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Hypocholesterolemic effects of phenolic-rich extracts of *Chemlali* olive cultivar in rats fed a cholesterol-rich diet

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Abstract—This study was designed to test the lipid-lowering and the antioxidative activities of green and black olive phenolic extracts. Wistar rats fed a standard laboratory diet or a cholesterol-rich diet for 16 weeks were used. The serum lipid levels, the malondialdehyde (MDA) and the activity of superoxide dismutase (SOD) as well as that of catalase (CAT) were examined. The cholesterol-rich diet induced hypercholesterolemia that was manifested in the elevation of total cholesterol (TC) and low density lipoprotein cholesterol (LDL-C). Administration of aqueous methanol and ethyl acetate extracts of green olives and ethyl acetate extract of black olives significantly lowered the serum levels of TC and LDL-C, while increasing the serum level of high density lipoprotein cholesterol (HDL-C). Furthermore, the content of MDA in liver, heart and kidney decreased significantly after oral administration of green and black olive extracts compared with those of rats fed a cholesterol-rich diet. In addition, olive extracts increased CAT and SOD activities in liver. These results suggested that the hypocholesterolemic effect of green and black olive extracts might be due to their abilities to lower serum cholesterol level as well as to slow down the lipid peroxidation process and to enhance the antioxidant enzyme activity.

1. Introduction

Recently, the physiological effects of polyphenol-rich foods, such as fruits, vegetables, and beverages including fruit juices, wine, tea, coffee, chocolate, and olive oil have been receiving much attention as dietary sources of antioxidants that are valuable for human health. Many epidemiological studies have strongly suggested the existence of a correlation between intake of polyphenol-rich foods and low mortality due to coronary heart disease (CHD).[1–3] CHD, such as myocardial infarction and ischemic stroke, which is closely related to atherosclerosis, is a major cause 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.

Several studies have suggested oxidative damage as an important etiologic factor in atherosclerosis. In particular, according to the oxidative stress theory, oxidative modification of LDL was found to play a key role in the development of atherosclerosis.[4,5] Therefore, inhibiting this process is considered to be an important therapeutic approach. Indeed, vitamin E and probucol were reported to prevent LDL oxidation and to delay the development of atherosclerotic plaques in animal models,[6,7] suggesting the effectiveness of antioxidants for the treatment and prevention of atherosclerosis.

Olive oil, which is the principal source of fat in many Mediterranean diets, is obtained by pressing the olive fruit. This fruit contains notable amounts of minor constituents, most of which are phenolic in nature. These phenolic compounds give extra virgin olive oil its particular taste. Oleuropein is known to be the most prominent individual phenolic component of olive fruit and responsible for their intense bitterness.[8] The

Keywords: *Chemlali* olive; Phenolic extracts; Rat; Atherosclerosis; Antioxidant enzymes.
Abbreviations: CHD, coronary heart disease; TC, total cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; AI, atherosclerotic index; TG, triglycerides; CAT, catalase; SOD, superoxide dismutase; MDA, malondialdehyde; HPLC, high-performance liquid chromatography; LC–MS, liquid chromatography–mass spectrum; HCD, cholesterol-rich diet; CD, control diet; ACAT, acyl-CoA, cholesterol acyltransferase; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase.
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concentration of oleuropein varies with the olive varieties and declines with physiological development of the fruit.[8] An indirect relationship between oleuropein content in olive fruit and hydroxytyrosol, which is yielded by oleuropein hydrolysis, was observed.[9] Several studies have reported that oleuropein and hydroxytyrosol are the main antioxidant components in olive derivatives. Experiments in vitro and in vivo on laboratory animals have demonstrated that LDL-C oxidation was inhibited by olive oil constituents.[10,11] It was also observed that olive oil affects lipid peroxidation and antioxidant parameters, and lead to favorable changes in the plasma lipid status.[12] Numerous in vitro studies have shown that olive oil phenolics are able to inhibit platelet aggregation, lipoxygenases and ecasnoid production.[13,14] Recently, the good bioavailability of olive oil polyphenols has been reported.[15,16]

Most of the research on the possible cholesterol-lowering effect of olive oil has been undertaken, relative to oleic acid. However, a few studies have focused on the effect of polyphenols contained in olives on cholesterol metabolism.

The present study was designed to compare the effect of green and black olive phenolic extracts on the cholesterol metabolism and antioxidative status in the rats fed a hypercholesterolemic diet.

2. Results

2.1. Identification of phenolic compounds in olive extracts

The identification was based on comparisons of the chromatographic retention time and UV absorbance spectra of compounds in olive extracts with those of authentic standards. A representative chromatogram of the HPLC analysis of olive extracts is given in Figures 1 and 2. These chromatograms showed that oleuropein and hydroxytyrosol were the major compounds detected. Other phenolic compounds were present at lower concentrations in the olive extracts, such as tyrosol. The identification of oleuropein and hydroxytyrosol was also confirmed by using an LC–MS apparatus in the positive mode. The spectra exhibited a molecular ion at *m/z* 541 with fragments at *m/z* 137, 165, 225, 243, 361 and 379 for oleuropein and a molecular ion at *m/z* 155 with fragments at *m/z* 137, 119, 99 and 91 for hydroxytyrosol. Flavonoid compounds were also found in aqueous methanol extracts especially of black olives. Thus, eleven flavonoid aglycons and glycosides

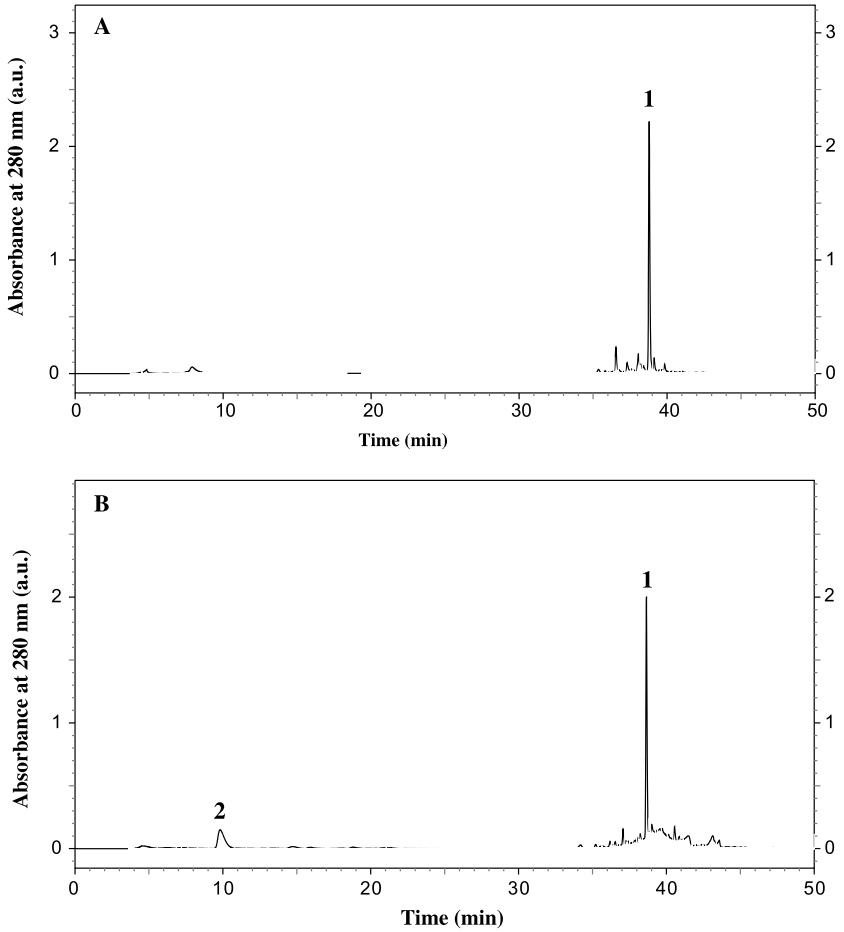

Figure 1. Chromatogram profile of aqueous methanol (A) and ethyl acetate extract (B) of *Chemlali* green olives at 280 nm. **1**: oleuropein, **2**: hydroxytyrosol.

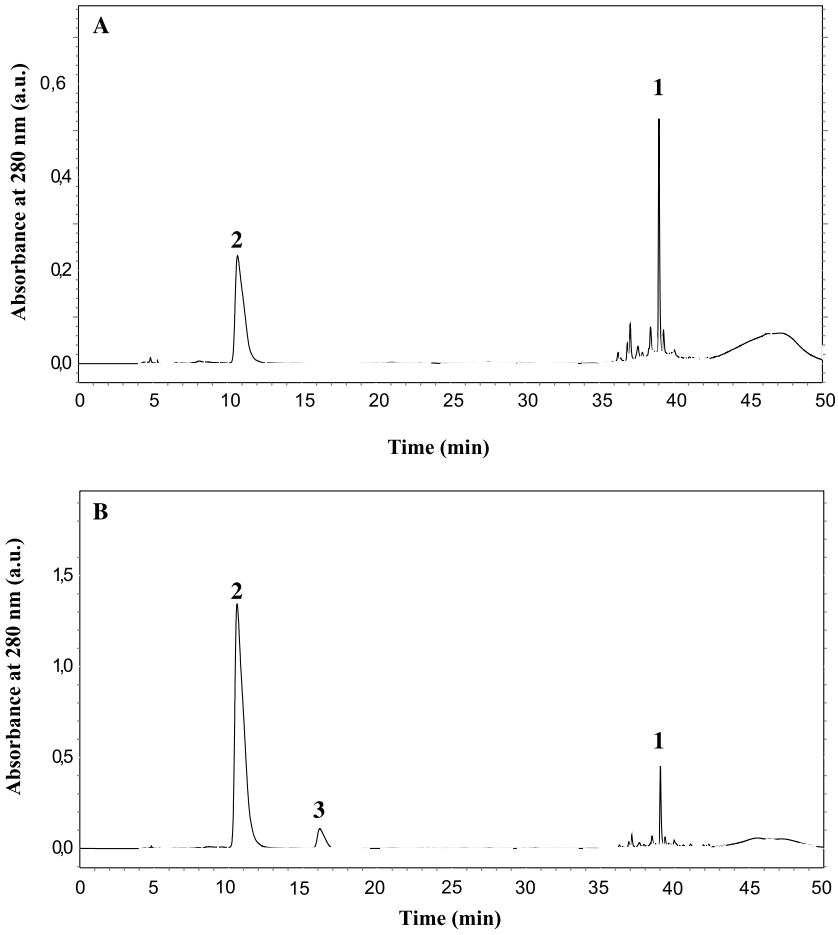

Figure 2. Chromatogram profile of aqueous methanol (A) and ethyl acetate extract (B) of *Chemlali* black olives at 280 nm. **1**: oleuropein, **2**: hydroxytyrosol, and **3**: tyrosol.

were identified in this extract. Only flavonoid glycosides were detected in the green olive aqueous methanol extract. The identification was carried out using a combination of HPLC and LC–MS apparatus on the basis of R_f values, UV spectra at 335 nm, mass spectra and by comparison of the spectra with those of available authentic standards. Table 1 lists each of the identified flavonoids in elution order. The structure assignment of flavonoids for which no standards were available was based on a systematic search for molecular ions using extracted ion mass chromatograms and comparing those with data in the literature.

Table 1. Flavonoids detected in aqueous methanol extracts of *Chemlali* olive cultivar with their retention times, UV spectra and mass spectral data

No.	Flavonoid	Retention time (min)[a]	UV λ_{max} (nm)	$[M+H]^+$ (*m*/*z*)[b]	$[I+H]^+$ (*m*/*z*)[c]	$[A+H]^+$ (*m*/*z*)[d]
1[e]	Luteolin 7-*O*-glucoside	13.2	350, 267sh, 255	449	—	287
2[e]	Luteolin 7-*O*-rutinoside	13.4	351, 266sh, 255	595	449	287
3[e]	Quercetin 3-*O*-glucoside	13.6	355, 256	465	—	303
4[e]	Rutin	13.7	355, 256	611	465	303
5[e]	Apigenin 7-*O*-rutinoside	14.4	338, 266	579	433	271
6[e]	Chrysoeriol 7-*O*-glucoside	14.9	350, 267sh, 253	463	—	301
7[e]	Luteolin 4′-*O*-glucoside	15.1	339, 268, 247sh	449	—	287
8[f]	Quercetin	17.3	371, 255	303		
9[f]	Luteolin	18.0	351, 266sh, 254	287		
10[f]	Apigenin	19.5	337, 267	271		
11[f]	Chrysoeriol	19.6	345, 267sh, 252	301		

[a] For HPLC solvent system, see Section 4.
[b] APCI-MS (positive mode) data for the protonated molecular ion.
[c] APCI-MS (positive mode) data for protonated intermediate molecular ions.
[d] APCI-MS (positive mode) data for the protonated aglycone ion.
[e] Flavonoids identified in aqueous methanol extracts of green and black olives.
[f] Flavonoids identified only in aqueous methanol extract of black olives.

Table 2. Oleuropein and hydroxytyrosol composition in olive extracts *Chemlali* cultivar (% in weight of dry ethyl acetate and methanol water extract)

Compounds	Green olive		Black olive	
	Aqueous methanol extract	Ethyl acetate extract	Aqueous methanol extract	Ethyl acetate extract
Oleuropein	89.65	81.73	30.27	25.22
Hydroxytyrosol	3.67	16.74	9.20	46.54

2.2. Quantification of major compounds

The concentrations of the major phenolic compounds (oleuropein and hydroxytyrosol) in olive extracts are given in Table 2. Table 2 shows that a high concentration of oleuropein was obtained in the first stage of maturation, that is, green olives. It reached 89.65% and 81.73% in aqueous methanol and ethyl acetate extract, respectively. The level of oleuropein decreased and reached a minimum of 30.27% at the last stage of maturation, that is, black olives. Chemically, oleuropein is the ester of oleoside 1,1-methyl ester and 3,4-dihydroxyphenylethanol (hydroxytyrosol). Hydroxytyrosol is the principal product of oleuropein degradation during the maturation of fruit. Indeed, hydroxytyrosol concentration began at a low level and increased as the maturation of the olive progressed. Table 2 shows that the extraction yield of hydroxytyrosol was higher in the ethyl acetate extract of black olives than green olives. More precisely, hydroxytyrosol content in olive methanol and ethyl acetate extracts varied from 3.67% to 16.74% for green olives and from 9.2% to 46.54% of black olives, respectively. The existence of a higher amount of hydroxytyrosol in ethyl acetate extracts than in aqueous methanol extracts can be explained by the fact that ethyl acetate is a less polar solvent than methanol/water. It means that monomeric phenols are well soluble in ethyl acetate, while aqueous solvents are an adequate medium for glycoside polyphenol compounds.

2.3. Body and organ weights

The body weight increased in all groups throughout the treatment without any significant differences between them (data not shown). There were no differences in the heart and kidney:body weight ratios (Fig. 3). However, the liver:body weight ratio increased in rats fed a cholesterol-rich diet (HCD) compared with the rats fed a control diet (CD) (Fig. 3). In HCD groups of rats, the liver:body weight ratio decreased significantly in the presence of phenolic compounds extracted from olives compared with those of HCD group.

2.4. Serum lipids

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 cholesterol-rich diet (HCD) showed a significant increase compared with the rats fed normal diet (CD). However, a decrease of HDL-C concentration of rats in the HCD group was observed ($P < 0.05$). Rats having received an oral administration of green and black olive extracts had lower concentrations of TC and LDL-C than those of rats receiving an HCD. In particular, the administration of ethyl acetate and aqueous methanol extracts of green olives and ethyl acetate extract of black olives reduced

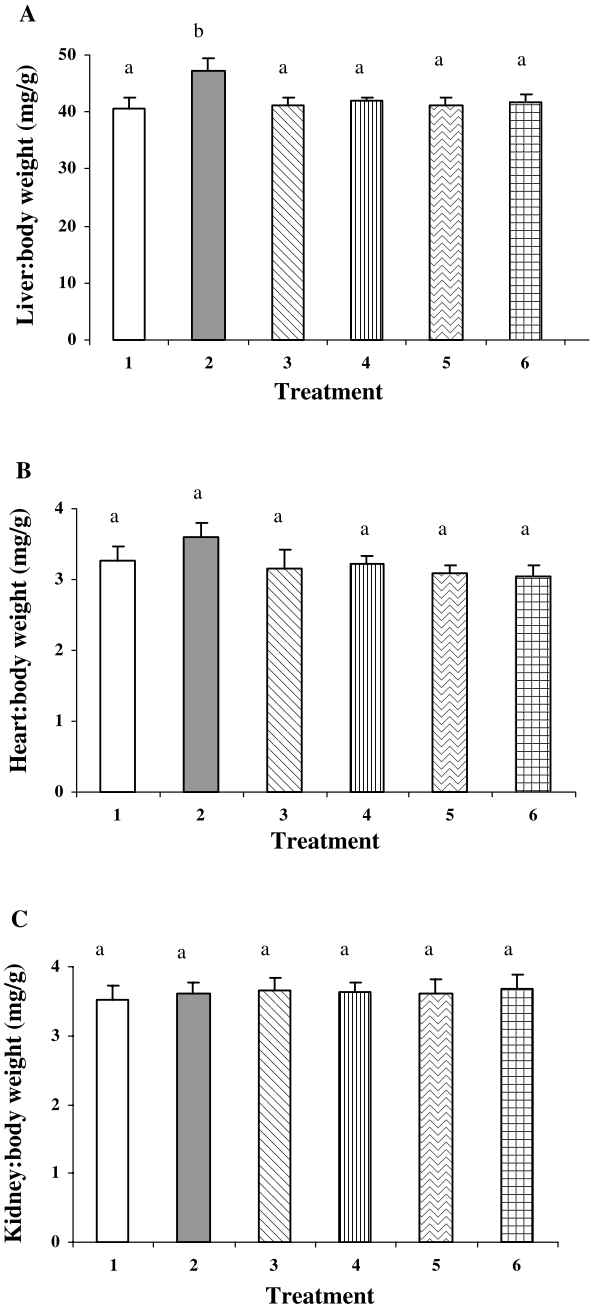

Figure 3. Effect of olive extracts on the liver (A), heart (B) and kidney (C):body weight ratios. **1**: control diet (CD) (standard diet), **2**: high cholesterol diet (HCD), **3**: HCD + methanol extract of green olives, **4**: HCD + methanol extract of black olives, **5**: HCD + ethyl acetate extract of green olives, and **6**: HCD + ethyl acetate extract of black olives. Each bar represents mean ± SE from eight rats. Bars with different letters differ, $P < 0.05$.

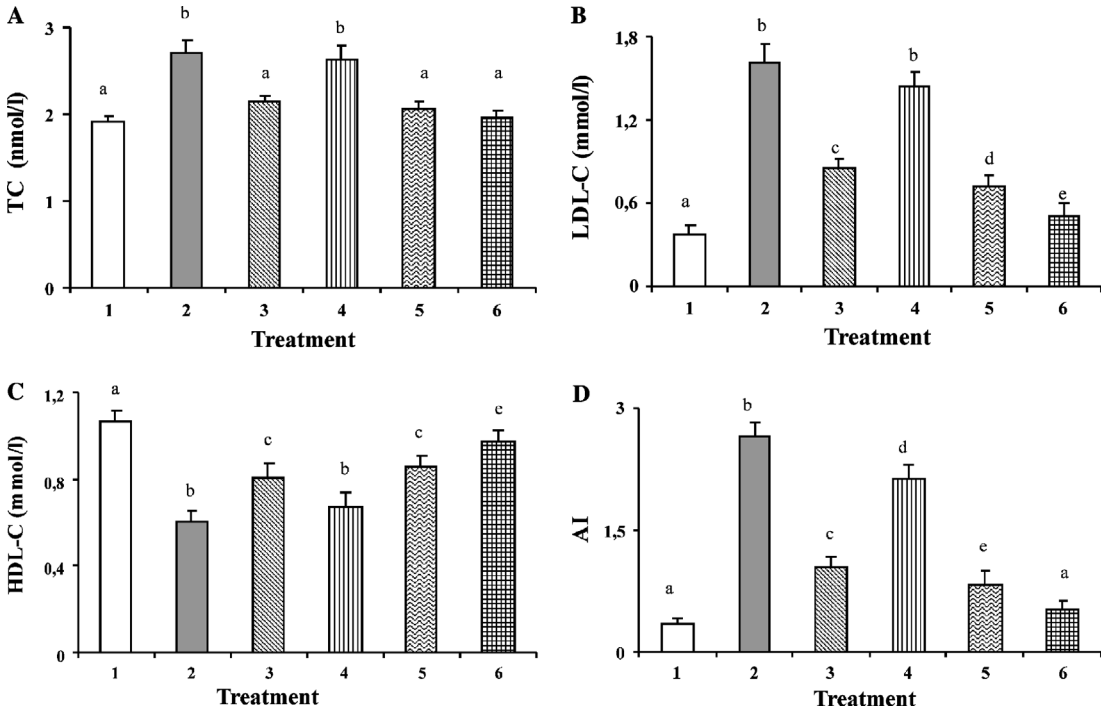

Figure 4. Effects of olive extracts on rat TC (A), LDL-C (B), HDL-C (C) and AI (D) levels. **1**: control diet (CD) (standard diet), **2**: high cholesterol diet (HCD), **3**: HCD + methanol extract of green olives, **4**: HCD + methanol extract of black olives, **5**: HCD + ethyl acetate extract of green olives, and **6**: HCD + ethyl acetate extract of black olives. (D) AI, atherogenic index, AI = LDL-C/HDL-C. Each bar represents mean ± SE from eight rats. Bars with different letters differ, $P < 0.05$.

the TC and LDL-C levels by 24.2%, 55.9%; 20.5%, 47.1% and 27.1, 68.8%, respectively. Although the concentration of HDL-C in phenolic-treated rats never exceeded that of rats fed the standard laboratory diet, the concentration of HDL-C of rats treated with ethyl acetate and aqueous methanol extracts of green olives and ethyl acetate extract of black olives increased significantly compared with those of rats in the HCD group ($P < 0.05$). The atherogenic index (AI) was significantly reduced by orally administering phenolic compounds extracted from green and black olives. The TC concentrations and AI of animals treated with ethyl acetate extract of black olives were comparable to that of the control group ($P > 0.05$).

The difference in the triglycerides level between the CD group and HCD groups of rats was not significant (data not shown).

2.5. Hepatic antioxidant enzyme activities

The CAT and SOD activities significantly decreased (−24%) in livers of rats fed a cholesterol-rich diet compared to control diet group. The decrease was significantly restored ($P < 0.05$) in the HCD rats supplemented with ethyl acetate and aqueous methanol extracts of green olives and ethyl acetate extract of black olives (Fig. 5).

2.6. MDA levels

The MDA levels were significantly increased ($P < 0.05$) in liver, heart and kidneys of the animals fed high cholesterol diet compared to the control diet group. In HCD groups of rats, this increase was significantly reduced in the presence of extracts rich in phenolics, such as ethyl acetate and methanol extracts of green olives and ethyl acetate extract of black olives (Fig. 6).

3. Discussion

The present study investigated the effect of green and black olive phenolic extracts on the cholesterol metabolism and antioxidative status in the rats fed a hypercholesterolemic diet. The results suggested that serum lipid-lowering and antioxidative effects of olive phenolics supplements were very potent in cholesterol-rich diet fed rats.

In the current study, only ethyl acetate and methanol extracts of green olives and ethyl acetate of black olives exhibited a protective effect against atherosclerosis. The methanol extract of black olives, which contained fewer hydroxytyrosol and oleuropein, showed very low activity. The hypocholesterolemic and antioxidative effects of olive extracts could be related to its hydroxytyrosol and oleuropein content. Indeed, previous reports have shown that those phenolics are the main antioxidant components in olives, olive oil and olive byproducts.[17,18]

Other phenolic compounds, such as flavonoid glycosides and aglycones, were detected in methanolic extracts of green and black olives at a concentration lesser than 1%. The mean daily dose of flavonoids contained in

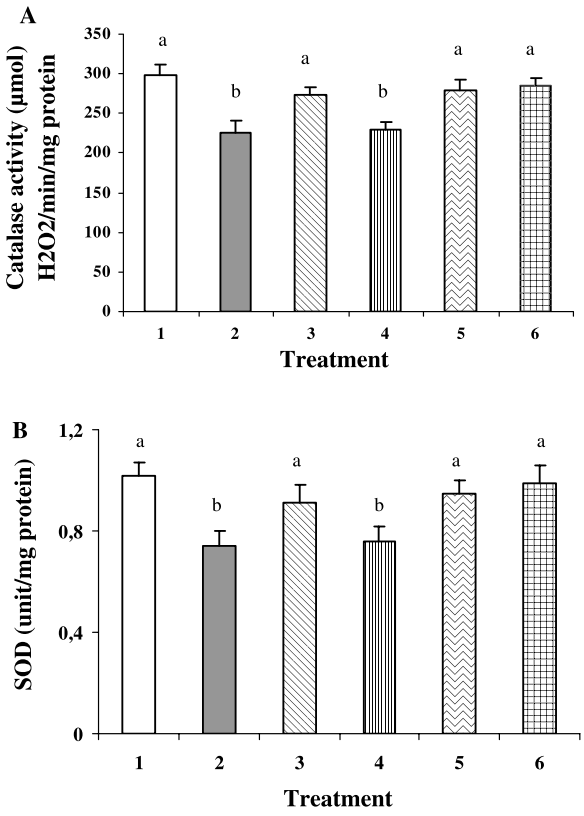

Figure 5. Effects of olive extracts on rat hepatic catalase (A) and superoxide dismutase (B) activities. **1**: control diet (CD) (standard diet), **2**: high cholesterol diet (HCD), **3**: HCD + methanol extract of green olives, **4**: HCD + methanol extract of black olives, **5**: HCD + ethyl acetate extract of green olives, and **6**: HCD + ethyl acetate extract of black olives. Each bar represents mean ± SE from eight rats. Bars with different letters differ, $P < 0.05$.

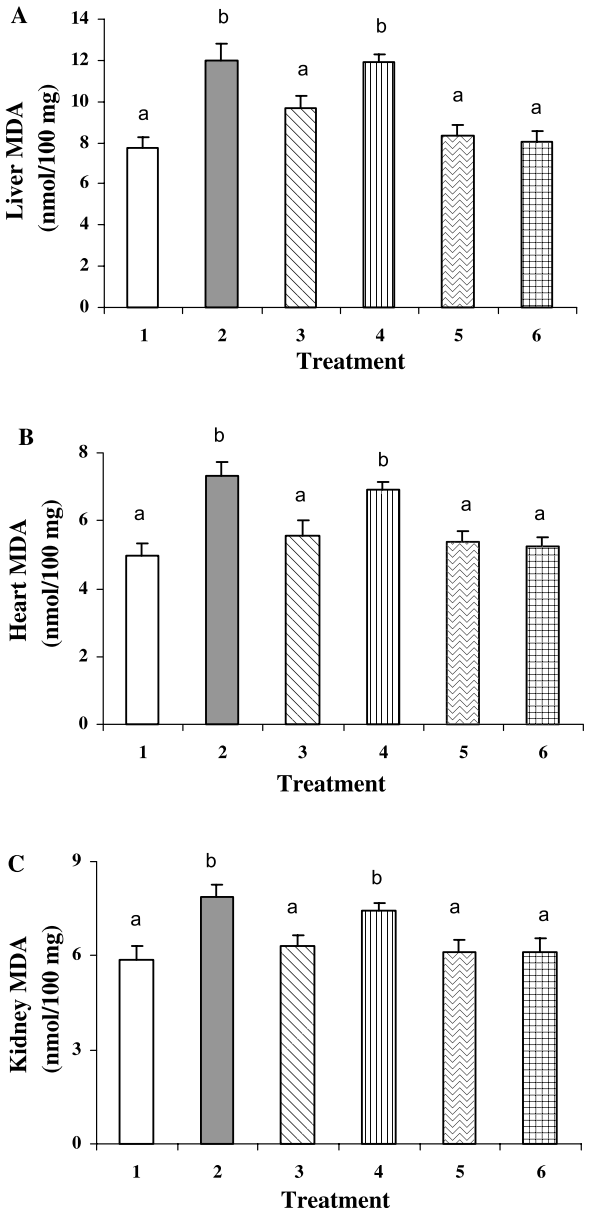

Figure 6. Effects of olive extracts on rat liver (A), heart (B) and kidney (C) MDA levels. **1**: control diet (CD) (standard diet), **2**: high cholesterol diet (HCD), **3**: HCD + methanol extract of green olives, **4**: HCD + methanol extract of black olives, **5**: HCD + ethyl acetate extract of green olives, and **6**: HCD + ethyl acetate extract of black olives. Each bar represents mean ± SE from eight rats. Bars with different letters differ, $P < 0.05$.

the orally administered extracts in the treated rats was lower than 0.1 mg/kg body weight. Several studies have brought in evidence that flavonoids possess hypocholesterolemic activity and antioxidant properties when they are administered at high doses. For instance, Bagchi et al.[19] reported that a grape seed extract rich in flavonoids and supplemented to hypercholesterolemic hamsters in 50 to 100 mg/kg doses reduces their cholesterol levels by 25 and 23%, respectively. Furthermore, the oral administration of rutin at 1 g/kg was previously found to lower the plasma cholesterol in cholesterol fed rats.[20] In this study, the aqueous methanol extract of black olives was administered at 5 mg/kg, which is a lower dose than those previously reported.

The results of the diet intake and growth after a 16-week treatment showed no significant difference among all groups, suggesting that neither the cholesterol-rich diet nor olive extracts had an adverse effect on the growth of rats. The rats in the HCD group had higher concentrations of TC and LDL-C in serum than those of the control group, indicating that the hypercholesterolemic model was successfully established.

Most of cholesterol is an essential structure element of biological membranes; the rest transited through blood or functions as the starting material for the synthesis of bile acid, steroid hormones and vitamin D. However, increased concentrations of serum TC and LDL-C raise the risk of developing CHD.[21,4] Our data demonstrated that oral administration of green and black olive extracts in rats fed a cholesterol-rich diet reduced the levels of TC and LDL-C. The reduction of TC induced by olive phenolics might be attributed to their increase of faecal bile acid and cholesterol excretion or decrease of cholesterol biosynthesis. The two key enzymes involved in regulating cholesterol metabolism are 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, the rate-limiting enzyme in the cholesterol biosynthetic pathway, and acyl-CoA:

cholesterol acyltransferase (ACAT), the cholesterol-esterifying enzyme in tissue, including the liver. The inhibition of HMG-CoA reductase reduces cholesterol synthesis and its inhibitors are very effective in lowering serum cholesterol in most animal species, including humans.[22–24] ACAT is primarily responsible for the esterification of cholesterol in all mammalian cells.[25] Inhibition of ACAT was shown to limit atherosclerotic lesion development, cholesteryl ester enrichment, and monocyte-macrophage foam cell involvement.[26] Although we did not determine the output of faeces and the HMG CoA reductase and ACAT activities, similar effects were observed in phenolic compounds from other sources.[20,27]

It is widely accepted that reduction in plasma HDL is a risk factor for developing atherosclerosis. HDL facilitates the translocation of cholesterol from the peripheral tissue, such as arterial walls to liver for catabolism. The increase in HDL may slow down the atherosclerotic process.[28] Our results showed that hydroxytyrosol, oleuropein supplemented-diet increased the concentration of serum HDL-C when compared with the cholesterol-rich diet. Atherosclerotic index (AI), defined as the ratio of LDL-C and HDL-C, is believed to be an important risk factor of atherosclerosis. Since LDL-C was significantly suppressed and administering ethyl acetate and methanol extracts of green olives and ethyl acetate of black olives resulted in increased HDL-C, the value of AI was significantly decreased. This decrease in AI can be said to be another positive change after olive phenolics treatment.

MDA, the product of lipid peroxidation, is an index of the level of oxygen free radicals. A decrease in lipid peroxidation leads to a reduction of atherosclerosis caused by hypercholesterolemia.[29] The content of MDA in rats fed a cholesterol-rich diet was increased compared to rats fed standard laboratory diet, suggesting that hypercholesterolemia could enhance the process of lipid peroxidation. The oral administration of ethyl acetate and aqueous methanol extracts of green olives and ethyl acetate extract of black olives prevented a cholesterol-rich diet induced elevation of MDA and resulted in a significantly decreased content of MDA in liver, heart and kidney. The obtained data suggested that olive phenolics might be capable of lowering or slowing down oxidative-stress-related lipid peroxidation.

A cholesterol-rich diet brings about remarkable modifications in antioxidant defence mechanisms. Studies have shown that hypercholesterolemia diminishes the antioxidant defence system and decreases the activities of SOD and CAT, elevating the lipid peroxide content.[30] In the present study, the activities of SOD and CAT in the liver of HCD group were significantly decreased compared with those of control rats. Administration of ethyl acetate and aqueous methanol extracts of green olives and ethyl acetate extract of black olives to the rats fed cholesterol-rich diet significantly elevated the activities of SOD liver. These results have suggested that phenolic compounds extracted from olives could improve the efficiency of superoxide anion to hydrogen peroxide due to increased SOD activity, which catalyzes dismutation of superoxide anion into hydrogen peroxide. Phenolic extracts of green olives and black olives also increased the activity of CAT in liver, which, in turn, detoxifies hydrogen peroxide and converts lipid hydroperoxides to nontoxic substances.

In summary, we found that in this model of experimental atherogenesis, the supplementation with phenolic compounds extracted from green and black olives exhibited an antihyperlipedemic action, reduced the lipid peroxidation process and enhanced the antioxidant defence system. These effects may constitute an additional explanation of the ability of the Mediterranean diet, in which olive and olive oil are major components, to reduce the frequency of cardiovascular diseases.

4. Materials and methods

4.1. Olive extract

Chemlali olives were harvested from Sfax (Tunisia). The green olive samples were collected at the beginning of fruit development, at the beginning of July and the black ones were collected at the end of olive morphology, at the end of December. The olives were selected randomly from different parts of olive trees, and were immediately transferred to the laboratory and freeze-dried. After that, the olives were blended and extracted with methanol/water (8:2) and ethyl acetate. The solution was stirred at 120 rpm for 24 h at room temperature. Then, it was filtered using GF/F filter paper and transferred to a flask, which was evaporated to dryness.

4.2. Reagents and standards

Phenolic compounds including tyrosol and pyrogallol were obtained from Sigma-Aldrich Chemie GmbH Steinheim, Germany. Oleuropein was purchased from Extrasynthèse, Genay, France. Hydroxytyrosol was prepared, as described previously.[31] Apigenin, luteolin, luteolin 7-*O*-glucoside, quercetin and rutin were obtained from Apin (Abingdon, U.K.). Thiobarbituric acid was purchased from Fluka Chemie GmbH Steinheim. All phenolic compound solutions were made in a mixture methanol/water 80/20 (v/v). Double distilled water was used in the HPLC mobile phase. Pure HPLC solvents were used in all cases.

4.3. High-performance liquid chromatography

Phenolic monomer identification was carried out by HPLC analysis. It was performed on a Shimadzu apparatus composed of an LC-10ATvp pump and an SPD-10Avp detector. The column used was a C-18 (4.6×250 mm) Shim-pack VP–ODS. The temperature was maintained at 40 °C. The mobile phase used was 0.1% phosphoric acid in water (A) versus 70% acetonitrile in water (B) for a total running time of 50 min. The elution conditions applied were: 0–25 min, 10–25% B; 25–35 min, 25–80% B; 35–37 min, 80–100% B; and 37–40 min, 100% B. Finally, washing and reconditioning steps of the column were included (40–50 min)

with a linear gradient of 100–10% B. The flow rate was 0.6 ml/min and the injection volume was 50 μl.

4.4. LC–MS analysis

The LC–MS system used was a Waters apparatus composed of a 600 E pump, a Merck-Hitachi L-400 UV detector and Merck Lichrosphere 100 RP-18 column (4 × 250 mm). Positive-ion APCI–MS mode was obtained with a quadripole ion trap instrument (Finnegan-MAT LCQ), as described previously.[32] Identification of compounds by LC–MS analysis was carried out by comparing retention times and mass spectra of the unknown peaks to those of standards.

4.5. Animals and diets

Forty-eight male Wistar rats weighing between 180 and 200 g were purchased from 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 h periods of light and darkness. The rats were randomly divided into six groups of eight rats each. Group 1 was fed on a normal diet (CD) (Table 3). Group 2 was fed a high cholesterol diet (HCD) (normal diet supplemented with 1% cholesterol and 0.25% bile salts). Groups 3 and 4 received HCD and, respectively, methanol and ethyl acetate extracts of green olives harvested in July (5 mg/kg of body weight). Groups 5 and 6 received HCD and, respectively, methanol and ethyl acetate extracts of black olives harvested in December (5 mg/kg of body weight). Phenolic compounds were orally administered drinking water. The duration of the treatment was 12 weeks. The animals were given food and water ad libitum during the experimental period. The body weight was measured every day. At the end of the experimental period, the rats were killed by decapitation. Blood samples were collected to determine the plasma lipid profile. The livers, hearts and kidneys were removed and rinsed with physiological saline. All samples were stored at −80 °C until analyzed.

Table 3. Composition of the control diet (g/kg)

Diet ingredient	Concentration
Casein	200
DL-Methionine	3
Corn oil	155
Cornstarch	393
Sucrose	154
Cellulose	50
Mineral mix[a]	35
Vitamin mix[b]	10

[a] Mineral mixture contained the following (mg/kg of diet): $CaHPO_4$, 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 the following (mg/kg of diet): Retinol, 12; cholecalciferol, 0.125; thiamine, 40; riboflavin, 30; pantothenic acid, 140; pyridoxine, 20; inositol, 300; cyanocobalamin, 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).

4.6. Serum lipids

Concentrations of TC, TG and HDL-C in serum were determined by enzymatic colorimetric methods using commercial kits (Sigma). Concentration of LDL-C was accomplished according to the procedures described by Friedwald et al.[33]

4.7. Antioxidant enzyme activities

The preparation of the enzyme source fraction in the liver tissue was as follows. One gram of liver tissue was homogenized in 10 ml KCl 1.15% and centrifuged at 8000 rpm 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 Bradfort[34] using bovine serum albumin as standard.

CAT activity was measured using the method of Regoli and Principato.[35] Twenty microlitres of the supernatant was added to a cuvette containing 780 μl of a 50 M potassium phosphate buffer (pH 7.4), and 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 at 240 nm for 1 min on a spectrophotometer. A molar extinction coefficient of 0.0041 mM^{-1} cm^{-1} was used to determine the catalase activity. The activity was defined as the μ mole H_2O_2 decrease/mg protein/min.

SOD was measured according to the method of Park et al. [20] 100 μl of the supernatant was mixed with 1.5 ml of a Tris–EDTA–HCl buffer (pH 8.5) and 100 μl of 15 mM pyrogallol, and then incubated at 25 °C for 10 min. The reaction was determined by adding 50 μl of 1 N HCl, and then the activity was measured at 440 nm. One unit was determined as the amount of enzyme that inhibited the oxidation of pyrogallol by 50%. The activity was expressed as units/mg protein.

4.8. Malondialdehyde (MDA) assay

As a marker of lipid peroxidation production, the MDA concentration was measured using the method of Park et al.[20] Briefly, 200 μl of a 10% (w/v) solution of the tissue homogenate was mixed with 600 μl distilled H_2O and 200 μl of 8.1% (w/v) SDS, vortexed and then incubated 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 mixture, 1.0 ml of distilled water and 5.0 ml of a butanol/pyridine (15:1) solution were added and vortexed. This solution was centrifuged at 4000 rpm for 15 min and the resulting colored layer was measured at 532 nm using malonaldialdehyde (MDA) prepared by the hydrolysis of 1,1,3,3-tetramethoxypropane as the standard.

4.9. Statistical analysis

All data presented are means ± SE. Statistical differences were calculated using a one-way analysis of variance

(ANOVA), followed by Student's *t*-test. Differences were considered significant at $P < 0.05$.

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