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Phenolic extract and purified hydroxytyrosol recovered from olive mill wastewater prevent in vivo low-density lipoprotein oxidation and modulates lipid metabolism in rats fed a cholesterol-rich diet

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Abstract - Atherosclerosis, the principal contributor to the pathogenesis of myocardial and cerebral infarctions, is known to be one of the leading causes of morbidity and mortality worldwide. Hyperlipidemia and elevated plasma low-density lipoprotein cholesterol (LDLC) resulting from lipid metabolic changes are an important risk factor for the development and progression of atherosclerosis. Phenolic compounds from various sources have been reported to prevent LDL oxidation in vitro and show marked hypolipidemic activity in vivo, suggesting the effectiveness of polyphenols for the prevention and treatment of atherosclerosis.

In this study, a phenolic-rich extract of olive mill wastewaters (OMW) was prepared under optimal conditions and hydroxytyrosol was purified from the obtained extract. Moreover, the effects of these phenolic on the susceptibility of human LDL to oxidation and on plasma lipids in rats fed a cholesterol-rich diet were studied.

First, lipoproteins were isolated by sequential ultracentrifugation from the plasma of healthy subject. After dialysis, LDL was incubated in the presence of Cu SO4. The oxidation was followed by the kinetics of appearance of the conjugated dienes and quantification of lipid peroxidation products (TBARS). For *in vivo* effect, Wistar rats were divided into three groups (n = 8). Group 1 was fed a standard laboratory diet (CD). Group 2 was fed a High cholesterol diet (HCD). Groups 3 and 4 received HCD with Hydroxtyrosol at 2.5 and 5 mg/kg respectively. Groups 5and 6 received HCD with OMW extract at 5 and 10 mg/kg respectively. Serum and tissues were collected for biochemical and histological studies.

Hydroxytyrosol and OMW extract significantly prolonged the lag time of LDL oxidation and reduced The TBARS formation. *In vivo* experiments, the cholesterol-rich diet induced hypercholesterolemia that was manifested in the elevation of total cholesterol (TC), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-C). Administration of Hydroxytyrosol and OMW extract decreased the serum levels of TC, TG, and LDL-C significantly and increased the serum level of high-density lipoprotein cholesterol (HDL-C).

From this data, it can be implied that OMW phenolics possess both antioxidant effects against LDL oxidation and hypolipidemic effects in vivo.

Keywords: olive mill wastewaters extract; hydroxytyrosol; Antioxidant; cholesterol-fed rat; LDL oxidation

1. Introduction

Atherosclerosis is a disease of blood vessels and known colloquially as “hardening of the arteries”. It is characterized by the accumulation of fatty substance, cholesterol, cellular waste products, calcium and other substances in the inner lining of an artery. Major complications of atherosclerosis include angina pectoris, myocardial infarction and stroke, which are recognized as leading causes of morbidity and mortality in Western countries. It has been reported that the oxidative modified LDL might be important in the progression of atherosclerosis, due to the observations that oxidized LDL is cytotoxic, chemotactic, and chemostatic. Monocyte macrophages in an environment of oxidized LDL would

avidly remove LDL from the interstitium and generate macrophage foam cells, a major cell type present within fatty streaks and fibrous plaque (Napoli et al. 1997; Steinberg 1997). Therefore, it has been proposed that inhibition of the generation of the oxidative LDL-generated foam cells and reductions in the level of triglyceride, cholesterol, and LDL, by naturally occurring compounds, would result in retardation of atherosclerostic lesion development. Phenolic compounds from various sources have been reported to prevent LDL oxidation in vitro and show marked hypolipidemic activity in vivo, suggesting the effectiveness of polyphenols for the prevention and treatment of atherosclerosis (Dufull et al, 2003, Koshy et al, 2001). During olive oil extraction in Mediterranean area, large volumes of water are generated and subsequently discarded. These olive mill wastewaters (OMW) contain notable amounts of polar phenolic compounds which are major contributors to the toxicity of OMW (Pekin et al, 2010). However, these phenolic compounds are endowed with several biological activities such as antioxidant properties (Obeid et al, 2005). Numerous studies have shown that OMW phenolics have various biochemical roles, including anti-inflammatory (De la Puerta et al, 1999), antithrombotic (Petroni et al, 1995) and hypoglycemic activities (Hamden et al, 2009). In this study, a phenolic-rich extract of OMW was prepared under optimal conditions and hydroxytyrosol was purified from the obtained OMW extract. Moreover, the effects of these phenolic on the susceptibility of human LDL to oxidation and on plasma lipids in rats fed a cholesterol-rich diet were studied.

2. Material and methods

2.1. Preparation of OMW extract.

OMW extracts were prepared from OMW as previously reported (Allouche et al, 2004). Briefly, continuous countercurrent extractions of OMW were conducted at ambient temperature in a polyethylene mixer settler unit of Robatel design (mixer volume, 35 ml; settler volume, 200 ml). The total feed flow rate ranged from 2 to 5 L/h. For each run, the steady state was confirmed by phenolic monomer analysis in the organic stream and by verification of the mass flow rate balance. The maximum deviation of the latter was 2%. The organic extract was evaporated under vacuum at 40 °C in rotary evaporator. The residue was redissolved in a minimum volume of solvent and analyzed by high-performance liquid chromatography (HPLC).

2.2. Purification of Hydroxytyrosol

Hydroxytyrosol was purified from OMW as previously reported (Allouche et al, 2004). Briefly, 1 g of OMW extract was chromatographed on a C-18 silica gel (liChroprep RP-18; 25-□□□m) column (2.5 mm x70 mm) under medium pressure. Phenolic compound elution was carried out with the same gradient solvent as used in the HPLC. The flow rate was adjusted to 0.3 ml/min, and 4.5 ml fractions were collected. These fractions were measured by optical density at 280 nm, and the chromatogram (optical density versus fraction number) was represented (data not shown). The first separated peak corresponds to pure hydroxytyrosol.

2.3. Inhibitory effect on Cu^{2+} induced LDL oxidation

Human plasma was obtained from EDTA-treated blood and separated by low-speed centrifugation at 2330 g at 4 ◦C for 10 min. The plasma sample was stored at −20 ◦C and used within 1 week for LDL isolation. LDL with a density of 1.019–1.063 g/ml was isolated by a sequential ultracentrifugation method (Redgrave et al, 1975) and dialyzed against 10Mm phosphate buffered saline (PBS) pH 7.4, at 4 ◦C for 24 h. Protein concentration in the LDL sample was determined by the modified method of Lowry (Markwell et al, 1978). To measure the effect of hydroxytyrosol and OMW extract on conjugated diene (CD) formation, LDL, adjusted to 200 µg protein/ml with 5 mM PBS pH 7.4 was preincubated at 37 ◦C for 1 h with various concentrations of OMW phenolics (2, 5, 10 and 20 µg/ml). Oxidation reaction of LDL was initiated by adding freshly prepared 5µM CuSO4 solution and incubated at 37 ◦C for 6 h. CD formation during oxidation of LDL was continuously monitored by a spectrophotometric method based on the changes in absorbance at 234 nm. To determine the formation of thiobarbituric acid reactive substances (TBARS), an aliquot of the reaction mixture was removed and the reaction was terminated with the addition of 50 µl of 100mM butylated hydroxytoluene (BHT). Afterwards, 1ml of 10% trichloroacetic acid (TCA), 0.5 ml of 5mM disodium ethylenediamine (Na_2EDTA), 1.5 ml of 8% sodium dodecyl sulfate (SDS) and 1.5 ml of 0.6% thiobarbituric acid (TBA) were added to each 1ml aliquot of the reaction. After incubation at 80 ◦C for

1 h, TBARS formation was measured by using a spectrofluorometer at an excitation wavelength of 515 nm and an emission wavelength of 553 nm.1,1,3,3-tetraethoxypropane (TEP) was used as standard.

2.4. Animals and diets

48 male Wistar rats weighing between 180 and 200g were purchased from the Pasteur Institute (Tunis). The animals were individually housed in stainless steel cages in a room at a 24°C -controlled temperature and lighting alternating 12-hour periods of light and darkness. The rats were randomly divided into 6 groups of 8 rats each. Group 1 was fed on a normal diet (CD) (Table 1). Group 2 was fed on a high cholesterol diet (HCD) (normal diet supplemented with 1% cholesterol and 0.25% bile salts). Group 3 and 4 received HCD and hydroxytyrosol at 2.5 and 5 mg/kg of body weight respectively. Group 5 and 6 received HCD and OMW extract at 5 and 10 mg/ kg of body weight respectively. Phenolic compounds were administrated orally in drinking water. The duration of the treatment was 16 weeks. At the end of the experimental period, the rats were killed by decapitation. Blood samples were collected to determine the plasma lipid profile. The aortas were removed, rinsed with physiological saline and was fixed in 10% formalin for the histopathological examination.

Table 1. Concentration of normal diet

diet ingredient	concentration
casein	200
DL-methionine	3
cornstarch	393
sucrose	154
cellulose	50
mineral mix	35
vitamin mix	10

2.5. Serum Lipids

Concentrations of TC, TG, and HDL-C in serum were determined by enzymatic colorimetric methods using commercial kits (Sigma). LDL-C was accomplished according to the procedures described by Friedwald et al. 1972. The atherosclerotic index (AI) was defined as the ratio of LDL-C, and HDL-C was also calculated for different groups.

2.6. Histopathological Analysis

At the time of sacrifice, the aorta tissues were removed, cut into small pieces, and fixed in 10% formaldehyde solution. The washed tissues were dehydrated in the descending grades of ethanol and finally cleared in toluene. The tissues were then embedded in molten paraffin wax. Sections were cut at 5 mm thicknesses and stained with hemeatoxylin and eosin. The sections were then viewed under light microscope for histopathological changes.

2.7. Statistical Analysis

All data presented are the means ± standard errors (SEs). Statistical differences were calculated using a one-way analysis of variance, followed by Student's test. Differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Extraction and analysis of OMW extract.

The HPLC chromatogram of the final extract, obtained from OMW using the continuous countercurrent extractor under the optimal conditions, is shown in Figure 1. Hydroxytyrosol and tyrosol were the major compounds detected (Table 2). Their concentrations in the extract were 1225 and 345 mg/L, respectively. para-Hydroxyphenyl acetic acid, cafeic acid, and p-coumaric acid were present at lower concentrations. 3,4-Dihydroxyphenylacetic acid and ferulic acid were also detected at the same concentration,70 mg/L (Table 2). Protocatechuic acid, vanillic acid, and synergic acids and other compounds were detected but not quantified.

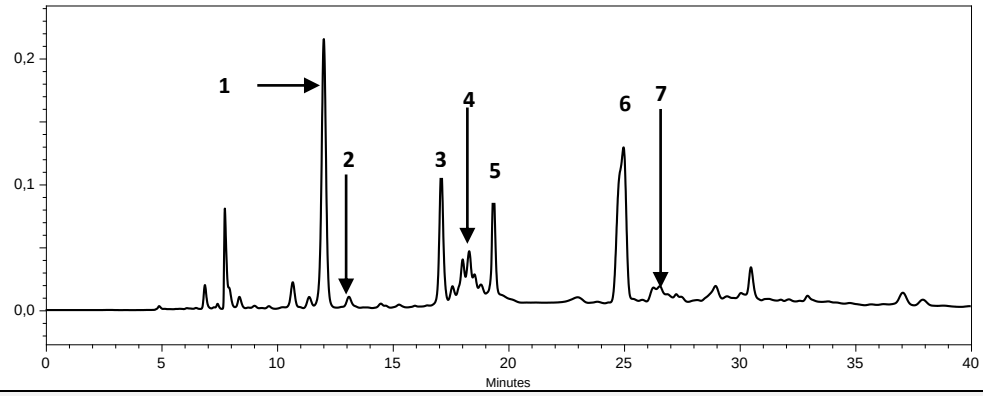

Figure 1. HPLC-chromatogram of the liquid-liquid continuous ethyl acetate extract of olive mill wastewater 1: hydroxytyrosol; 2: 3,4-dihydroxyphenyl acetic acid; 3: tyrosol; 4: para-hydroxyphenyl acetic acid; 5: cafeic acid; 6: para-coumaric acid; 7: ferulic acid

Table 2. HPLC evaluation of major phenolic compounds identified in OMW extract (mg/l).

Phenolic monomers	**Concentration in OMW extract (mg/l)**
Hydroxytyrosol	1225.6
Tyrosol	345
3,4-dihydroxyphenyl acetic acid	70.2
para-hydroxyphenyl acetic acid	198
cafeic acid	256.7
para-coumaric acid	169
ferulic acid	70.2

3.2. Effects of Hydroxytyrosol and OMW extract on CD formation during LDL oxidation.

We measured the dynamics of CD formation in human LDL in the absence or presence of OMW phenolics. It was demonstrated that the lag phase in lipid peroxidation processes reflects the antioxidant status of membranes and lipoproteins, and, as a corollary, their resistance to oxidation (Yoshida and Kisugi, 2010). In this study, incubation of human LDL (200µg protein/ml) with 5µM CuSO4 induced CD formation with a lag-time (the duration between time 0 to time when CD formation started) of 46.66±1.76 min. In the presence of hydroxytyrosol and OMW extract at a final concentration of 2, 5, 10 and 20 µg/ml, the lag-time of CD formation was increased to 62.0±1.15, 80.66±4.41, 136.67±1.67, 238.33±1.66 min, and 47. 41 ±1.45, 55.27±2.15, 83±2.15, 137 ±3.47 respectively (Figure 2).

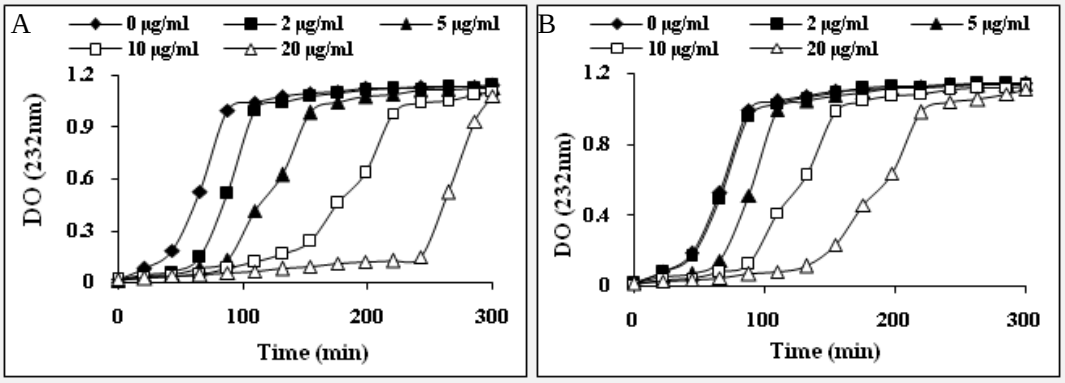

Figure 2. The effects of hydroxytyrosol (A) and OMW extract (B) on conjugated diene formation during human LDL oxidation.

3.3. Effects of Hydroxytyrosol and OMW extract on TBARS formation during LDL oxidation

During LDL oxidation, the lag phase is followed by the propagation phase of rapid LDL oxidation giving rise to lipid peroxides. Lipid peroxides undergo decomposition phase when breaking of the double bonds gives rise to malondialdehydes (MDA). Nucleophilic substitution reaction between MDA and TBA used in the experimentation produced "MDA:TBA adduct," also called "thiobarbituric acid reactive substances (TBARS)" (Lefèvre et al.1998).The effect of OMW phenolics on the formation of TBARS resulting from the oxidation of human LDL was shown in Figure 3. Incubation of 200 µg protein/ml of human LDL with 5 µM CuSO4 at 37 ◦C for 6-h induced TBARS formation. In the presence of hydroxytyrosol and OMW extract at a final concentration of 2, 5, 10 and 20 µg/ml, the TBARS levels were reduced by 33, 72, 82, 90 % and 6, 29, 43 and 71 %, respectively. These findings indicate that hydroxytyrosol and OMW extract could suppress the initiation and propagation of lipid peroxidation, and they may help suppress atherosclerosis by scavenging hydrogen oxide radicals.

It has been reported that during free radical-induced oxidative modification of LDL, peroxidation of polyunsaturated fatty acids generates lipid hydroperoxides. Further decomposition of hydroperoxides augments the peroxidation process until there occurs a deflection through reduction of the alkoxyl and/or peroxyl radicals to alkoxides and/or hydroperoxides, respectively (Rice-Evans et al. 1997). Chain-breaking phenolic antioxidants come into play in this situation and shield against the vicious cycle of oxidation and peroxidation (Rice-Evans et al.1997)

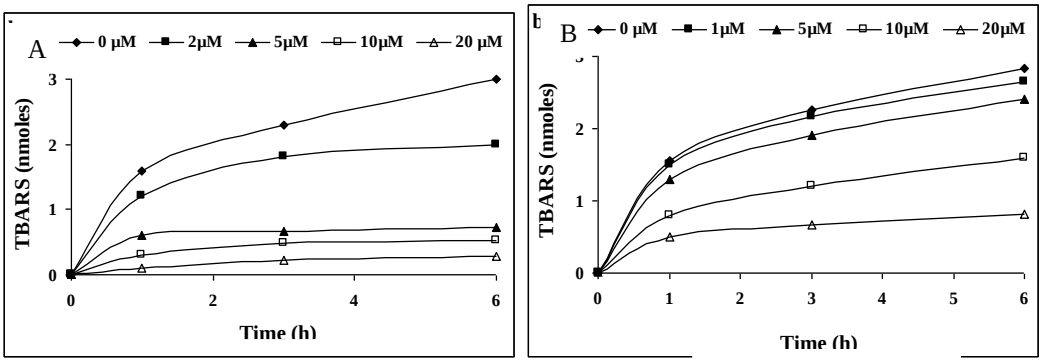

Figure 3. The effects of hydroxytyrosol (A) and OMW extract (B) on the formationof TBARS during human LDL oxidation.

3.4. In vivo hypolipidemic effects of Hydroxytyrosol and OMW extract

Figure 4 shows the serum lipid levels at the end of the experiment. After 16 weeks of treatment, the TC and LDL-C concentrations of rats fed a HCD showed a significant increase as compared with the rats fed normal diet (CD). However, a decrease of HDL-C concentration of rats in the HCD group was observed. Rats that received an oral administration of hydroxytyrosol and OMW extract had lower concentrations of TC and LDL-C than those of rats that received an HCD. In particular, the administration of hydroxytyrosol at 2.5 and 5 mg/kg and OMW extract at 5 and 10 mg/kg reduced the TC and LDL-C levels by 30, 69%; 26, 48%; 19, 16%; and 27, 60%, respectively. Moreover, the concentration of HDL-C of rats treated with hydroxytyrosol and OMW extract increased significantly as compared with those of rats in the HCD group. Lowering levels of TC and LDL-C and improving level of HDL-C has been linked to a lower risk of coronary heart disease (Libby et al. 2000). In fact, it was reported that the decrease in LDL-C concentration and the increase of HDL-C level could fasten the removal of cholesterol from peripheral tissues to liver for catabolism and excretion (Young et al. 2004). Also, high HDL-C levels may compete with LDL receptor sites on arterial smooth muscle cells and thus inhibit the uptake of LDL (Young et al, 2004). In addition, the increase in HDL-C concentration could protect the LDL against oxidation in vivo because lipids in HDL are preferentially oxidized before those in LDL (Young et al. 2004). Therefore, the results of the present study indicate that olive leaf phenolics may reduce the incidence of coronary heart disease. These data are in agreement with the study of Gorinstein et al. (2002) who reported that polyphenols from olive oil decrease plasma LDL-C levels and prevent their oxidation in vivo. The mechanism of this hypocholesterolemic action may be due to the inhibition of dietary cholesterol absorption in the

intestine or its production by liver (Krzeminski et al. 2003) or stimulation of the biliary secretion of cholesterol and cholesterol excretion in the faeces (Prasad and Kalra 1993).

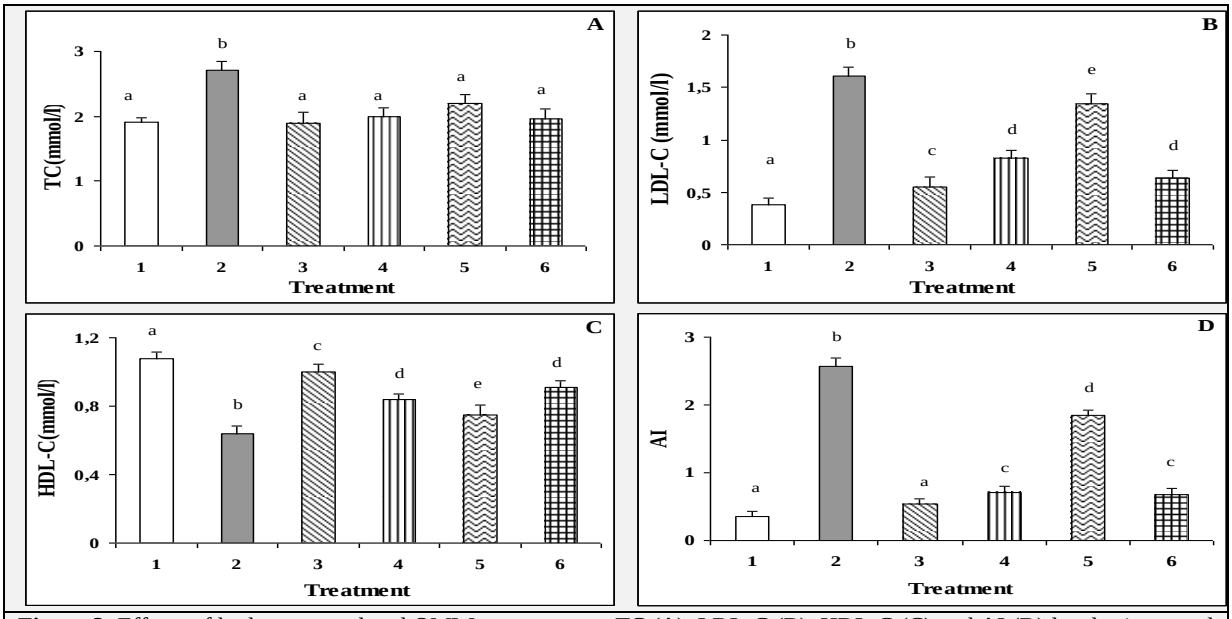

Figure 3. Effects of hydroxytyrosol and OMW extract on rat TC (A), LDL-C (B), HDL-C (C) and AI (D) levels. 1: control diet (CD) (standard diet), 2: high cholesterol diet (HCD), 3: HCD + hydroxytyrosol (2.5 mg/ kg), 4: HCD + hydroxytyrosol (5 mg/ kg), 5: HCD + OMW extract (5 mg/ kg), 6: HCD + OMW extract (10 mg/ kg). Each bar represents mean □ SE from eight rats. Bars with different letters differ, $p < 0.05$.

3.5. Histopathologies of aortic wall

The histopathological examination of cholesterol-fed rats revealed the presence of lesion in the aortic wall when compared with the aorta of the control group (Figure 4A, B). However, these signs were significantly improved in the hydroxytyrosol (2.5 mg/kg) and OMW extract (10 mg/kg) treated group (Figure 4C).

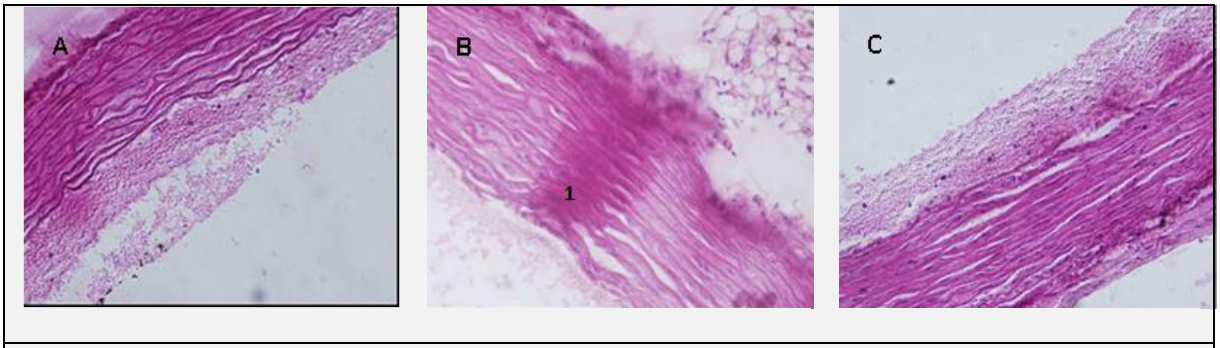

Figure 4. Microscopic views of transverse sections of aorta in normal (A), high cholesterol-fed (B), and hydroxytyrosol or OMW extract supplements rats (C) (Hematoxylin-Eosin H&E staining, 400 x). Key: 1, lesion of aortic wall.

4. Conclusion

Our study clearly demonstrated that phenolic extract and hydroxytyrosol recovered from OMW possess strong antioxidant activities against in vitro LDL oxidation and had very pronounced hypocholesterolemic effects in vivo. These effects highlighted olive tree by-products as a source of antioxidants able to reduce the frequency of cardiovascular diseases.

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