JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Effects of Hydroxytyrosol and Hydroxytyrosol Acetate Administration to Rats on Platelet Function Compared to Acetylsalicylic Acid

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Virgin olive oil (VOO) contains the polyphenols hydroxytyrosol (HT) and hydroxytyrosol acetate (HT-AC). This study investigated the antiplatelet effect of HT and HT-AC in healthy rats and compared their effects to acetylsalicylic acid (ASA). All compounds were administered orally for 7 days. HT and HT-AC inhibited platelet aggregation in whole blood, with a 50% inhibitory dose (ID_{50}) of 48.25 mg/kg per day for HT, 16.05 mg/kg per day for HT-AC, and 2.42 mg/kg per day for ASA. Platelet synthesis of thromboxane B_2 was inhibited by up to 30% by HT and 37% by HT-AC; the ID_{50} of this effect for ASA was 1.09 mg/kg per day. Vascular prostacyclin production was inhibited by up to 27.5% by HT and 32% by HT-AC; the ID_{50} of this effect for ASA was 6.75 mg/kg per day. Vascular nitric oxide production was increased by up to 34.2% by HT, 66% by HT-AC, and 64% by ASA. We conclude that HT and HT-AC administered orally inhibited platelet aggregation in rats and that a decrease in thromboxane synthesis along with an increase in nitric oxide production contributed to this effect.

KEYWORDS: Hydroxytyrosol acetate; olive oil polyphenols; platelet aggregation; prostanoids; nitric oxide

INTRODUCTION

Interventional studies published to date indicate that the Mediterranean diet is effective in reducing cardiovascular risk factors and is associated with a reduction in cardiovascular events or death. The traditional Mediterranean diet is based on a number of food items that have health benefits, including olive oil as the main source of fats. The healthy effects of olive oil on many cardiovascular risk factors have been documented scientifically as beneficial for the lipid and thrombotic profile, insulin-mediated glucose metabolism, blood pressure, hemostasis, endothelial function, inflammation, and oxidative stress (*1, 2*).

Although different components in virgin olive oil (VOO), such as oleic acid, can show properties that benefit health, there is a general consensus that polyphenols are one of the components responsible for these benefits (*1–5*). The polyphenol present at highest concentrations in VOO is hydroxytyrosol (3,4-dihydroxyphenylethanol) (HT), if considered the sum of HT as a simple phenol and the HT derived from oleuropein aglycone hydrolysis; its concentration is known to vary depending upon the variety of olive, ripeness, method of oil pressing, and geographical region, among other factors (*6*). In general, the Manzanilla variety contains 3−4-fold as much HT as the Hojiblanca, Picual, Cornicabra, or Arbequina varieties.

The effects of polyphenols on physiopathological mechanisms of cardiovascular disease have merited particular attention. Among these effects are inhibition of platelet aggregation (*7, 8*), inhibition of oxidation of low-density lipoproteins (LDLs) (*9*), stimulation of nitric oxide (NO) production (*10*) and down-regulation of the expression of endothelial adhesion molecules (*11*). Because platelet aggregation is one of the main events in arterial thrombosis, insights into the mechanisms of action of VOO polyphenols in the inhibition of platelet aggregation are potentially useful in the search for new approaches to cardiovascular medicine and dietary interventions that favor cardiovascular health.

Although HT is the main polyphenol in VOO, other polyphenols are also present, including hydroxytyrosol acetate (HT-AC) (*6*). Dependent upon the variety of olive, the concentration of HT-AC ranges from approximately equal to HT (in the Arbequina variety), one-third to one-fourth the concentration

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10.1021/jf801502z CCC: $40.75
Published on Web 08/16/2008

of HT (Manzanilla and Hojiblanca varieties), or twice as high as HT (Picual variety) (*6*).

The aim of this study was to evaluate the effects of the oral administration of HT-AC (7 days) on platelet aggregation compared to HT and acetylsalicylic acid (ASA), chosen for comparison because ASA is the drug used most widely to prevent ischemic cardiovascular disease, owing to its antiplatelet action.

MATERIALS AND METHODS

Materials. Thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$ enzyme immunoassay kits were from Amersham International plc (Little Chalfont, Buckinghamshire, U.K.). The nitrite/nitrate enzyme-linked immunosorbent assay (ELISA) kit and HT were obtained from Cayman Chemical (Ann Arbor, MI). Collagen was obtained from Menarini Diagnóstica S.A. (Barcelona, Spain). All other reagents were from Sigma Chemical Corp. (St. Louis, MO). Hydroxytyrosol acetate was supplied by the Department of Organic Chemistry and Pharmaceutics, School of Pharmacy, University of Seville (Seville, Spain). Hydroxytyrosol acetate was prepared by a procedure under patent (Alcudia, F.; Cert, A.; Espartero, J. L.; Mateo, R.; Trujillo, M. Method of preparing hydroxytyrosyl esters, esters thus obtained and use of same. PCT WO 2004/005237 A1). To a solution of hydroxytyrosol in ethyl acetate, crude pancreatic porcine lipase (50 mg) was added, and the mixture was stirred for 2 days. The resulting suspension was filtered through celite, and the solvent was eliminated in vacuo, yielding pure hydroxytyrosol acetate.

Study Design. Male Wistar rats weighing 200−250 g were used. All animals were housed at the University of Malaga Centro de Experimentación Animal under standard temperature and light/dark conditions, with access to food and water *ad limitum*. The rats were used in accordance with current Spanish legislation for animal care, use, and housing (RD 223/1998, based on European Directive 86/609/CEE). The recommendations in Principles of Laboratory Animal Care (NIH publication 86-23, revised 1985) were followed, as was the Spanish Law on the Protection of Animals, where applicable.

A total of 16 groups of animals ($N = 6$ animals per group) were used according to the dose of each compound: a control group (treated with isotonic saline solution p.o.), six groups treated with HT (1, 5, 10, 20, 50, and 100 mg/kg per day p.o.), six groups treated with HT-AC (1, 5, 10, 20, 50, and 100 mg/kg per day p.o.), and three groups treated with ASA (1, 5, and 10 mg/kg per day p.o.). All compounds were given once per day for 7 days via an endogastric cannula at 10:00 h. The last dose was given 1 h before the animals were killed.

At the end of the treatment period, all rats were anesthetized with ethyl ether and immobilized in a supine position. The abdominal cavity was opened; the intestinal packet was moved to the right side; and the abdominal aorta was dissected at the level of its bifurcation into the iliac arteries. Blood was obtained via puncture of the abdominal aorta with 3.8% sodium citrate at a proportion of 1:10 as the anticoagulant. Part of the blood sample was centrifuged at 1500*g* for 25 min at 25 °C; the plasma was frozen at −80 °C until laboratory analysis for biochemical parameters.

The thoracic and abdominal portions of the aorta were carefully washed in isotonic saline solution and placed in containers with buffer consisting of NaCl (100 mM), KCl (4 mM), $NaHCO_3$ (25 mM), Na_2SO_4 (2.1 mM), sodium citrate (20 mM), glucose (2.7 mM), and Tris (50 mM) (pH 8.3).

Laboratory Analyses. Samples of blood, plasma, and aorta were used in the following analyses:

Platelet Aggregation. We tested whole blood with 3.8% sodium citrate at a proportion of 1:10 by electric impedance with a Chrono-Log 540 aggregometer (Chrono-Log Corp., Haverton, PA), using collagen (10 μg/mL) to induce platelet aggregation. After 10 min of aggregation at 37 °C, we measured maximum aggregation as the maximum change in electric impedance after the addition of collagen to the sample.

Platelet Thromboxane B_2 Production. We incubated samples of whole blood with calcium A23187 ionophore (10 μM) for 30 min at 37 °C. Samples were then centrifuged at 10000*g* for 3 min at 4 °C, and the supernatant was frozen at −80 °C until use to measure thromboxane B_2 (stable metabolite of thromboxane A_2) by an enzyme immunoassay.

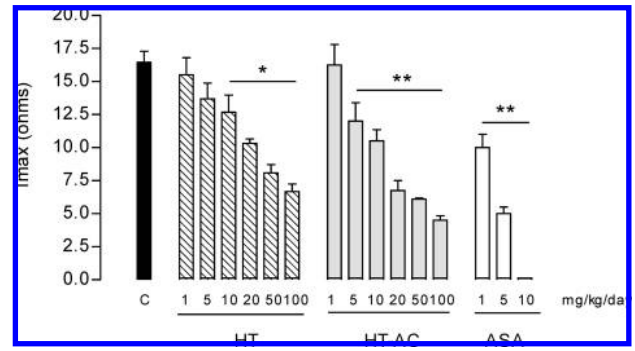

Figure 1. Maximum intensity of platelet aggregation (Imax) in whole blood induced with collagen from rats treated with saline (C), hydroxytyrosol (HT), hydroxytyrosol acetate (HT-AC), or acetylsalicylic acid (ASA) during 7 days ($n = 6$ rats per group). (*) $p < 0.01$ and (**) $p < 0.0001$, with respect to the control group.

Vascular 6-Keto-$PGF_{1\alpha}$ Production. The concentration of 6-keto-$PGF_{1\alpha}$ was measured with two methods. Plasma was obtained by centrifuging whole blood with the anticoagulant at 2500*g* for 10 min at 18 °C. The plasma was frozen at −80 °C until use to measure de 6-keto-$PGF_{1\alpha}$ (stable metabolite of prostacyclin) by enzyme immunoassay.

A segment of the aorta measuring approximately 50 mg was incubated in fresh buffer at 37 °C for 3 min, after which 6-keto-$PGF_{1\alpha}$ production was induced with 10 μM calcium ionophore A23187 for 30 min. After this period, the arterial tissue was weighed and the supernatant was frozen at −80 °C until use to measure 6-keto-$PGF_{1\alpha}$ by enzyme immunoassay.

Nitric Oxide Production. The concentration of nitrite plus nitrate was quantified as an indirect indicator of NO production. Nitrite plus nitrate was determined twice in each sample of plasma (same samples as were used to measure 6-keto-$PGF_{1\alpha}$) with a commercial enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI).

Statistical Analysis. The data in the text, tables, and figures are expressed as the mean ± standard error of 10 experiments. All statistical analyses were performed with the Statistical Program for Social Sciences version 14.0 (SPSS Co., Chicago, IL). One-way analysis of variance (ANOVA) followed by Bonferroni transformation was used, and differences were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

All three compounds studied here inhibited collagen-induced platelet aggregation in whole blood in a dose-dependent way (**Figure 1**). By extrapolation of the dose of each compound that inhibited platelet aggregation by 50 (ID_{50}) in the control group, the resulting ID_{50} values were 48.25 mg/kg per day for HT, 16.05 mg/kg per day for HT-AC, and 2.42 mg/kg per day for ASA.

The antiplatelet effect of ASA has been widely reported, and the results reported here are similar to values found in earlier studies in rats (*12, 13*). Some phenolic compounds in VOO have been shown to inhibit platelet functioning, e.g., HT (*7, 8*), oleuropein (*7*), and phenolic isochromans (*14*). Studies that demonstrated this effect were performed in vitro; in contrast, the values we report here for the antiplatelet aggregating effect of HT and HT-AC were obtained in rats given these compounds orally. Moreover, feeding with VOO in rats and rabbits significantly reduced collagen-induced platelet aggregation (*15, 16*). Our findings confirm this effect of HT and show further that the antiplatelet aggregating effect of HT-AC is stronger than that of HT. The ability of HT-AC to influence platelet activity has notable implications, because ASA is the drug used most

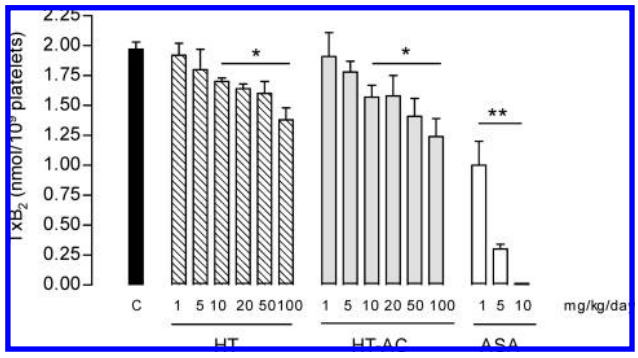

Figure 2. Platelet thromboxane B_2 (TxB_2) production induced with calcium ionophore A23187 from rats treated with saline (C), hydroxytyrosol (HT), hydroxytyrosol acetate (HT-AC), or acetylsalicylic acid (ASA) during 7 days ($n = 6$ rats per group). (*) $p < 0.01$ and (**) $p < 0.0001$, with respect to the control group.

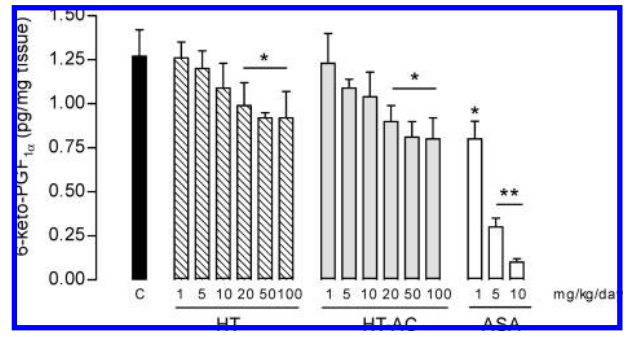

Figure 3. Aortic 6-keto-$PGF_{1\alpha}$ production induced with calcium ionophore A23187 from rats treated with saline (C), hydroxytyrosol (HT), hydroxytyrosol acetate (HT-AC), or acetylsalicylic acid (ASA) during 7 days ($n = 6$ rats per group). (*) $p < 0.01$ and (**) $p < 0.0001$, with respect to the control group.

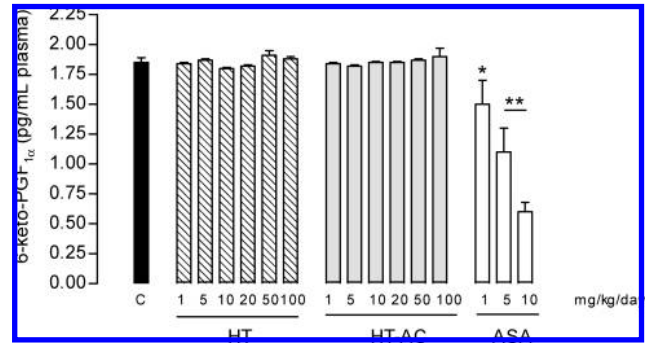

Figure 4. Plasma concentration of 6-keto-$PGF_{1\alpha}$ from rats treated with saline (C), hydroxytyrosol (HT), hydroxytyrosol acetate (HT-AC), or acetylsalicylic acid (ASA) during 7 days ($n = 6$ rats per group). (*) $p < 0.01$ and (**) $p < 0.0001$, with respect to the control group.

widely throughout the world for prophylaxis against thromboembolism, owing to its antiplatelet effect.

The range of doses of ASA that we tested in rats comprises an equivalent dose in humans for oral treatment as prophylaxis against thrombotic events (75 mg/day for a body weight of 75 kg), a moderately high dose (350 mg/day), and an anti-inflammatory dose in humans (750 mg/day). For HT and HT-AC, we tested a range that comprised a dose expected to have little anti-aggregating effect to a dose expected to have a clear inhibitory effect. This range is above the dose humans would receive by consuming VOO as part of their daily diet. However, olive oil contains a complex of polyphenols and other antioxidants, which may have synergistic effects, as shown earlier for their antioxidant action (*17*). This makes it advisable to use higher doses when each compound is used separately. Moreover, some differences in the pharmacokinetics of HT between rats and humans have been demonstrated (*18*); these differences refer to a high basal excretion of HT in rats. This makes it advisable to use higher doses when each compound is used separately.

The antiplatelet effect of ASA is directly related to two fundamental mechanisms: inhibition of platelet thromboxane synthesis (*19*) and increased calcium-dependent NO production (*20–22*). This relationship led us to test how HT and HT-AC modified these two parameters in animals. In the thromboxane synthesis assays (**Figure 2**), HT and HT-AC inhibited synthesis in a dose-dependent way, although the effect was weaker in quantitative terms than that of ASA. The ID_{50} for thromboxane B_2 synthesis was 1.09 mg/kg per day for ASA, but inhibition was less than 50% with the highest doses of HT and HT-AC. The greatest inhibition that we observed at a dose of 100 mg/kg per day was 30% for HT and 37% for HT-AC. Earlier experiments with HT showed that incubating collagen- or thrombin-induced whole blood with 400 μM of this polyphenol reduced thromboxane production by approximately 75% (*7*); however, there are no published studies on the potential effect of HT-AC. The inhibitory effect of HT on the thromboxane production in vivo in humans has been described after administration of HT-rich olive oil wastewater extract to patients with type I diabetes (*23*) or after administration of extra virgin olive oil to mildly dyslipidemic patients (*24*).

Thromboxane synthesis may be inhibited by blockade of cyclooxygenase or thromboxane synthetase activity. In the light of our findings, we hypothesized that cyclooxygenase inhibition was involved, because vascular prostacyclin production was also inhibited (**Figure 3**). The ID_{50} for ASA was 2.32 mg/kg per day. As we found for thromboxane synthesis, neither HT nor HT-AC achieved 50% inhibition; maximum inhibition was 27.5% for the former and 32% for the latter. However, plasma concentrations of 6-keto-$PGF_{1\alpha}$ were not significantly modified by treatment with HT or HT-AC, in contrast to the inhibitory effect seen in animals treated with ASA (**Figure 4**). The ID_{50} of ASA for 6-keto-$PGF_{1\alpha}$ production was 6.75 mg/kg per day. The differences that we observed between the effect of HT and HT-AC in plasma and aortic tissue may be due to our methodology; in plasma, we measured prostacyclin produced by vessels, leukocytes, the vascular wall, and tissues, such as the kidney, whereas in aortic rings, we measured only calcium-dependent cyclooxygenase activity. Moreover, a reduction in the intracellular peroxide-tone should be considered to assess a possible responsibility of HT in the inhibition of eicosanoid synthesis (*24*). It is also possible that HT exerts in platelets and/or arteries an effect similar to that described in rat colon mucosa, in which a reduction in the arachidonic acid concentration (main precursor of eicosanoids) has been observed after the administration of extra virgin olive oil (*25*).

The difference in the ability of ASA to inhibit prostacyclin versus thromboxane production has been explained as reflecting competition between ASA and salicylic acid produced when ASA passes through the liver (presystemic circulation) (*26, 27*). Platelets lack a nucleus and cannot synthesize cyclooxygenase de novo, whereas the vascular wall can synthesize prostacyclin once ASA disappears from plasma. This mechanism may account for the differences in effect observed between plasma and the vascular wall, because in the latter, this enzyme is stimulated and what is measured is its immediate product, whereas in plasma, the amount of 6-keto-$PGF_{1\alpha}$ measured may comprise prostacyclin synthetase synthesized de novo.

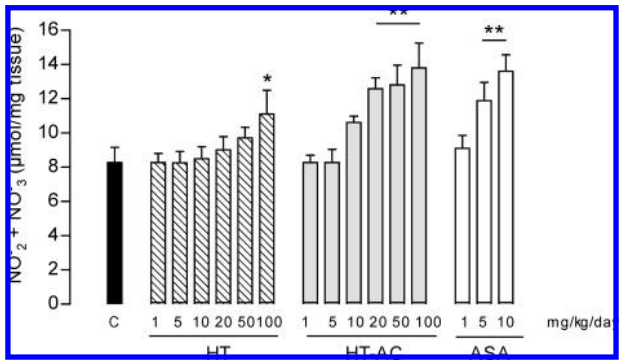

Figure 5. Aortic nitrite plus nitrate (NO_2^- plus NO_3^-) production induced with calcium ionophore A23187 from rats treated with saline (C), hydroxytyrosol (HT), hydroxytyrosol acetate (HT-AC), or acetylsalicylic acid (ASA) during 7 days ($n = 6$ rats per group). (*) $p < 0.01$ and (**) $p < 0.0001$, with respect to the control group.

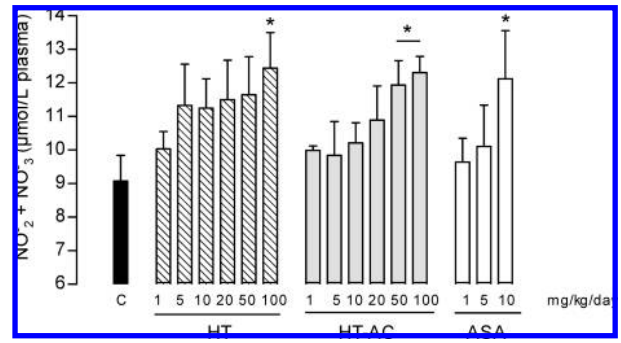

Figure 6. Plasma concentration of nitrite plus nitrate (NO_2^- plus NO_3^-) from rats treated with saline (C), hydroxytyrosol (HT), hydroxytyrosol acetate (HT-AC), or acetylsalicylic acid (ASA) during 7 days ($n = 6$ rats per group). (*) $p < 0.05$, with respect to the control group.

One important parameter to consider in studies of cyclooxygenase-inhibiting drugs is the prostacyclin/thromboxane ratio, which was 1.55 ± 0.18 in control rats, 2.94 ± 0.16 in rats treated with ASA, 1.72 ± 0.19 in animals treated with HT, and 1.34 ± 0.15 in those treated with HT-AC. In all groups and at all doses, we measured thromboxane B_2 and 6-keto-$PGF_{1\alpha}$ production after induction with calcium ionophore A23187. This means that ASA disrupted the ratio between the two prostanoids in favor of a proportionally greater inhibition of prostacyclin production, whereas HT and HT-AC spared this ratio and resulted in values similar to those in control animals. The implications of this finding merit attention because they provide evidence that these polyphenols inhibit platelet aggregation while sparing, to a large extent, prostacyclin synthesis, i.e., one of the main endogenous mechanisms that also inhibits platelet aggregation.

Although the inhibition of thromboxane synthesis clearly parallels the inhibition of platelet aggregation by ASA, no such connection was seen for HT and HT-AC, whose effect on platelet aggregation was proportionally greater. This raises the possibility that an additional mechanism is involved in the ability of VOO polyphenols to inhibit platelet aggregation. The effect of ASA on overall inhibition of aggregation in vivo has been shown to involve stimulation of calcium-dependent NO (*20–22*), which inhibits platelet functioning. We therefore investigated the possible effect of VOO polyphenols on NO production. Our results show that nitrite plus nitrate production was stimulated in aortic ring preparations induced with calcium ionophore A23187 in all three treatment groups (**Figure 5**) and that plasma concentrations of nitrite plus nitrate increased in a dose-dependent way (**Figure 6**). As discussed above in connection with the effects of treatment on vascular prostacyclin, stimulation of aortic tissue represents an indirect approach to investigating constitutive (calcium-dependent) nitric oxide synthase activity, while plasma concentrations of nitrite plus nitrate are an indicator of overall NO production (*28*). Our findings show that both polyphenols stimulated NO production in a manner proportionally similar to ASA. An earlier analysis of phytochemicals in VOO showed that HT increased cytoplasmic concentrations of calcium, thereby increasing NO synthesis in endothelial cells (*3*). Here, we document that both HT and HT-AC were able to increase NO concentrations ex vivo.

However, these results should be taken into account with precaution because of the differences in the pharmacokinetics of HT between rats and humans (*18*); for that reason, the results obtained in our experiments could be in some aspects different to that obtained in humans. Moreover, according to the pharmacokinetic parameters of HT in rats, an acute effect of HT or HT-AC cannot be ruled out and it must be due to its metabolites in our experimental conditions. Moreover, it is possible that HT-AC could be affected by a hydrolysis in the intestinal tract, and this could improve its bioavailability; however, it requires further studies to conclude this affirmation. Finally, another possible limitation of this study is related to the dose, because it has been demonstrated that administration of 10 mg/kg per day as a beverage to Apo-E-deficient mice could enhance experimental atherosclerotic lesions (*29*); however, the differences in the animal model must be taken into account.

We conclude that HT and HT-AC, when given orally to rats, inhibit platelet aggregation and that among the mechanisms involved in this effect are decreased thromboxane synthesis and increased NO production. These results may offer an explanation for the beneficial effects of VOO in preventing cardiovascular events and open new perspectives toward the potential use of these polyphenols as an alternative to ASA in the prevention of arterial thrombotic events.

ABBREVIATIONS USED

ASA, acetylsalicylic acid; HT, hydroxytyrosol; HT-AC, hydroxytyrosol acetate; LDL, low-density lipoprotein; NO, nitric oxide; 6-keto-$PGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$; TxB_2, thromboxane B_2; VOO, virgin olive oil.

ACKNOWLEDGMENT

We thank A. Pino Blanes for his invaluable technical assistance and K. Shashok for translating parts of the manuscript into English.

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Received for review May 14, 2008. Revised manuscript received July 16, 2008. Accepted July 17, 2008. This study was supported by a grant from the Ministerio de Ciencia y Tecnología, Spain (AGL-04-7935-C03-02).

JF801502Z