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Hypolipidimic and antioxidant activities of oleuropein and its hydrolysis derivative-rich extracts from Chemlali olive leaves

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ABSTRACT

Oleuropein-rich extracts from olive leaves and their enzymatic and acid hydrolysates, respectively rich in oleuropein aglycone and hydroxytyrosol, were prepared under optimal conditions. The antioxidant activities of these extracts were examined by a series of models *in vitro*. In this study the lipid-lowering and the antioxidative activities of oleuropein, oleuropein aglycone and hydroxytyrosol-rich extracts in rats fed a cholesterol-rich diet were tested. Wistar rats fed a standard laboratory diet or cholesterol-rich diets for 16 weeks were used. The serum lipid levels, the thiobarbituric acid reactive substances (TBARS) level, as indicator of lipid peroxidation, and the activities of liver antioxidant enzymes (superoxide dismutase (SOD) and catalase (CAT)) were examined. The cholesterol-rich diet induced hyperlipidemia resulting in the elevation of total cholesterol (TC), triglycerides (TG) and low-density lipoprotein cholesterol (LDL-C). Administration of polyphenol-rich olive leaf extracts significantly lowered the serum levels of TC, TG and LDL-C and increased the serum level of high-density lipoprotein cholesterol (HDL-C). Furthermore, the content of TBARS in liver, heart, kidneys and aorta decreased significantly after oral administration of polyphenol-rich olive leaf extracts compared with those of rats fed a cholesterol-rich diet. In addition, these extracts increased the serum antioxidant potential and the hepatic CAT and SOD activities. These results suggested that the hypocholesterolemic effect of oleuropein, oleuropein aglycone and hydroxytyrosol-rich extracts might be due to their abilities to lower serum TC, TG and LDL-C levels as well as slowing the lipid peroxidation process and enhancing antioxidant enzyme activity.

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1. Introduction

Recently, the physiological effects of polyphenol-rich foods, such as fruits, vegetables, and beverages including fruit juices, wine, tea, coffee, and chocolate, have been receiving a lot of attention as dietary sources of antioxidants that are valuable for human health. Many epidemiological studies have strongly suggested a correlation between the intake of polyphenol-rich foods and low mortality due to coronary heart disease (CHD) [1–3]. CHD, such as myocardial infarction and ischemic stroke, closely related to atherosclerosis, are major causes of death in developed countries. Therefore, it is worth studying the impact of the daily consumption of polyphenol-rich foods and the extent of the effects of such foods on atherosclerosis.

Recent studies have pointed out oxidative damage as an important etiologic factor in atherosclerosis. Especially, according to the oxidative stress theory, oxidative

Abbreviations: ABTS, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; AIa, therosclerotic index; TG, triglycerides; CAT, catalase; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances; HPLC, high-performance liquid chromatography; HCD, cholesterol-rich diet; CD, control diet.

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modification of low-density lipoprotein (LDL) is thought to play a key role in the development of atherosclerosis [4,5]. Therefore, the inhibition of such process is considered to be an important therapeutic approach. For instance, vitamin E and probucol have been reported to prevent LDL oxidation and delay the development of atherosclerotic plaques in animal models, suggesting the effectiveness of antioxidants for the treatment and prevention of atherosclerosis [6,7].

In the Mediterranean area, olive leaves are one of the by-products of farming of the olive grove; they can be found in high amounts in the olive oil industries (10% of the total weight of the olives) and they accumulate during pruning of the olive trees [8]. Olive leaves are considered as a cheap raw material which can be used as a useful source of high-added value products (phenolic compounds) [9]. The main phenolic compounds in olive leaves are the glycosylated forms of oleuropein and ligstroside [10,11]. Numerous *in vitro* studies have shown that oleuropein and its derivatives have various biochemical roles [12,13], including anti-inflammatory and antithrombotic activities. Moreover, it was reported that these polyphenols are able to prevent low-density lipoprotein oxidation [13] and platelet aggregation [14] and to inhibit lipoxygenases and eicosanoid production [14,15].

Hydroxytyrosol is the principal oleuropein derivative; it is used to prevent diseases because it is endowed with an important antioxidant property [16]. Moreover, oleuropein aglycone is obtained by enzymatic hydrolysis of oleuropein by β-glucosidase-induced deglucosilation of the iridoid glycoside moiety of oleuropein [17]. Oleuropein aglycone has many biological activities and it has been shown that it is more efficient than hydroxytyrosol and oleuropein as anti-proliferative and pro-apoptotic agent in the prevention of some cancer [18].

In our previous studies, oleuropein, oleuropein aglycone and hydroxytyrosol-rich extracts from olive leaf extract and its enzymatic and acid hydrolysis product were prepared under optimal conditions. The antioxidant activities of these extracts were examined by a series of models *in vitro* [19,20]. Therefore, the aim of this study was to investigate the effects of these olive leaf extracts on serum lipid levels and antioxidant enzymes activities in rats fed a cholesterol-rich diet.

2. Materials and methods

2.1. Extraction of phenolic compounds

The extraction was carried out on *Olea europaea* L. c.v Chemlali leaves from the south of Tunisia. Samples of fresh green leaves were used. Leaves were dried and powdered for the extraction. A mixture of methanol and water (200 ml, 4:1 v/v) was added to the olive leaf powder (50 g) and the mixture was kept under agitation for 24 h. Subsequently, the solution was filtered using GF/F filter paper. The extract was concentrated by evaporation to dryness at 40 °C, and the residue obtained was stored in glass vials, at 0 °C in the dark until HPLC analysis. Four gram (4 g) sample was dissolved in methanol (10 ml) and extracted three times with ethyl acetate (Prolabo, France) (40 ml) to prepare the oleuropein-rich extract.

2.2. Acid hydrolysis

One gram of the ethyl acetate extract was dissolved in 10 ml of a MeOH/H_2O (4:1) mixture in sealed vial. The solution was hydrolysed at 100 °C for 1 h using 5 ml of a 2-M HCl solution (Prolabo, France). After hydrolysis, the reaction was cooled and diluted with water (10 ml) and the hydrophobic fraction was extracted by a separatory funnel, three times with 25 ml of ethyl acetate, which was subsequently removed by evaporation.

2.3. Enzymatic hydrolysis

Enzymatic hydrolysis was carried out using β-glucosidase from almond (Sigma). The reaction was carried out as previously described by Bouaziz and Sayadi [20].

2.4. HPLC analysis

A reverse-phase high-performance liquid chromatography technique was developed to identify and quantify the major phenolic compounds contained in the hydrolysate extract. Concentrations were calculated based on peak areas compared to those of external standards. The HPLC chromatograph was a Schimadzu apparatus equipped with a (LC-10ATvp) pump and a (SPD-10Avp) detector. The column was (4.6 mm × 250 mm) (Shim-pack, VP-ODS) and the temperature was maintained at 40 °C. The flow rate was 0.5 ml/min. The mobile phase used was 0.1% phosphoric acid in water (A) versus 70% acetonitrile in water (B) for a total running time of 40 min, and the gradient changed as follows: solvent B started at 20% and increased immediately to 50% in 30 min. After that, elution was conducted in the isocratic mode with 50% solvent B within 5 min. Finally, solvent B decreased to 20% until the end of running time.

2.5. Experimental design

Fifty male Wistar rats weighing between 150 and 170 g were purchased from Pasteur Institut (Tunis, Tunisia). The animals were maintained in individual stainless steel cages under controlled conditions (23 ± 1 °C, 12-h light–dark cycle, and had access to a standard laboratory diet (SICO, Sfax, Tunisia) and drinking water). The animals were housed according to the EEC 609/86 Directives regulating the welfare of experimental animals.

The rats were randomly divided into five experimental groups (*n* = 10). Group 1 was fed a standard laboratory diet (CD) (Table 1). Group 2 was fed a cholesterol-rich diet (HCD) (normal diet supplemented with 1% cholesterol and 0.25% bile salts). Groups 3, 4 and 5 received HCD with, respectively; olive leaf extract, acid hydrolysate extract and enzymatic hydrolysate extract (quantity succeeding to 3 mg/kg of body weight oleuropein, hydroxytyrosol and oleuropein aglycone, respectively) which was dissolved in drinking water. The experiment was conducted over a period of 16 weeks. The body weight was measured every

Table 1
Composition of the control diet (g/kg)

Diet ingredient	Concentration
Casein	200
DL-Methionine	3
Cornstarch	393
Sucrose	154
Cellulose	50
Mineral mix[a]	35
Vitamin mix[b]	10

[a] Mineral mixture contained (mg/kg of diet) the following: $CaHPO_4$, 17,200; KCl, 4000; NaCl, 4000; MgO, 420; $MgSO_4$, 2000; Fe_2O_3, 120; $FeSO_4{\cdot}7H_2O$, 200; trace elements, 400; $MnSO_4{\cdot}H_2O$, 98; $CuSO_4{\cdot}5H_2O$, 20; $ZnSO_4{\cdot}7H_2O$, 80; $CoSO_4{\cdot}7H_2O$, 0.16; KI, 0.32; sufficient starch to bring to 40 g (per kg of diet).

[b] Vitamin mixture contained (mg/kg of diet) the following: retinol, 12; cholecalciferol, 0.125; thiamine, 40; riboflavin, 30; pantothenic acid, 140; pyridoxine, 20; inositol, 300; cyano-cobalamin, 0.1; menadione, 80; nicotinic acid, 200; choline, 2720; folic acid, 10; *p*-aminobenzoic acid, 100; biotin, 0.6; sufficient starch to bring to 20 g (per kg of diet).

day. At the end of the experimental period, rats were killed by decapitation and blood samples were collected to determine the plasma lipid profile. The livers, hearts, kidneys and aorta were removed and rinsed with physiological saline solution. All samples were stored at −80 °C until analyzed.

2.6. Serum lipids

Concentrations of total cholesterol (TC), total triglycerides (TG), low-density lipoprotein (LDL-C) and high-density lipoprotein HDL-C in serum were determined by enzymatic colorimetric methods using commercial kits (Kyokuto Pharmaceutical, Japan). The atherosclerotic index (AI), defined as the ratio of LDL-C, and HDL-C was calculated for all the groups.

2.7. Antioxidant enzyme activities

CAT and SOD activities were evaluated in liver tissue. In practice, 1 g of liver tissue was homogenized in 10 ml KCl 1.15% and centrifuged at 7740 × *g* for 15 min. The supernatants were removed and stored at −80 °C for analysis. The amount of protein in supernatant was measured, according to the method of Bradford [21] using bovine serum albumin as standard. CAT activity was measured using the method of Regoli and Principato [22]. Briefly, 20 μl of the supernatant was added to a cuvette containing 780 μl of a 50-M potassium phosphate buffer (pH 7.4), then, the reaction was initiated by adding 200 μl of 500 mM H_2O_2 to make a final volume of 1.0 ml at 25 °C. The decomposition rate of H_2O_2 was measured spectrophotometrically at 240 nm for 1 min. A molar extinction coefficient of 0.0041 $mM^{-1}\,cm^{-1}$ was used to determine the CAT activity. One unit of CAT was defined as the 1 mole H_2O_2 decrease/((mg protein) min). SOD activity was measured according to the method of Marklund and Marklund based on pyrogallol oxidation by superoxide anion (O^{2-}) and its dismutation by SOD [23]. 25 μl of the supernatant was mixed with 935 μl of Tris–EDTA–HCl buffer (pH 8.5) and 40 μl of 15 mM pyrogallol. The activity was measured after 45 s at 440 nm. One unit was determined as the amount of enzyme inhibiting the oxidation of pyrogallol by 50%. The activity was expressed as units/mg protein.

2.8. ABTS assay in the serum samples

The Trolox equivalent antioxidant capacity (TEAC) assay, measuring the reduction of the ABTS radical cation by antioxidants, was derived from the method previously described [24] with minor modifications. Briefly, ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. For the study $ABTS^{\bullet+}$ solution was diluted with phosphate buffer saline pH 7.4 (PBS) to an absorbance of 0.70 (±0.02) at 734 nm. After addition of 2.0 ml of diluted $ABTS^{\bullet+}$ solution to 50 μl of serum, or Trolox standard, the reaction mixture was incubated for 6 min in a glass cuvette at 30 °C. The decrease in absorbance was recorded at 734 nm. All measurements were performed in triplicate. The free radical scavenging capacity of the biological sample, calculated as inhibition percentage of $ABTS^{\bullet+}$, was equated against a Trolox standard curve prepared with different concentrations (1.5–30 μmol/l). The results are expressed as μM of Trolox equivalents.

2.9. TBARS assay

As a marker of lipid peroxidation, the TBARS (thiobarbituric acid-reactive substances) concentrations were measured in liver, heart, kidneys and aorta homogenates using the method of Park et al. [25]. Briefly, 200 μl of a 10% (w/v) tissue homogenate solution was mixed with 600 μl of distilled H_2O and 200 μl of 8.1% (w/v) SDS, vortexed, and incubated for 5 min at room temperature. The reaction mixture was heated at 95 °C for 1 h after the addition of 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% (w/v) TBA. After cooling the reaction, 1.0 ml of distilled water and 5.0 ml of butanol:pyridine (15:1) solution were added and vortexed. The mixture was centrifuged at 1935 × *g* for 15 min and the resulting coloured layer was measured at 532 nm using malondialdehyde (MDA) made by the hydrolysis of 1,1,3,3-tetramethoxypropane as standard.

2.10. Histopathological analysis

At the time of sacrifice, the liver, heart and aorta tissues were removed and fixed in 10% formaldehyde solution. The washed tissues were dehydrated in increasing gradient of ethanol and finally cleared in toluene. The tissues were then embedded in molten paraffin wax. Sections were cut at 5 μm thickness and stained with haematoxylin and eosin. The sections were then viewed under light microscope to detect eventual histopathological changes.

2.11. Statistical analysis

Data are given as means ± S.E. Statistical differences were calculated using a one-way analysis of variance (ANOVA), followed by Student's *t*-test. Differences were considered significant at $P < 0.05$.

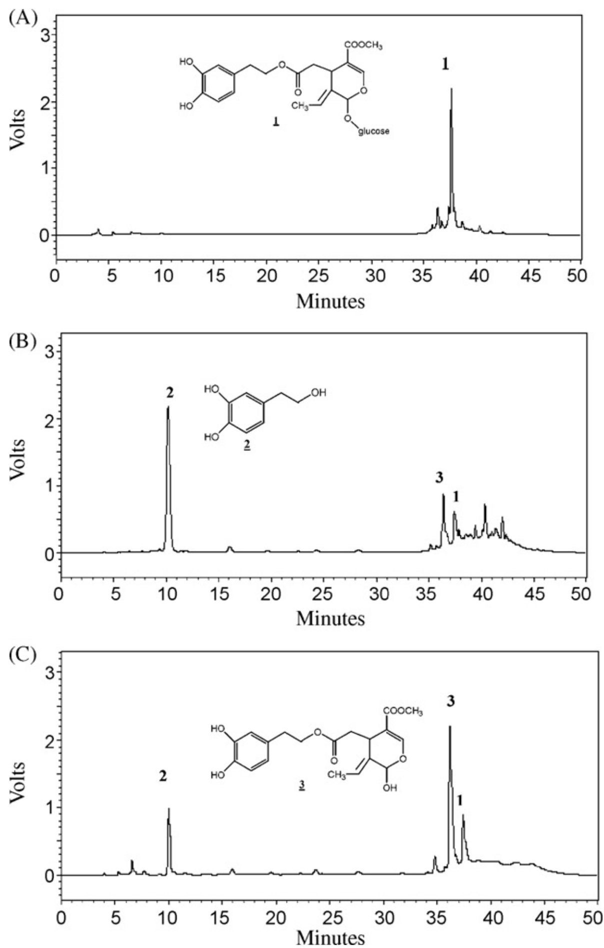

Fig. 1. HPLC chromatograms at 280 nm of olive leaf extracts (A) olive leaf extract after acid hydrolysis (B) and olive leaf extract after enzymatic hydrolysis (C). (1): Oleuropein; (2) hydroxytyrosol; (3) oleuropein aglycone.

3. Results

3.1. Olive leaves and hydrolysate extract characterization

Fig. 1 shows that a high concentration of oleuropein (4.32 g/100 g dry weight) was obtained from Chemlali olive leaves. This concentration was far in excess compared to the other phenolics. The identification of oleuropein was based on comparison of the chromatographic retention time and UV absorbance spectra with those of an authentic standard, and confirmed by using an LC–MS system operating in positive mode. The mass spectrum exhibited a molecular ion at m/z 541 with fragments at m/z 137, 165, 225, 243, 361 and 379, which was consistent with the known fragmentation scheme for oleuropein [26]. Acid treatment of aqueous methanolic leaf extracts induced hydrolysis of the more complex phenolic molecules. The most notable effect seen was the increase in hydroxytyrosol concentration, coupled with the concomitant disappearance of oleuropein. Chemically, oleuropein is the ester of oleoside 11-methyl ester and hydroxytyrosol. The later is the principal product of oleuropein degradation. Indeed, the HPLC profile of phenols in the olive leaf extract after acid hydrolysis showed that hydroxytyrosol was the major compound in the hydrolysate extract (Fig. 1) where its concentration reached 1.4 g/100 g dry weight. The identification of hydroxytyrosol was confirmed by LC–MS. The mass spectrum exhibited a molecular ion at m/z 155 with fragments at m/z 137, 119, 99, and 91, which was consistent with the known hydroxytyrosol fragmentation scheme previously reported [20].

The biotransformation of olive leaf extract by β-glucosidase carried out at 37 °C and pH 7 showed a small amount of hydroxytyrosol and high concentration of oleuropein aglycone after 2 h of incubation time (Fig. 1). The concentration of the latter reached 3.82 g/100 g dry weight. The products obtained by this procedure were identified by LC–MS in the negative mode, showing the presence of hydroxytyrosol and oleuropein aglycone. The mass spectrum in Fig. 2 showed a peak m/z 377 at 9.2 corresponding to oleuropein aglycone. The fragmentation of such a peak led to fragments having molecular masses m/z 345, 307 and 275 resulting from consecutive with those published by Savarese et al. [27].

3.2. Body and organ weights

There were no significant differences between the body weight increases in all groups throughout the treatment (data not shown). Also, no differences were observed in the heart and kidney/body weight ratios. However, the liver/body weight ratio increased in rats fed a cholesterol-rich diet (HCG) compared with those fed a control diet (CD) (Fig. 3). Olive leaf extract and its acid and enzymatic hydrolysate extracts significantly decreased the liver/body weight ratio compared to the HCD group.

3.3. Serum lipids

After the treatment, the TC, TG and LDL-C concentrations of rats fed a cholesterol-rich diet (HCD) showed a significant increase compared with control group (CD) (Fig. 4). However, a decrease of HDL-C concentration of rats in the HCD group was observed ($P < 0.05$). Rats orally administrated with olive leaf extract, acid hydrolysate extract and enzymatic hydrolysate extract had lower concentrations of TC, TG and LDL-C than rats receiving an HCD. Olive leaves, chemical hydrolysate and enzymatic hydrolysate extracts reduced the TC, TG and LDL-C levels by 37%, 21%, 37%; 44%, 23%, 38% and 36%, 19.22%, 35.97%, respectively. Moreover, the treatment of HCD fed groups with phenolic extracts significantly re-established their HDL-C level ($P < 0.05$). As a consequence, the atherogenic index (AI) was significantly reduced by oral administration of phenolic compounds present in the olive leaf extracts and its corresponding hydrolysates ($P < 0.05$). In fact phenolic compounds reduced the AI by 58%, 59% and 51%, respectively.

3.4. Hepatic antioxidant enzyme activities

The CAT and SOD activities significantly decreased in livers of rats fed a cholesterol-rich diet compared to those fed a control diet. The decrease was significantly restored

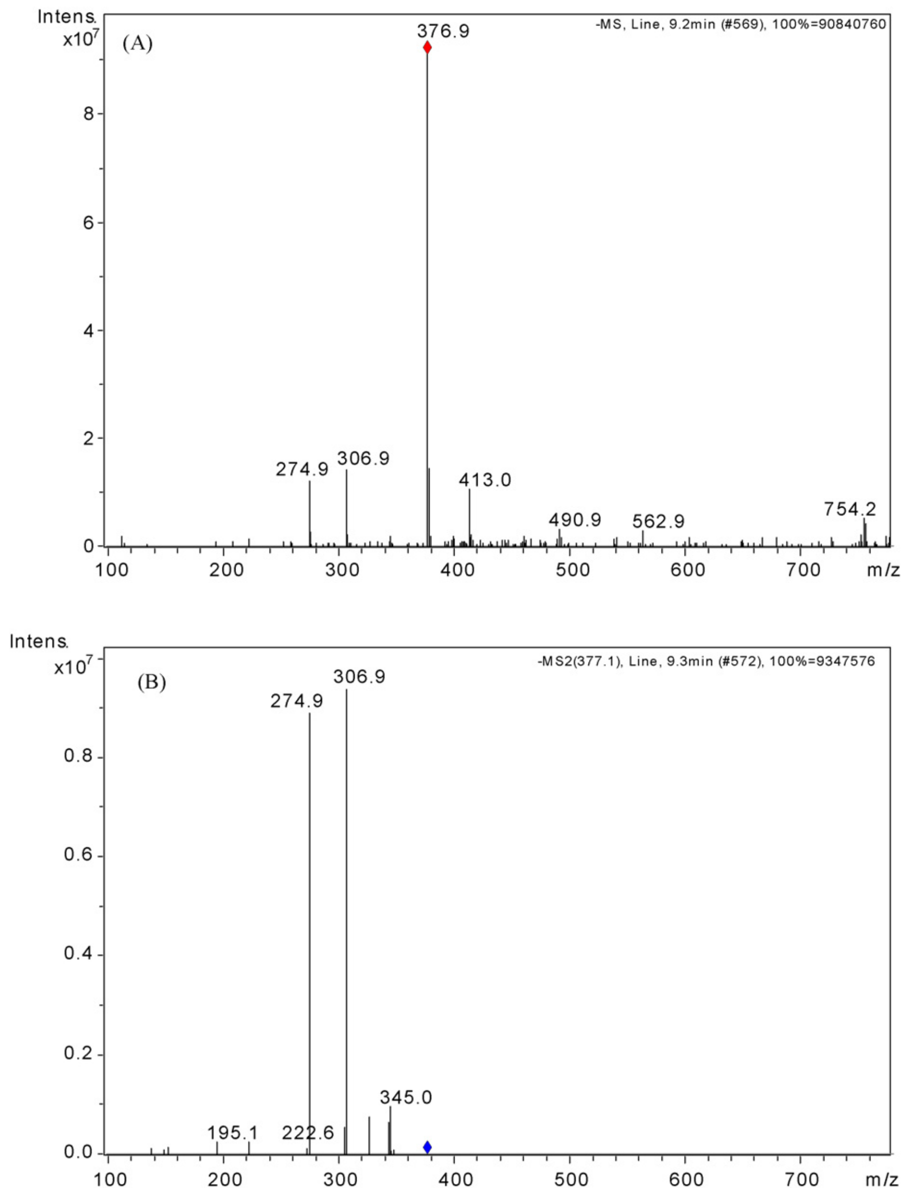

Fig. 2. Mass spectrum of 9.2 min total ion chromatographic peak in enzymatic hydrolysate leaf extract of Chemlali cultivar: (A) MS and (B) MS^2.

($P < 0.05$) in the presence of the olive leaves and the hydrolysate extracts (Fig. 5).

3.5. Serum antioxidant potential

ABTS radical cation scavenging ability (Fig. 6) in serum of a cholesterol-rich diet fed rats was significantly low in comparison to rats fed normal diet (CD). Oral administration of olive leaf extract and hydrolysate extracts allowed to repair the impairment between both groups.

3.6. TBARS levels

The olive leaf-deriving preparations were revealed to slow down the lipid peroxidation (Fig. 7). The TBARS levels were significantly increased ($P < 0.05$) in liver, heart, kidneys and aorta within animals fed high cholesterol diet compared to the control diet group. This increase was significantly reduced after treatment with phenolic extracts from olive leaves. In fact TBARS concentrations in liver, heart, kidneys and aorta were reduced by 15.97%, 14.8%, 12.85% and 22.19%, 17.91%, 15.3%, 14.37 and 20.63% and 16.81%, 15.31%, 13,33% and 21.72%, respectively in groups 3, 4 and 5.

3.7. Histopathological analysis

The abnormal cardiac histology in response to the hypercholesterolemic diet is presented in Fig. 8. The photomicrographs show thickening of cardiac muscle cells and

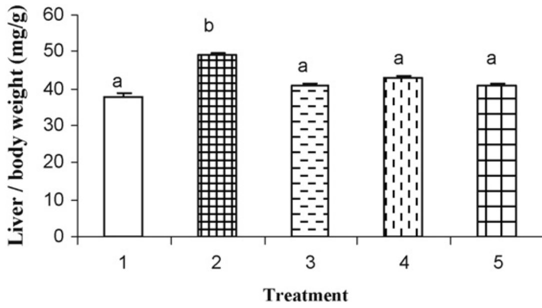

Fig. 3. Effects of olive leaf extracts on the liver:body weight ratio. Group: 1, CD (standard diet); 2, HCD; 3, HCD + olive leaf extract; 4, HCD + acid hydrolysate extract; 5, HCD + enzymatic hydrolysate extract. Each bar represents mean ± S.E. from 10 rats. Bars with different letters differ significantly; $P < 0.05$.

the onset of cardiac muscle hypertrophy. The figure depicts a more or less normal cellular architecture of the heart muscle cells, with notable absence of cardiac muscle hypertrophy in rats fed hypercholesterolemic diet supplemented with phenolic extracts.

In the same way, liver tissue was also influenced by olive leaf extracts. The photomicrographs pointed out vacuolated hepatocytes with the nucleus being pushed to the periphery, and fatty cyst; an overall picture of fatty liver. Polyphenol-rich extracts allowed to a certain extent, to overcome the hepatic architecture aberrations with the preservation of parenchymal structure and occasional appearance of fat cells (Fig. 9).

The high cholesterol diet was also responsible for the development of lesion in the aortic wall. Such anomalies were significantly improved in the olive leaf extract-treated group (Fig. 10).

Fig. 4. Effects of olive leaf extracts on rat total cholesterol (TC) (A), triglycerides (TG) (B), low-density lipoprotein cholesterol (LDL-C) (C), high-density lipoprotein cholesterol (HDL-C) (D) and atherogenic index (AI) (E) levels. Group: 1, CD (standard diet); 2, HCD; 3, HCD + olive leaf extract; 4, HCD + acid hydrolysate extract; 5, HCD + enzymatic hydrolysate extract. Each bar represents mean ± S.E. from 10 rats. Bars with different letters differ significantly; $P < 0.05$.

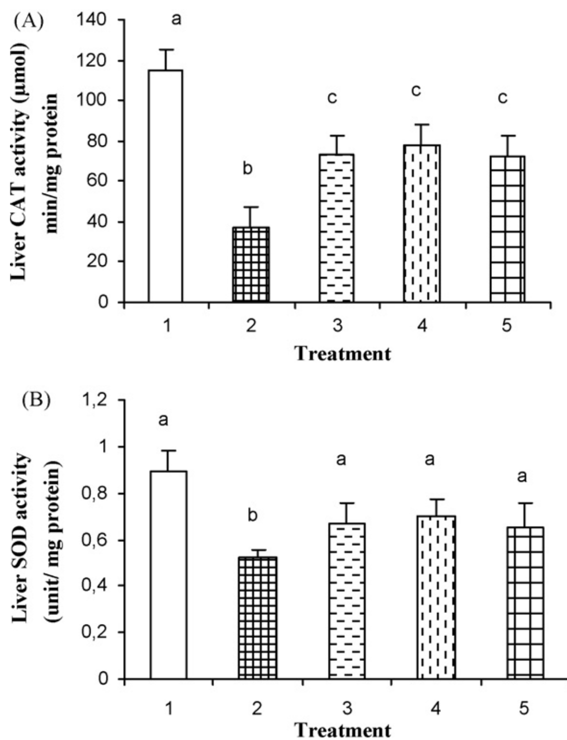

Fig. 5. Effects of olive leaf extracts on CAT (A) and SOD (B) activities in liver. Group: 1, CD (standard diet); 2, HCD; 3, HCD + olive leaf extract; 4, HCD + acid hydrolysate extract HCD + acid hydrolysate extract; 5, HCD + acid hydrolysate extract. Each bar represents mean ± S.E. from 10 rats. Bars with different letters differ significantly; $P < 0.05$.

4. Discussion

In this study, Wistar rats fed a cholesterol-rich diet for 16 weeks were used to study the effects of polyphenol-rich olive leaf extracts on serum lipid levels and antioxidant enzymes activities in liver. Oleuropein, oleuropein aglycone and hydroxytyrosol-rich extracts recovered from olive leaves were tested for their lipid-lowering, antioxidative and protective effects against hypercholesterolemia and atherosclerosis. Olive leaf derivatives have been recognized

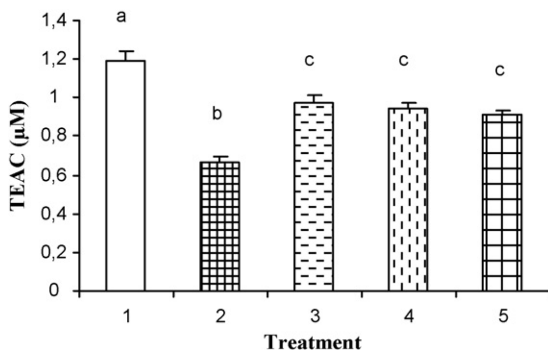

Fig. 6. Antioxidant activity in control and treated rat's serum. Group: 1, CD (standard diet); 2, HCD; 3, HCD + olive leaf extract; 4, HCD + acid hydrolysate extract; 5, HCD + enzymatic hydrolysate extract. Each bar represents mean ± S.E. from 10 rats. Bars with different letters differ significantly; $P < 0.05$.

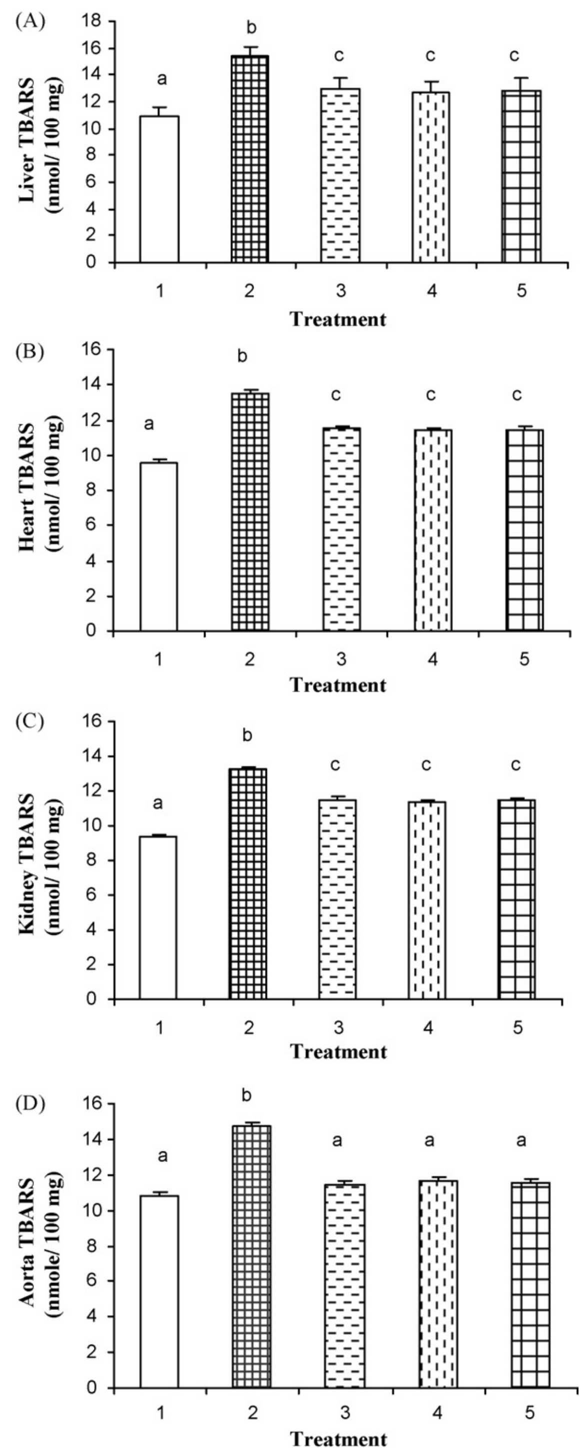

Fig. 7. Effects of olive leaf extract (3) and hydrolysate extracts (4 and 5) on rat liver (A), heart (B), kidney (C), and aorta (D) TBARS levels. Each bar represents mean ± S.E. from 10 rats. Bars with different letters differ significantly; $P < 0.05$.

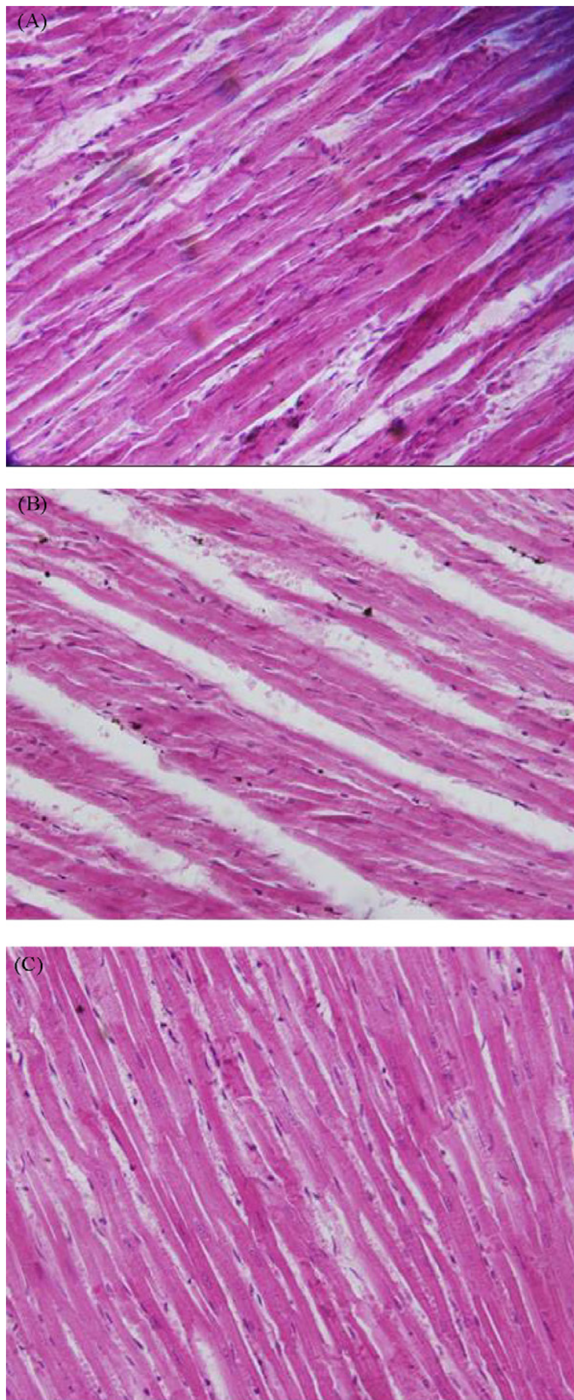

Fig. 8. Histopathological observation of heart tissue: (A) group 1: control, showing normal myocardium; (B) group 2: HCD-fed rats showing marked hypertrophy with cellular and nuclear enlargements; (C) groups 3, 4 and 5: olive leaf-and hydrolysate extract-supplemented rats showing near normal myocardium with moderate reversal of hypertrophic changes (hematoxylin–eosin, H&E staining, 400×).

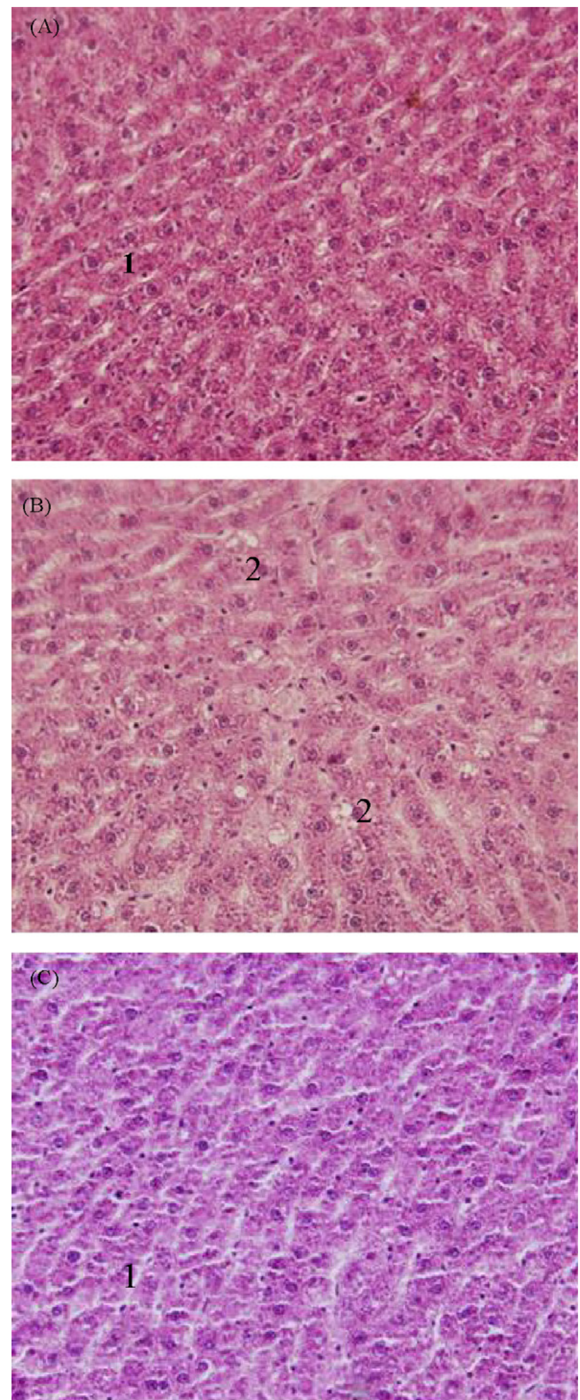

Fig. 9. Microscopic views of transverse sections of liver in normal (A), high-cholesterol fed (B), and olive leaf extract- or hydrolysate extract-supplemented rats (C) (hematoxylin–eosin, H&E staining, 400×). Key: 1, normal hepatocyte; 2, hepatic lipid droplet.

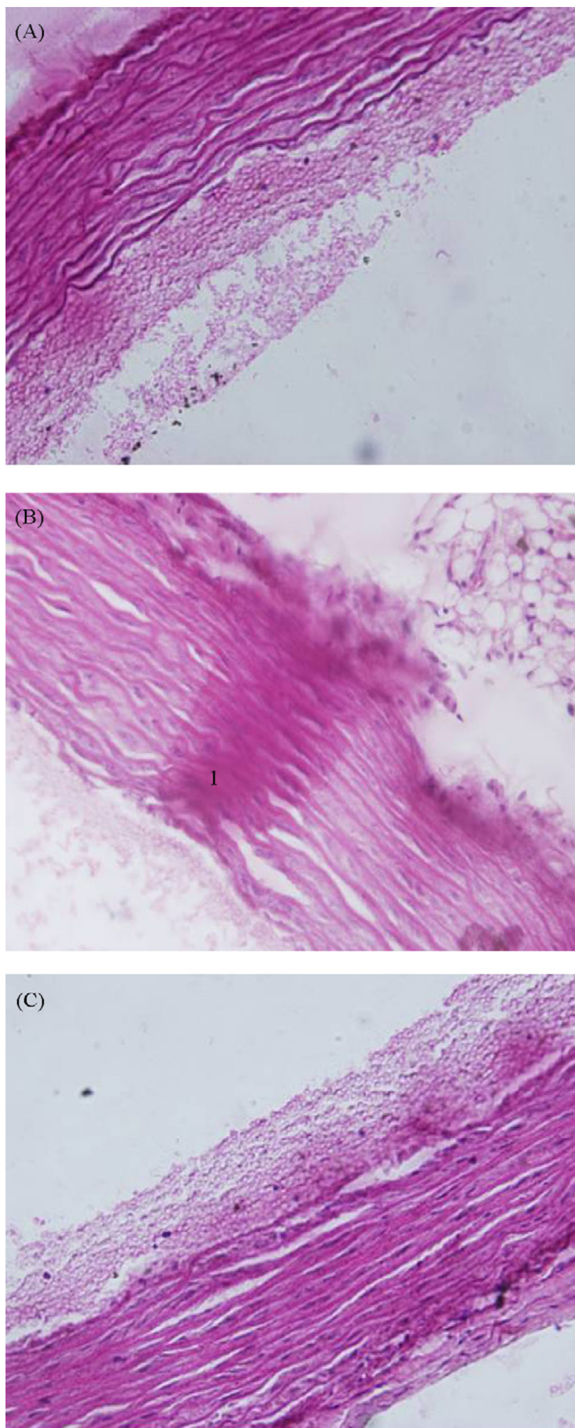

Fig. 10. Microscopic views of transverse sections of aorta in normal (A), high-cholesterol fed (B), and olive leaf extract- or hydrolysate extract-supplemented rats (C) (hematoxylin–eosin, H&E staining, 400×). Key: 1, lesion of aortic wall.

as important components of a healthy diet because of their phenolic content. The principal active component in olive leaf extract is oleuropein, a natural product of the secoiridoid group. Its concentration in olive leaf extract was high compared to the other phenolics. Various extraction methods were used to extract this compound and its amount varied with the olive cultivar [20]. Acid hydrolysis of olive leaf extract leads to a high quantity of hydroxytyrosol. Indeed, there are many sources of hydroxytyrosol in olive leaves, such as hydroxytyrosol-4-β-D-glucoside, oleuroside, verbascoside, demethyloleuropein, and oleuropein as the major component [19]. In this context, several methods have been developed to produce hydroxytyrosol because it is not commercially available. The method developed by Bouaziz et al. [19] consisted in C-18 silica gel chromatography using an FPLC system. These researchers have produced 2.3 g of pure hydroxytyrosol per 100 g of fresh olive leaves. On the other hand, enzymatic hydrolysis of olive leaf extract showed that hydroxytyrosol and oleuropein aglycone were the major obtained compounds. The aglycone, recovered by oleuropein hydrolysis, is well known as pharmacologically active molecule. In fact it is described for its potential application as an antioxidant and antimicrobial agent in some fairly common diseases of olive trees [19,28].

Olive leaf extract and its corresponding acid and enzymatic hydrolysate extracts were tested for their lipid-lowering and antioxidant activities in hypercholesterolemic rats. In effect, cholesterol virtually synthesized by all tissues in humans assumes a number of essential functions in the body. For instance, it is a component of cell membrane and it controls the membrane fluidity, as well as permeability to small ions and molecules [29]. Cholesterol also functions as a precursor for the synthesis of bile acid, steroid hormones and vitamin D [29]. It is therefore of critical importance for body tissues to be assured a continuous supply of cholesterol. However, dietary cholesterol has been reported to contribute significantly to elevation of plasma cholesterol [30]. The presence of increased cholesterol levels in the diet has been demonstrated to elevate serum and aortic tissue cholesterol and, as such, increase aortic atherosclerosis [30]. Similarly, Li et al. [31] established a linear correlation between dietary cholesterol intake and mortality from coronary heart disease (CHD). Furthermore, cholesterol- and cholic acid-supplemented diets feeding has been shown to accelerate the formation of aortic plaques in the apolipoprotein E null mutant mouse [32].

In the present study, rat body weight gains were not significantly different among the groups investigated. This observation is in accord with the study of Ander et al. [33] who showed that the body weight of hypercholesterolemic rats was not significantly different from that of control animals. Data related with organs weight showed no significant difference among all groups except the liver:body weight ratio which significantly increased in high-cholesterol fed rats. This could be related to an accumulation of lipids such as cholesterol and triglycerides in the liver [34].

In this investigation, simultaneous administration of oleuropein, oleuropein aglycone and hydroxytyrosol-rich extracts with cholesterol resulted in reductions in serum

TC, TG and LDL-C levels compared with cholesterol-fed rats. Similarly, these phenolics increased the HDL-C concentration by over 60%. Lowering levels of TC and LDL-C and improving level of HDL-C has been linked to a lower risk of CHD [29]. 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 [35]. Also, high HDL-C levels may compete with LDL receptor sites on arterial smooth muscle cells and thus inhibit the uptake of LDL [35]. 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 [35]. Therefore, the results of the present study indicate that olive leaf phenolics may reduce the incidence of CHD. These data are in agreement with the study of Gorinstein et al. [36] 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 [37] or stimulation of the biliary secretion of cholesterol and cholesterol excretion in the faeces [38].

Nowadays, it is established that oxidative stress is one of the causative factors that link hypercholesterolemia with atherogenesis [39]. Hypercholesterolemia leads to increased cholesterol accumulation in cells thereby activating the production of oxygen free radicals. Thus, cells are extremely vulnerable to these oxidative challenges and hypercholesterolemia [40].

In the present study, a significant decrease in the serum antioxidant potential was observed in rats fed with cholesterol compared to control group. However, the decrease in the serum antioxidant status in rats treated with olive leaf phenolics was significantly less than in HCD animals. Moreover, the hepatic CAT and SOD activities were reestablished by olive leaves and its hydrolysate extracts. It has been reported that a high fat diet lowered the activities of antioxidant enzymes, which is possibly due to their increased implication in fighting excessive oxidative stress in hypercholesterolemic rats [41]. The increase of hepatic antioxidant enzyme activities and the serum antioxidant potential observed in olive leaf phenolic-supplemented rats may be due to the removal toxic reactive species resulting from the high cholesterol feeding.

These results can be supported by the fact that TBARS levels in liver, heart, kidney, and aorta were significantly lowered in the olive leaf extract-supplemented groups compared to the cholesterol fed group. TBARS are the major oxidation products of peroxidized polyunsaturated fatty acids; thus, increased TBARS content is an important indicator of lipid peroxidation [42]. The reduced lipid peroxidation observed in the olive leaf extract-treated animals may be attributed to the important role of oleuropein, oleuropein aglycone and hydroxytyrosol as antioxidants. This power may be attributed to their ability to decompose free radicals by quenching reactive oxygen species and by trapping radicals before reaching their cellular targets [43].

The obtained results revealed that the administration of oleuropein-, oleuropein aglycone- and hydroxytyrosol-rich extracts induced a protective effect against experimental atherogenesis. Oleuropein aglycone and oleuropein could be converted *in vivo* by esterases into hydroxytyrosol. The later is responsible for protecting animals from atherosclerosis due to its highest antioxidant activity [44,45]. In fact, the beneficial effects of the acid hydrolysate extract seem to be due to the major compound which is hydroxytyrosol. Indeed, recently it has reported that pure hydroxytyrosol had an important hypocholesterolemic and antioxidant effect which confirms the high activity of the hydrolysate extract [46].

In conclusion, we demonstrate that polyphenols recovered from olive leaf extracts, oleuropein, hydroxytyrosol as well as oleuropein aglycone, exhibited a pronounced hypolipidemic effect, reduced the lipid peroxidation process and enhanced the antioxidant defence system in experimental atherogenic model. The activity of the used phenolic may be attributed to the 3,4-dihydroxyphenyl ethanol group which is present in the three molecules. These effects highlighted olive tree by-products as a source of antioxidants able to reduce the frequency of cardiovascular diseases.

Conflict of interest

None.

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