JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Lipid-Lowering and Antioxidant Effects of Hydroxytyrosol and Its Triacetylated Derivative Recovered from Olive Tree Leaves in Cholesterol-Fed Rats

HEDYA JEMAI,[†] INES FKI,[†] MOHAMED BOUAZIZ,[†] ZOUHAIER BOUALLAGUI,[†] ABDELFATTAH EL FEKI,[§] HIROKO ISODA,[#] AND SAMI SAYADI*,[†]

Laboratoire des Bioprocédés, Pôle d'Excellence Régional AUF, Centre de Biotechnologie de Sfax, B.P. «K» 3038 Sfax, Tunisia; Laboratoire d'Ecophysiologie animale, Faculté des Sciences de Sfax, B.P. 802, 3018 Sfax, Tunisia; and Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan

This study was designed to test the lipid-lowering and antioxidative activities of triacetylated hydroxytyrosol compared with its native compound, hydroxytyrosol, purified from olive tree leaves. Wistar rats fed a standard laboratory diet or a cholesterol-rich diet for 16 weeks were used. The serum lipid levels, the thiobarbituric acid-reactive substances (TBARS) level, as an indicator of lipid peroxidation, 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), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-C). Administration of hydroxytyrosol and triacetylated hydroxytyrosol (3 mg/kg of body weight) decreased the serum levels of TC, TG, and LDL-C significantly and increased the serum level of high-density lipoprotein cholesterol (HDL-C). Furthermore, the content of TBARS in liver, heart, kidney, and aorta decreased significantly when hydroxytyrosol and its triacetylated derivatives were orally administered to rats compared with those fed a cholesterol-rich diet. In addition, triacetylated hydroxytyrosol and hydroxytyrosol increased CAT and SOD activities in the liver. These results suggested that the hypolipidemic effect of triacetylated hydroxytyrosol and hydroxytyrosol might be due to their abilities to lower serum TC, TG, and LDL-C levels as well as to their antioxidant activities preventing the lipid peroxidation process.

KEYWORDS: Triacetylated hydroxytyrosol; hydroxytyrosol; cholesterol-fed rat; antioxidant enzymes; hypolipidemic; serum lipid levels

INTRODUCTION

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 (*1*). Hyperlipidemia resulting from lipid metabolic changes is a major cause of atherosclerosis. Hypercholesterolemia, or more specifically elevated plasma low-density lipoprotein cholesterol (LDL-C), is an important risk factor for the development and progression of atherosclerosis (*2*). Moreover, 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 (*3, 4*). 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 (*5, 6*).

Among the different phenolic compounds, particular attention has been focused on hydroxytyrosol (*7*), which occurs naturally in olive oil (*8*), in olive mill solid–liquid wastes from two-phase olive oil processing (*9*), and in olive mill wastewaters. This *o*-diphenol has been proven to be a potent scavenger of superoxide anion and hydroxyl radical (*10, 11*), and it is more active than antioxidant vitamins (*12*) as well as the synthetic

* Corresponding author (telephone/fax 216 74 874 452; e-mail sami.sayadi@cbs.rnrt.tn).
[†] Centre de Biotechnologie de Sfax.
[§] Faculté des Sciences de Sfax.
[#] University of Tsukuba.

10.1021/jf072589s CCC: $40.75
Published on Web 04/02/2008

antioxidants (*13*). Clear epidemiological and biochemical evidence indicates that hydroxytyrosol is endowed with significant antithrombotic, antiatherogenic, and anti-inflammatory activities (*14, 15*). Recently, the hypocholesterolemic potential of hydroxytyrosol has been demonstrated (*16–18*).

In this respect, however, a major problem is that hydroxytyrosol is chemically unstable, unless preserved dried in the absence of air and in the dark. Therefore, the efficiency of this molecule added in its native form to biological matrices as a protective agent against reactive oxygen species could not be guaranteed. On the basis of these considerations, it would be useful to conveniently produce hydroxytyrosol in chemically more stable derivatives able to be biochemically converted in vivo into its original active form. Considering that the acetyl group is a ubiquitous substrate in the biochemical processes and that the acetylating agents are very common and manageable, we have planned and succeeded in preparing hydroxytyrosol acetyl derivatives.

The aim of this study is to examine the effect of the triacetylated hydroxytyrosol on the cholesterol metabolism and antioxidative status in hypercholesterolemic diet fed rats compared with the parent purified hydroxytyrosol from *Olea europaea* L. leaves.

MATERIALS AND METHODS

Olive Leaf Extract Preparation. The extraction was carried out on Chemlali olive leaves. Samples of fresh green leaves were used. Leaves were dried and powdered for the extraction. A mixture of methanol and water (80:20 v/v) was added to the olive leaf powder, and the mixture was left to stand under agitation for 24 h and then was filtered.

Acid Hydrolysis. Hydroxytyrosol-rich extract was prepared as follows: 1 g of the olive leaf extract was dissolved in 10 mL of a MeOH/H_2O (4:1) mixture in a sealed vial. The solution was hydrolyzed at 100 °C for 1 h using 5 mL of HCl (2 M) (Prolabo, France). After 1 h, the sample was cooled and diluted with water (10 mL) and the hydrophobic fraction was extracted by a separatory funnel three times with 50 mL of ethyl acetate (Prolabo, France), which was subsequently removed by evaporation.

HPLC Analysis. A reversed-phase high-performance liquid chromatographic (HPLC) technique was developed to identify and quantify the major phenolic compounds contained in the hydrolyzed extract. For this purpose, a standard mixture solution of phenolic compounds was analyzed. Sample concentrations were calculated on the basis of peak areas compared to those of each of the external standards. The HPLC chromatograph was a Shimadzu apparatus equipped with a (LC-10ATvp) pump and a (SPD-10Avp) detector. The column was 4.6 × 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 the running time.

Chromatographic Purification of Hydroxytyrosol. Hydrolyzed extract (1 g) was chromatographed on a C-18 silica gel (liChroprep RP-18; 25–40 μm) column (2.5 × 70 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 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).

Hydroxytyrosol Acetylation. One hundred milligrams of hydroxytyrosol (0.65 mmol) was dissolved in diethyl ether (20 mL) and mixed with pyridine (165 μL) in a glass vial equipped with a magnetic stirrer. Then 1654 μL (2.3 mmol) of acetyl chloride in 10 mL of diethyl ether was added dropwise. The mixture was stirred at 0 °C for 10 h, and a white precipitation appeared (pyridine chloridrate). The formed precipitate was filtered, and the obtained solution was dried at 35 °C under vacuum to give triacetylated hydroxytyrosol as a pale brown residue.

Table 1. Composition of the Control Diet

diet ingredient	concn (g/kg)
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: $CaHPO_4$, 17200; KCl, 4000; NaCl, 4000; MgO, 420; $MgSO_4$, 2000; Fe_2O_3, 120; $FeSO_4 \cdot 7H_2O$, 200; trace elements, 400 ($MnSO_4H_2O$, 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, thiamin, 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).

Purification of Triacetylated Hydroxytyrosol. The material consisted of an AKTA basic system (Biosciences-Amersham) equipped with a UV detector and a C18-Bioscale column eluted with the same gradient as in HPLC.

GC-MS Analysis. GC-MS analysis was performed with a HP model 5975B inert MSD, equipped with a capillary HP5MS column (30 m length, 0.25 mm i.d., 0.25 mm film thickness, Agilent Technologies, J&W Scientific Products). The carrier gas was used at 1 mL min^{-1} flow rate. The oven temperature program was as follows: 1 min at 100 °C, from 100 to 260 at 4 °C min^{-1} and 10 mn at 260 °C. OMW samples (40 mL) were acidified at pH 2 by HCl (1 N) and extracted with ethyl acetate (4/40 mL). The organic layer was collected and reduced to 10 mL by rotary evaporation (37 °C) and then silylated. For the silylation procedure, a mixture of pyridine (40 μL) and BSTFA (200 μL) was added and vortexed in screw-cap glass tubes and consecutively placed in a water bath at 80 °C for 45 min. From the silylated mixture 1 μL was directly analyzed by GC-MS.

Animals and Diets. Forty male Wistar rats weighing between 150 and 170 g were purchased from the Pasteur Institute (Tunis). During the treatment, the animals were individually housed in stainless steel cages in a controlled room temperature at 24 °C, under a 12 h light/12 h dark cycle and with free access to food and water. The rats were randomly divided into four 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 and 4 received HCD with hydroxytyrosol and triacetylated hydroxytyrosol (3 mg/kg of body weight), respectively. Hydroxytyrosol and triacetylated hydroxytyrosol were dissolved in drinking water. The duration of the treatment was 16 weeks. 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, kidneys, and aortas were removed and rinsed with physiological saline solution. All samples were stored at −80 °C until analysis.

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

Antioxidant Enzyme Activities. CAT and SOD activities were evaluated in liver tissue. The preparation of the enzyme source fraction was as follows. One gram of liver tissue was homogenized in 10 mL of KCl (1.15%) and centrifuged at 7740*g* for 15 min. The supernatants were removed and stored at −80 °C for analysis. The protein content in supernatant was measured according to the method of Bradford (*19*) using bovine serum albumin as standard. CAT activity was measured

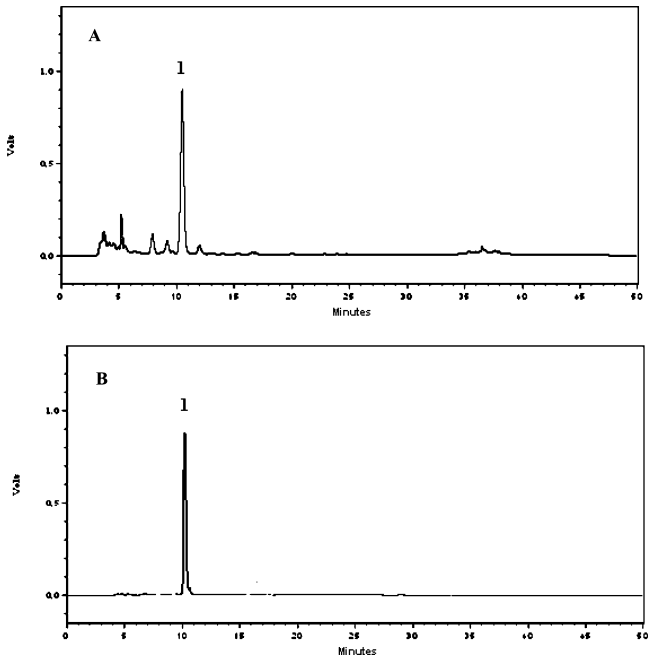

Figure 1. HPLC chromatogram at 280 nm of an olive leaf extract after acid hydrolysis (**A**) and purified hydroxytyrosol (**B**) (peak 1).

using the method of Regoli and Principato (*20*). Briefly, 20 μL 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 CAT activity. The activity was defined as the micromoles of H_2O_2 decrease per milligram of protein per minute.

SOD activity was measured according to the method of Marklund and Marklund (*21*). This method is based on pyrogallol oxidation by superoxide anion (O_2^-) and its dismutation by SOD. Briefly, 25 μL of the supernatant was mixed with 935 μL of a 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 that inhibited the oxidation of pyrogallol by 50%. The activity was expressed as units per milligram of protein.

Thiobarbituric Acid-Reactive Substances (TBARS) Assay. As a marker of lipid peroxidation product, the TBARS concentration was measured using the method of Park et al. (*22*). Briefly, 200 μL of a 10% (w/v) solution of the tissue homogenate was mixed with 600 μL of distilled H_2O and 200 μL of 8.1% (w/v) SDS, vortexed, and then incubated at room temperature for 5 min. 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 the mixture had cooled, 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 1935*g* for 15 min, and the resulting colored layer was measured at 532 nm using a malondialdehyde (MDA) standard curve.

Statistical Analysis. All data presented are the mean ± SE. Statistical differences were calculated using a one-way analysis of variance (ANOVA), followed by Student's test. Differences were considered to be significant at $p < 0.05$.

RESULTS

Purification and Acetylation of Hydroxytyrosol. The hydrolysis reaction of the olive leaf extracts was realized to produce large quantities of hydroxytyrosol. **Figure 1A** shows that the hydrolysate solution was composed of a single major phenolic compound, identified as hydroxytyrosol. The hydrolysate was submitted to the purification using the C-18 column under medium pressure. The first separated peak corresponds to pure hydroxytyrosol. The purity of hydroxytyrosol was further confirmed with HPLC analysis (**Figure 1B**).

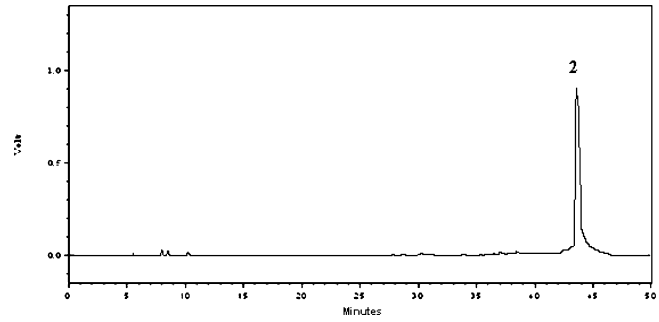

Figure 2. HPLC chromatogram at 280 nm of hydroxytyrosol after acetylation. Peak 2 represents triacetylated hydroxytyrosol.

Table 2. Abbreviated Mass Spectra of Hydroxytyrosol and Triacetylated Hydroxytyrosol

TMS derivatives of	mass spectra (*m*/*z* and % of the base peak)
hydroxytyrosol	370 (M^+, 39), 267 (90), 193 (25), 179 (12), 73 (100)
triacetylated hydroxytyrosol	280 (M^+, 5), 220 (6), 196 (3), 178 (18), 137 (10), 136 (100), 135 (5), 123 (10), 107 (2), 77 (2)

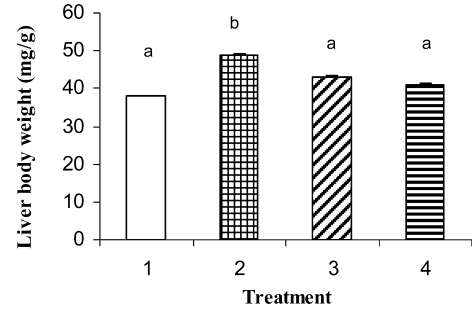

Figure 3. Effects of triacetylated hydroxytyrosol and hydroxytyrosol on the liver/body weight ratios: 1, standard diet (CD); 2, high-cholesterol diet (HCD); 3, HCD + hydroxytyrosol (3 mg/kg); 4, HCD + triacetylated hydroxytyrosol (3 mg/kg). Each bar represents the mean ± SE from 10 rats. Bars with different letters differ; $p < 0.05$.

To prepare the acetylated derivatives of hydroxytyrosol, acetyl chloride was used. Several conditions were tested including different quantities of acetyl chloride, pyridine, different temperatures, and different incubation times. Three hydroxyl groups exist in the hydroxytyrosol structure, and therefore different acetyl derivatives were expected. Under our experimental conditions, triacetylated derivative was obtained resulting in 96.8% initial hydroxytyrosol conversion. The acetylated raw material was further purified. A typical HPLC profile of triacetylated hydroxytyrosol derivative is shown in **Figure 2**. The identification of hydroxytyrosol and its acetylated derivative was confirmed by using the GC-MS apparatus (**Table 2**).

Body and Organ Weights. There was no significant difference in the body weight evolution in all groups throughout the treatment (data not shown). In the same way, there were no differences in the heart and kidney/body weight ratios. However, the liver/body weight ratio increased in rats fed a cholesterol-rich diet (HCD) compared with the rats fed a control diet (CD) (**Figure 3**). Triacetylated hydroxytyrosol and hydroxytyrosol decreased significantly the liver/body weight ratio compared with those of the HCD group.

Serum Lipids. Serum lipid levels were measured at the end of the experiment. After the treatment, the TC, TG, and LDL-C

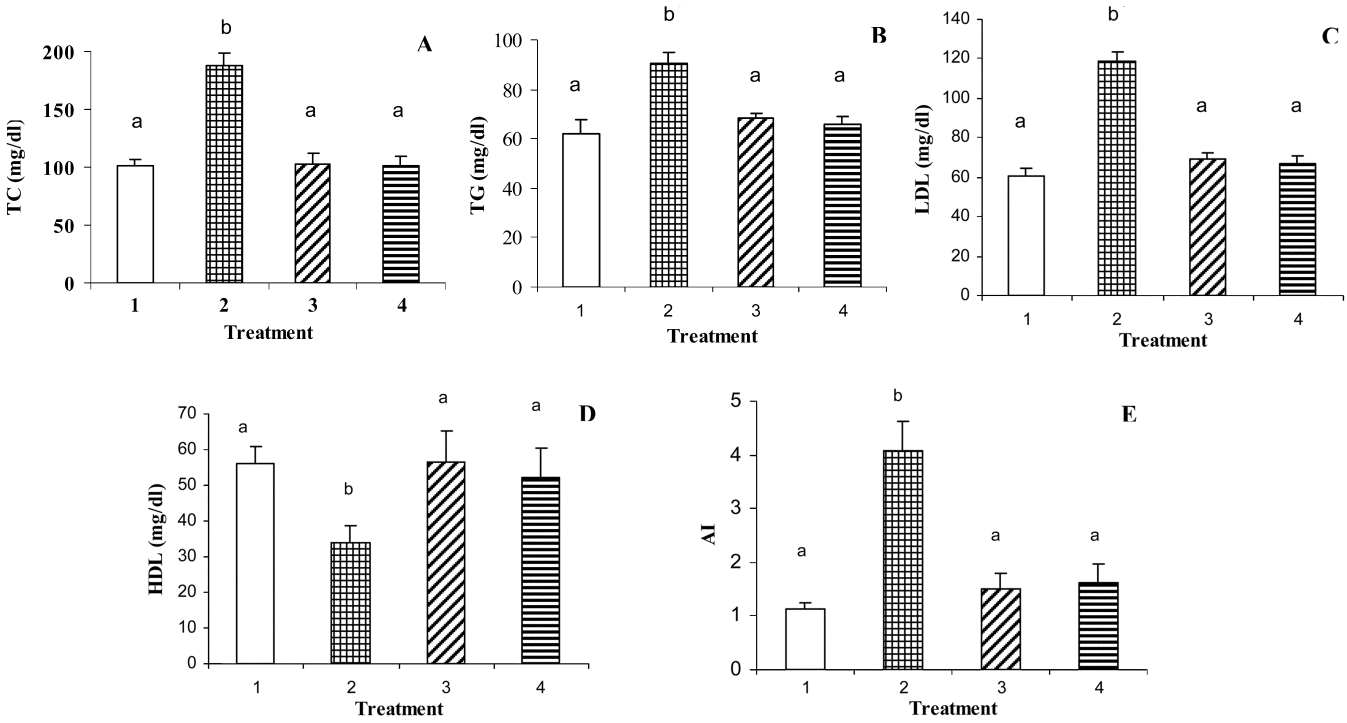

Figure 4. Effects of hydroxytyrosol and triacetylated hydroxytyrosol on rat total cholesterol (TC) (**A**), triglycerides (TG) (**B**), low-density lipoprotein cholesterol (LDL-C) (**C**), high-density lipoprotein cholesterol (HDL-C) (**D**) and atherogenic index (AI) (**E**) levels: 1, standard diet (CD); 2, high-cholesterol diet (HCD); 3, HCD + hydroxytyrosol (3 mg/kg); 4, HCD + triacetylated hydroxytyrosol (3 mg/kg). Each bar represents the mean ± SE from 10 rats. Bars with different letters differ; $p < 0.05$.

concentrations of rats fed a cholesterol-rich diet (HCD) showed a significant increase compared with the rats fed a normal diet (CD). However, a decrease of HDL-C concentration of rats in the HCD group was observed (**Figure 4**). Rats having received an oral administration of triacetylated hydroxytyrosol and hydroxytyrosol had lower concentrations of TC, TG, and LDL-C than those that received a HCD. Indeed, triacetylated hydroxytyrosol and hydroxytyrosol reduced the TC, TG, and LDL-C levels by 47, 28, and 44% and 45, 25, 42%, respectively. Moreover, the HDL-C of rats treated with triacetylated hydroxytyrosol and hydroxytyrosol increased significantly compared with those of rats in the HCD group ($p < 0.05$). The AI was significantly reduced by orally administering phenolic compounds ($p < 0.05$).

The supplementation of HCD-fed animals with triacetylated hydroxytyrosol and hydroxytyrosol was able to restore the lipid profile to the normal level of control group. In fact, the TC, TG, LDL-C, and HDL-C concentrations of animals treated with these phenolics were similar to those of the control group ($p < 0.05$).

Hepatic Antioxidant Enzyme Activities. The hepatic antioxidant enzyme activities significantly decreased in rats fed a cholesterol-rich diet compared to those fed a control diet (**Figure 5**). The decrease was significantly restored ($p < 0.05$) in the presence of triacetylated hydroxytyrosol and hydroxytyrosol.

TBARS Levels. The TBARS levels were significantly increased ($p < 0.05$) in the liver, heart, and kidneys of the animals fed the high-cholesterol diet compared to the control diet group. The treatment of HCD rats with triacetylated hydroxytyrosol and hydroxytyrosol significantly reduced the TBARS concentration (**Figure 6**).

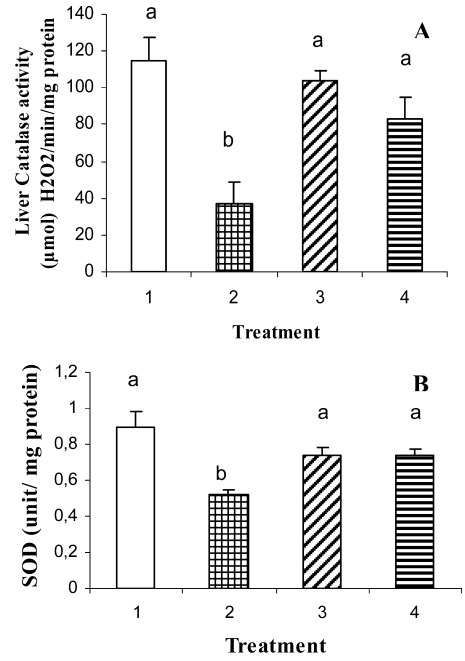

Figure 5. Effects of hydroxytyrosol and triacetylated hydroxytyrosol on CAT (**A**) and SOD (**B**) activities in liver: 1, standard diet (CD); 2, high-cholesterol diet (HCD); 3, HCD + hydroxytyrosol (3 mg/kg); 4, HCD + triacetylated hydroxytyrosol (3 mg/kg). Each bar represents the mean ± SE from 10 rats. Bars with different letters differ; $p < 0.05$.

DISCUSSION

Vascular disease is a prevalent disorder leading to coronary heart disease and strokes attributed to atherosclerosis, a complex disease process often initiated by hypercholesterolemia. A number of previous epidemiological studies have implied a role for polyphenols in reducing the risk of coronary heart disease based on the antioxidant activity of these compounds (*23, 24*).

The results reported in this paper represent the first evidence that triacetylated hydroxytyrosol, a chemically stable acetyl analogue of hydroxytyrosol, is as effective as the native compound in preventing hypercholesterolemia and oxidative stress in cholesterol fed rats.

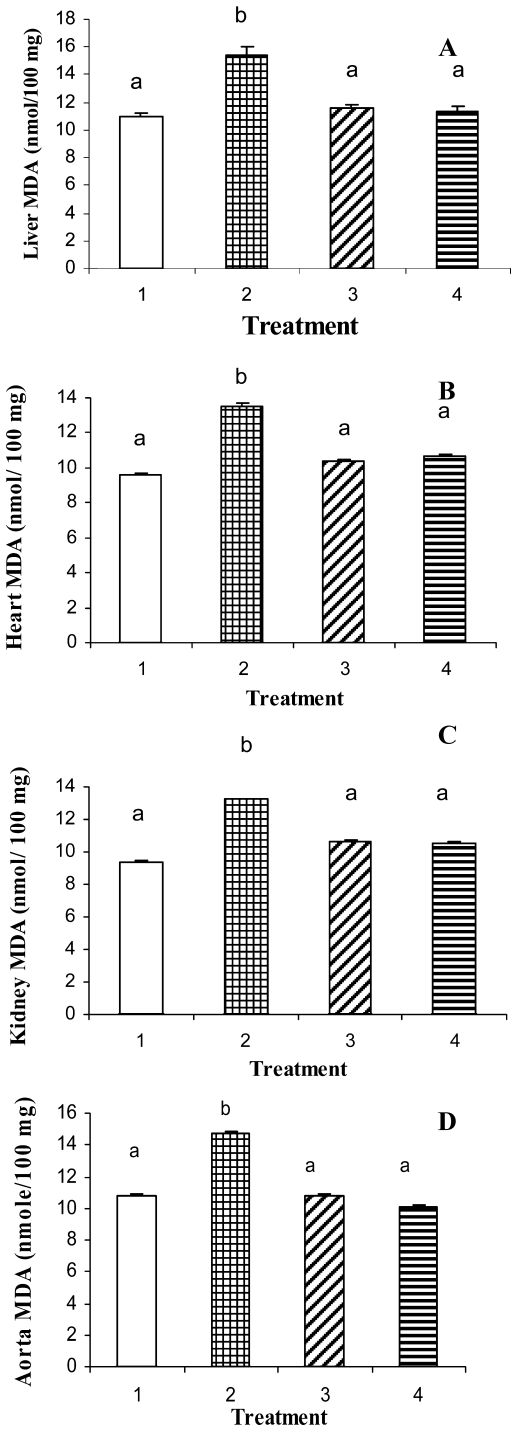

Figure 6. Effects of triacetylated hydroxytyrosol and hydroxytyrosol on rat liver (**A**), heart (**B**), kidney (**C**), and aorta (**D**) TBARS levels: 1, standard diet (CD); 2, high-cholesterol diet (HCD); 3, HCD + hydroxytyrosol (3 mg/kg); 4, HCD + triacetylated hydroxytyrosol (3 mg/kg). Each bar represents the mean ± SE from 10 rats. Bars with different letters differ; $p < 0.05$.

In this study, a relatively high amount of purified hydroxytyrosol was obtained in a short time by a simple hydrolysis reaction of *Olea europaea* leaf extract followed by purification using a C-18 silica gel column. There are several methods for the production of hydroxytyrosol, and recently several publications dealing with the production of such compound have been proposed. Hydroxytyrosol could be recovered from olive mill wastewaters (*25*) or from solid–liquid waste (*10*) or by chemical (*26*), biochemical (*27*), or biotechnological (*28*) synthesis starting from a synthetic precursor. However, because hydroxytyrosol is easily oxidized, it has to be dried and preserved in darkness in the absence of air. Therefore, the efficiency of hydroxytyrosol added in its native form to biological matrices as a protective agent against reactive oxygen species could not be guaranteed. For these reasons, triacetylated hydroxytyrosol derivative was prepared. It has been demonstrated that hydroxytyrosol acetyl derivatives offer two practical advantages: (i) increased efficiency when added to alimentary, pharmaceutical, or cosmetic matrices as a protective agent against reactive oxygen species (ROS) in human cells and (ii) possible exploitation as a nontoxic additive to lipophilic matrices (*29*). Moreover, it has been established that hydroxytyrosol acetyl derivatives showed a high free radical scavenging capacity, preventing protein oxidation and lipid peroxidation when cells ex vivo were exposed to active-oxygen substances and/or free radicals. This property makes them potentially useful in treating chronic pathological states associated with a high generation of active oxygen substances and/or free radicals (*30*). Our findings demonstrated that triacetylated hydroxytyrosol administration induced a protective effect against experimental atherogenesis. Triacetylated hydroxytyrosol could be converted in vivo by esterases into the native form, which is responsible for protecting animals from atherosclerosis. Indeed, it was recently reported that hydroxytyrosol acetyl derivative was as efficient as the parent compound in protecting human cells from oxidative stress-induced cytotoxicity, after metabolization by esterases in the intestinal tract (*31*).

In the current study, the high-cholesterol diet appeared to cause an increase of liver weights. This could be related to an accumulation of lipids such as triglycerides and cholesterol in the liver. In contrast to its inhibitory effect on cholesterol biosynthesis, dietary cholesterol was shown to stimulate hepatic fatty acid biosynthesis and the incorporation of newly synthesized fatty acid to hepatic TG (*32*). The decrease of liver weight, in triacetylated hydroxytyrosol and hydroxytyrosol groups, leads us to conclude that these phenolic compounds could reduce the accumulation of lipids in liver.

Results from the serum lipid status of the high-cholesterol-fed rats for 16 weeks showed increased concentrations of serum TC, TG, and LDL, whereas HDL was decreased. The elevations in serum total TC and TG levels observed in our study on HCD animals are in agreement with those reported in several studies (*33, 34*). The high levels of LDL-C found in HCD rats may be attributed to a down-regulation in LDL receptors by cholesterol included in the diet (*35*). Treatment of HCD-fed rats with triacetylated hydroxytyrosol and hydroxytyrosol showed a significant decrease in TC, TG, and LDL-C concentrations and an increase in HDL-C levels compared to the corresponding values of HCD group. A higher content of HDL-C is very important in humans because it is correlated with a reduced risk of coronary heart disease (*36*). The increased HDL facilitates the transport of cholesterol from the serum to the liver, where it is catabolized and excreted from the body.

The AI, defined as the ratio of LDL-C and HDL-C, is believed to be an important risk factor of atherosclerosis. Our data clearly demonstrate that triacetylated hydroxytyrosol and hydroxytyrosol significantly decrease the ratio. It has shown that abnormally high serum levels of LDL-C and low serum levels of HDL-C are associated with an increased atherosclerosis risk (*37*). Increasing the HDL-C concentrations and decreasing the LDL-C concentrations in HCD-fed rats indicates the antiatherogenic property of triacetylated hydroxytyrosol and hydroxytyrosol.

The mechanism of this hypocholesterolaemic action may be due to inhibition of the absorption of dietary cholesterol in the intestine or its production by the liver (*38*) or stimulation of the biliary secretion of cholesterol and cholesterol excretion in the feces (*39*).

Several studies have shown increased lipid peroxidation in clinical and experimental hypercholesterolemia. It has been established that hypercholesterolemia leads to increased production of oxygen free radicals (*40*), which exert their cytotoxic effect by causing lipid peroxidation, resulting in the formation of TBARS. In our study, hypercholesterolemic rats show significant rise in liver, heart, kidney, and aorta TBARS levels. Triacetylated hydroxytyrosol and hydroxytyrosol treatment along with cholesterol diet showed significant reduction of TBARS in all analyzed tissues. These data suggest that rats treated with triacetylated hydroxytyrosol and hydroxytyrosol are less susceptible to peroxidative damage under the challenge of oxidative stress such as a high-cholesterol diet.

It has been reported that oxidative stress is one of the causative factors that link hypercholesterolemia with the pathogenesis of atherosclerosis (*41*). This stress results from an imbalance between the production of free radicals and the effectiveness of the antioxidant defense system (*42*). Dietary polyphenols appear to have physiological antioxidant properties, which quench reactive oxygen and nitrogen species, thereby potentially contributing against the pathogenesis of cardiovascular disease (*43*). In the present study we have observed decreased activities of antioxidant enzymes SOD and CAT in the liver of rats fed a high-cholesterol diet as compared to those on normal diet. Our results are in agreement with reports of other workers which suggest that feeding a high-cholesterol diet to experimental animals depresses their antioxidant system due to increased lipid peroxidation and formation of free radicals (*44*). The treatment of cholesterol-fed rats with triacetylated hydroxytyrosol and hydroxytyrosol increased the SOD and CAT activities. The increase may have been due to the activation of both enzymes by triacetylated hydroxytyrosol and hydroxytyrosol, thereby resulting in a lower superoxide anion level. The higher CAT and/or SOD activity could lead to a reduced reactive oxygen species level in the triacetylated hydroxytyrosol and hydroxytyrosol supplemented group. These results suggest that triacetylated hydroxytyrosol and hydroxytyrosol reduce oxidative stress by preventing the generation of free radicals and finally inhibit development of atherosclerosis.

In conclusion, our results show that triacetylated hydroxytyrosol and hydroxytyrosol recovered from olive leaves are efficient in the protection against dyslipidemia by decreasing serum TC, TG, and LDL-C and increasing HDL-C, thus decreasing the AI. Moreover, they also improve antioxidant status by lowering lipid peroxidation and enhancing antioxidant enzymes.

ABBREVIATIONS USED

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; TBARS, thiobarbituric acid-reactive substances; HPLC, high-performance liquid chromatography; HCD, cholesterol-rich diet; CD, control diet.

ACKNOWLEDGMENT

We are grateful to A Gargoubi (Technician, CBS) and H. Auissaoui (Engineer, CBS) for technical assistance.

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Received for review August 30, 2007. Revised manuscript received January 23, 2008. Accepted January 29, 2008. This work received financial support from the Ministry of Higher Education, Scientific Research and Technology in Tunisia and Agence Universitaire Francophone (PER program).

JF072589S