate (Na_2HPO_4) and trichloroacetic acid were purchased from Sigma–Aldrich

**Corresponding author*: e-mail: hassan a46@yahoo.com

(St. Louis, MO, USA). All solvents used were of analytical grade. 2-thiobarbituric acid (TBA) was obtained from Fluka Chemie (Buchs SG, Switzerland).

Preparation of* ethanol *extracts of olive leaves

In brief, the air-dried olive leaves, weighting about 100g, were extracted in a soxhlet apparatus with ethanol at 40ºC, for 6h. The extracts were then filtered and concentrated in vacuo at 25ºC, yielding a waxy material (13.3 and 25.1% w/w, respectively). Finally, the extracts were lyophilized and kept at 4ºC until tested.

Blood sampling

Ten men were local participants in blood sampling. The protocols for the blood sampling were approved by the medical University of Lorestan ethics committee, and all men gave informed consent to participant. Fasting blood samples after an overnight fasting were collected in EDTA containing tubes (1.6 mg EDTA/ml blood) and then plasma was isolated by centrifugation (3000 rpm for 10 min at 4 °C). To minimize oxidation in vitro, sodium azide (0.06% wt/vol) was added to plasma immediately after collection.

Isolation of LDL

The LDL fraction was isolated from fresh plasma by single vertical discontinuous density gradient ultracentrifugation (Ani *et al*., 2007). The density of the plasma was adjusted to 1.21 g/ml by the addition of solid KBr (0.365 g/ml). Centrifuge tubes were loaded by layering 1.5 ml of density-adjusted plasma under 3.5 ml of 0.154 mol/L NaCl, and centrifuged in a Beckman L7-55 ultracentrifuge at 40000 rpm at 10°C for 2.5 hours. The yellow LDL band located in the upper middle portion of the tube was collected into a syringe by puncturing the tube. The isolated LDL was dialyzed for 48h at 4 °C against three changes of deoxygenated-PBS (0.01 mol/L Na_2HPO_4, 0.16 mol/L NaCl, pH 7.4) containing 0.01% NaN_3 and 0.01% EDTA.

Oxidation of LDL

Continuous monitoring of formation of conjugated dienes in LDL

After isolation of total LDL, the protein content of LDL was measured (Bradford, 1976). Vitamin E (100 µM) and various concentration of OLE (2, 20 and 200 µg/ml) was added to LDL with final concentration 150 µg/ml of LDL protein at 10 mM PBS, pH7.4. The oxidative modification of LDL was initiated by addition of freshly prepared 10 µM CuSO4 solution at 37°C in water bath for 5h. The kinetics of LDL oxidation was monitored every 10 minutes by measuring LDL samples absorbance at 234 nm. The lag phase was calculated from the oxidation profile of each LDL preparation by drawing a tangent to the slope of the propagation phase and extrapolation in to intercept the initial-absorbance axios. The lag phase represented the length of the antioxidant-protected phase during LDL oxidation by OLE in vitro. The lag time was measured as the time period until the conjugated dienes began to increase (Navder *et al*., 1999). The formation of conjugated dienes was calculated as conjugated dienes equivalent content (nmol/mg-protein) at 5h. The conjugated dienes concentration was calculated by using the extinction coefficient for diene conjugates at 234 nm (29500L/mol.cm).

Assay of the formation of thiobarbituric acid reactive substance (TBARS)

Lipid peroxidation end product was determined as TBARS. After initiating the oxidation process with $CuSO_4$, the sample mixtures were incubated at 37°C for 5h in a water bath and the reaction terminated by adding EDTA (2 mM). TBARS formation was measured in a spectrophotometer at 532 nm. The results were recorded as malondialdehyde (MDA) equivalent content (nmol/mg LDL-protein) (Seven *et al.,* 1999; Sheu *et al.,* 2003).

STATISTICAL ANALYSIS

Data were presented as mean ±SD of three experiments performed in duplicate. The variables used to describe the difference between the oxidation curves were lag time, conjugated dienes and MDA. These parameters were obtained using man Whitney test (using SPSS 13.0 statistical software) for independent data and differences were considered significant when $p < 0.05$.

RESULT

Continuous monitoring of formation of conjugated dienes in LDL and kinetics of $CuSO_4$-induced LDL oxidation

The effects of OLE and vitamin E on the kinetics of LDL oxidation are shown in fig. 1. It clearly shows that $CuSO_4$ dramatically increased oxidation of LDL. The formation of conjugated dienes, a marker of LDL oxidation decreased by vitamin E and OLE. Figs. 2 and 3 show the levels of conjugated dienes at 5h and lag time of all experimental groups. Thus OLE decreased the level of conjugated dienes and was significantly different from control. Vitamin E, as the positive control decreased conjugated dienes and increased lag time significantly from control.

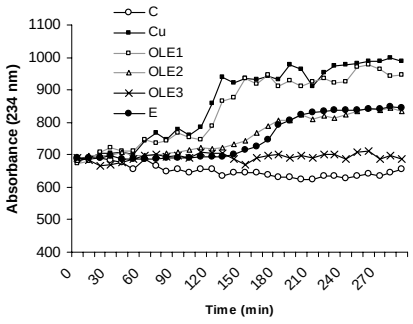

Fig. 1: The effects of OLE on LDL oxidation in l0 mM PBS, pH 7.4 at 37°C for 5h. (C) n-LDL, (Cu) n-LDL +

copper, (OLE 1) n-LDL + OLE (2 µg/ml), (OLE 2) n-LDL + OLE (20 µg/ml), (OLE 3) n-LDL + OLE (200 µg/m) and (E) n-LDL + Vitamin E (100 µM), Each point represents the mean of three experiments.

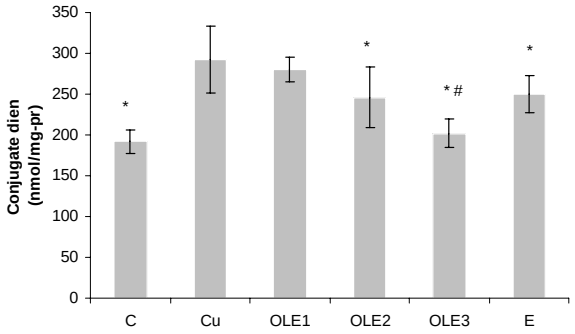

Fig. 2: The effects of OLE on the formation of conjugated dienes of LDL oxidation. Abbreviation as in figure 1. Each Point represents the means of three experiments. **p<0.05 in comparision with* (Cu) n-LDL + copper values, *#p<0.05 in comparision with* (OLE 1) n-LDL + OLE (2 µg/ml) values, by man Whitney test.

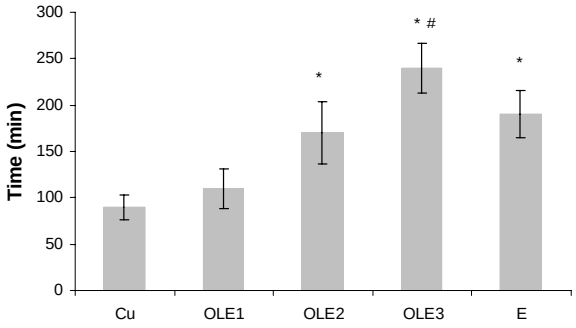

Fig. 3: The effects of OLE on lag time. Abbreviation as in figs. 1 and 2. Each Point represents the means of three experiments.

Olive leaves ethanol extract decreased final levels of conjugated dienes in the medium respectively. The inhibitor effects of the OLE on conjugated dienes formation and increase lag time were dose-dependent at concentrations ranging from 2 µg/ml to 200 µg/ml.

Assay of the formation of thiobarbituric acid reactive substances (TBARS)

The antioxidative effect of OLE and vitamin E on LDL was determined and expressed by measurement of MDA equivalent content. The levels of MDA after 5h of incubation in all experiment groups are shown in fig. 4. Olive leaves ethanol extract also decreased TBARS formation significantly. The inhibitor effects of the OLE on TBARS formation was dose-dependent at concentrations ranging from 2 µg/ml to 200 µg/ml. Vitamin E, as the positive control decreased TBARS formation significantly from control.

DISCUSSION

Our results showed that Vitamin E and OLE (20, 200 µg/ml) was increased lag time and inhibited the final levels of conjugated dienes in LDL oxidation *(p<0.05).* Also results showed that Vitamin E and OLE only at (200 µg/ml) decreased the formation of MDA in LDL oxidation (p<*0.05*). In addition, antioxidant activity of OLE at (200 µg/ml) similar to or better than vitamin E at (100 µM).

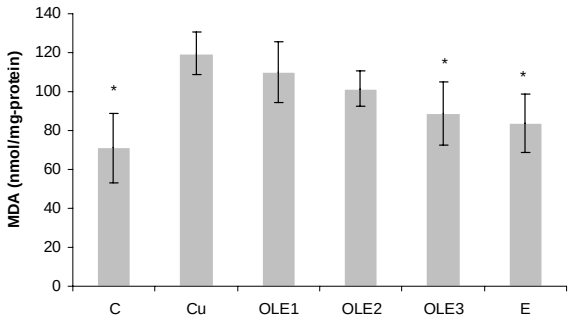

Fig. 4: The effects of OLE on the formation of MDA. Abbreviation as in figs. 1 and 2. Each Point represents the means of three experiments.

The balance between oxidants and antioxidants are principal key of oxidative modifications within the arterial wall that may initiate and/or contribute to atherogenesis. Therefore, it is important to consider sources of oxidants in the context of available antioxidants. Many substances may prevent, or significantly delay, the oxidation of other substrates. Many substances may prevent, or significantly delay, the oxidation of other substrates (Itabe, 2009). With regard to atherosclerosis, vascular antioxidants need to protect against oxidants, both within and outside cells. In the context of the oxidative modification hypothesis, antioxidant protection of LDL in the extracellular space deserves focus, as oxidized LDL has many potential proatherogenic activities (Berliner and Heinecke, 1996), and the cellular accumulation of oxidized LDL is considered a hallmark of atherosclerosis (Steinberg *et al*., 1989). In addition, cellular antioxidants are likely important in the context of the presence of heightened oxidative stress within the vessel wall and the known effect of oxidative events on key cellular activities such as ·NO-related bioactivities. The following briefly describes the antioxidant defenses in arterial wall cells and lipoproteins that counteract oxidative modifications (Itabe, 2009).

Olive Leaf may be beneficial in vaginal yeast infections, diabetes, fungus and yeast infections, asthma, frequent colds, rheumatoid arthritis, bacterial infections, herpes (Ercisli *et al*., 2009; Servili *et al*., 2009; Blekas., 2002; Visioli *et al*., 1998). In animal experiments *olive Leaf*

preparations demonstrate multiple properties including hypoglycemic, diuretic properties, bronchodilator, antiarrhythmic, antispasmodic, hypertensive, coronary dilator and antipyretic. The active constituent oleuropein has antioxidant activity and bacteriostatic (Singh *et al*., 2008; Sudjana *et al*., 2009; Pieroni *et al*., 1996; Soni *et al*., 2006; Bianco *et al*., 2006; Fernández-Cabanás *et al*., 2008). The effectiveness of antioxidants, such as vitamins E and C, urate flavonoids, plant phenolics and wheat bran extracts in suppressing LDL oxidation and delaying the development of heart diseases has been demonstrated (Liyanapathirana and Shahidi, 2004; Koba *et al*., 2007).

Olive leaf has some side effects such as the use of olive leaf with antibiotics can be inactivated antibiotics (Lee and Lee, 2010). Warfarin (Coumadin) is drug that can cause internal bleeding (Gasse *et al*., 2005). Olive leaf naturally relaxes blood vessels and capillaries (Zarzuelo *et al*., 1991). Therefore use of olive leaf with Warfarin which could increase Coumadin's side effect. Oleuropein can cause lower blood pressure and blood glucose levels (Panizzi, 1960; Fehri *et al*., 1994). Therefore Oleuropein may interact with pharmaceutical drugs that are designed to lower blood pressure or regulate diabetes. The interaction could result in a dangerous situation. Those who experience symptoms such as headache, nausea, flu-like symptoms, fainting, dizziness, and other life threatening symptoms should consult a physician immediately (Fehri *et al*., 1994).

The inhibitory effect of OLE on LDL Oxidation $CuSO_4$-induced LDL oxidation in vitro is first study. However, the mechanism by which the OLE inhibits LDL oxidation in vitro remains unclear. Laranjinha *et al*. suggested possible explanations for the protecting effects of compounds of extracts on LDL: "(i) Scavenging of various radical species in the aqueous phase, (ii) Interaction with peroxyl radicals at the LDL surface, (iii) Partitioning into the LDL particle and terminating chain-reactions of lipid peroxidation by scavenging lipid radicals, and (iv) Regenerating endogenous α-tocopherol back to its active antioxidative form" (Laranjinha *et al*., 1994).

Therefore, the major objective of this study was to determine the antioxidant effects of OLE using the *in vitro* model of $CuSO_4$-induced LDL oxidation. Our biochemical results clearly showed that OLE activity in $CuSO_4$-induced LDL oxidation by inhibiting the formation of conjugated dienes and TBARS and increase lag time of LDL in vitro in dose-dependent manners.

CONCLUSION

The results from this study clearly showed that OLE is found to posses good antioxidant activity and various concentration of OLE have a dose-dependent antioxidant activity against LDL oxidation by inhibiting the formation of conjugated dienes and TBARS and increase lag time. In conclusion, Olive leaves is a potent antioxidant and may be a good alternative to reduce the risk of atherosclerosis and coronary heart disease and other free radical associated health problems.

REFERENCES

Ani M. Moshtaghie AA and Ahmadvand H (2007). Comparative effects of copper, iron, vanadium and titanium on low density lipoprotein oxidation *in vitro*. *Iran Biomed. J*., **11**(2): 113-118.

Berliner JA and Heinecke JW (1996). The role of oxidized lipoproteins in atherogenesis. *Free Radic. Biol. Med*., **20**(5): 707-727.

Blekas G, Vassilakis C, Harizanis C, Tsimidou M and Boskou DG (2002). Biophenols in table olives. *J. Agric. Food Chem.,* **50**(13): 3688-3692.

Bianco A, Dezzi S, Bonadies F, Romeo G, Scarpati ML and Uccella N (2006). The variability of composition of the volatile fraction of olive oil. *Nat. Prod. Res.,* **20**(5): 475-478.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.,* **72**: 248-254.

Ercisli S and Barut EA (2009). Molecular characterization of olive cultivars using amplified fragment length polymorphism markers. *Genet. Mol. Res.,* **8**(2): 414-419.

Fehri B, Aiache JM, Memmi A, Korbi S, Yacoubi MT, Mrad S and Lamaison JL (1994). Hypotension, hypoglycemia and hypouricemia recorded after repeated administration of aqueous leaf extract of *Olea europaea* L. *J. Pharm. Belg.,* **49**(2): 101-108.

Fernández-Cabanás VM, Garrido-Varo A, Delgado-Pertiñez M and Gómez-Cabrera A (2008). Nutritive evaluation of olive tree leaves by near-infrared spectroscopy: Effect of soil contamination and correction with spectral pretreatments. *Appl. Spectrosc.,* **62**(1): 51-58.

Gasse C, Hollowell J, Meier CR and Haefeli WE (2005). Drug interactions and risk of acute bleeding leading to hospitalisation or death in patients with chronic atrial fibrillation treated with warfarin. *Thromb. Haemost.,* **94**(3): 537-543.

Itabe H (2009). Oxidative modification of LDL: Its pathological role in atherosclerosis. *Clin. Rev. Allergy Immunol.,* **37**(1): 4-11.

Koba K, Matsuoka A, Osada K and Huang YS (2007). Effect of loquat (*Eriobotrya japonica*) extracts on LDL oxidation. *Food Chem*., **104**(1): 308-316.

Laranjinha JA, Almeida LM and Madeira VM (1994). Reactivity of dietary phenolic acids with peroxyl radicals: Antioxidant activity upon low density lipoprotein peroxidation. *Biochem. Pharmacol.,* **48**(3): 487-494.

Lee OH and Lee BY (2010). Antioxidant and antimicrobial activities of individual and combined phenolics in Olea europaea leaf extract. *Bioresour. Technol.,* **101**(10): 3751-3754.

Lee OH, Lee BY, Lee J, Lee HB, Son JY, Park CS, Shetty K and Kim YC (2009). Assessment of phenolics-enriched extract and fractions of olive leaves and their antioxidant activities. *Bioresour. Technol.,* **100**(23): 6107-6113.

Leopold JA and Loscalzo J (2009). Oxidative risk for atherothrombotic cardiovascular disease. *Free Radic. Biol. Med.,* **47**(12): 1673-1706.

Liyanapathirana C and Shahidi F (2004). Antioxidant activity of wheat extracts as affected by *in vitro* digestion. *Biofactors.* **21**(1-4): 325-328.

Lobbes MB, Lutgens E, Heeneman S, Cleutjens KB, Kooi ME, van Engelshoven JM, Daemen MJ and Nelemans PJ (2006). Is there more than C-reactive protein and fibrinogen? The prognostic value of soluble CD40 ligand, interleukin-6 and oxidized low-density lipoprotein with respect to coronary and cerebral vascular disease. *Atherosclerosis,* **187**(1): 18-25.

Nariman F, Eftekhar F, Habibi Z and Falsafi T (2004). Anti-Helicobacter pylori activities of six Iranian plants. *Helicobacter.,* **9**(2): 146-151.

Navder KP, Baraona E, Leo MA and Lieber CS (1999). Oxidation of LDL in baboons is increased by alcohol and attenuated by polyenylphosphatidylcholine. *J. Lipid Res.,* **40**(6): 983-987.

Panizzi L (1960). The constitution of oleuropein, a bitter glucoside of the olive with hypotensive action. *Gazz. Chim. Ital*; **90**: 1449-1485.

Pieroni A, Heimler D, Pieters L, van Poel B and Vlietinck AJ (1996). *In vitro* anti-complementary activity of flavonoids from olive (*Olea europaea* L.) leaves. *Pharmazie.,* **51**(10): 765-768.

Servili M, Esposto S, Fabiani R, Urbani S, Taticchi A, Mariucci F, Selvaggini R and Montedoro GF (2009). Phenolic compounds in olive oil: Antioxidant, health and organoleptic activities according to their chemical structure. *Inflammopharmacol.,* **17**(2): 76-84.

Seven A, Civelek S, Inci E, Inci F, Korkut N and Burçak G (1999). Evaluation of oxidative stress parameters in blood of patients with laryngeal carcinoma. *Clin. iochem*., **32**(5): 369-373.

Sheu JY, Chen PH, Tseng WC, Chen CY, Tsai LY and Huang YL (2003). Spectrophotometric determination of a thiobarbituric acid-reactive substance in human hair. *Anal Sci.,* **19**(6): 957-960.

Singh I, Mok M, Christensen AM, Turner AH and Hawley JA (2008). The effects of polyphenols in olive leaves on platelet function. *Nutr. Metab. Cardiovasc. Dis.,* **18**(2): 127-132.

Soni MG, Burdock GA, Christian MS, Bitler CM, Crea R. Safety assessment of aqueous olive pulp extract as an antioxidant or antimicrobial agent in foods. Food Chem Toxicol. 2006; 44(7):903-15.

Steinberg D (1997). Low density lipoprotein oxidation and its pathobiological significance. *J. Biol. Chem.,* **272**(34): 20963-20966.

Steinberg D, Parthasarathy S, Carew TE, Khoo JC and Witztum JL (1989). Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med*., **320**(14): 915-924.

Sudjana AN, D'Orazio C, Ryan V, Rasool N, Ng J, Islam N, Riley TV and Hammer KA (2009). Antimicrobial activity of commercial *Olea europaea* (olive) leaf extract. *Int. J. Antimicrob. Agents,* **33**(5): 461-463.

Visioli F, Bellomo G and Galli C (1998). Free radical-scavenging properties of olive oil polyphenols. *Biochem. Biophys. Res. Commun.,* **247**(1): 60-64.

Willcox BJ, Curb JD and Rodriguez BL (2008). Antioxidants in cardiovascular health and disease: Key lessons from epidemiologic studies. *Am. J. Cardiol.,* **101**(10A): 75D-86D.

Witztum JL and Steinberg D (1991). Role of oxidized low density lipoprotein in atherogenesis. *J. Clin. Invest.,* **88**(6): 1785-1792.

Yekini I, Hammoudi F, Martin-Nizard F, Yous S, Lebegue N, Berthelot P and Carato P (2009). Antioxidant activity of benzoxazolinonic and benzothiazolinonic derivatives in the LDL oxidation model. *Bioorg. Med. Chem.*, **17**(22): 7823-7830.

Zarzuelo A, Duarte J, Jiménez J, González M and Utrilla MP (1991). Vasodilator effect of olive leaf. *Planta Med.,* **57**(5): 417-419.