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ORIGINAL CONTRIBUTION

Inhibitory and synergistic effects of natural olive phenols on human platelet aggregation and lipid peroxidation of microsomes from vitamin E-deficient rats

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Abstract

Purpose   This study explored the in vitro antioxidant and anti-platelet activities of hydroxytyrosol, hydroxytyrosol acetate, 3,4-dihydroxyphenylglycol and two phenolic olive extracts. These compounds and extracts were obtained from a new industrial process to hydrothermally treat the alperujo (160 °C/60 min), a by-product of olive oil extraction.

Methods   The extracts and the purified compounds were obtained chromatographically using both ionic and adsorbent resins. The antioxidant activity was determined by measuring inhibition of human platelet aggregation and inhibition of lipid peroxidation in liver microsomes of vitamin E-deficient rats.

Results   The positive effect of the extracts on the inhibition of platelet aggregation is showed, being higher in the case of hydroxytyrosol acetate up to 38 %, and for the first time, its synergist effect with hydroxytyrosol has been proved, obtaining more than double of inhibition. The phenolic extracts and the isolated phenols showed good results for inhibiting the lipid oxidation, up to 62 and 25 %, respectively. A synergistic effect occurred when the hydroxytyrosol acetate and the 3,4-dihydroxyphenylglycol were supplemented by hydroxytyrosol.

Conclusion   These results suggest the extract and these compounds obtained from a novel industrial process could

be natural alternatives for the prevention of diseases related to cardiovascular disorder or oxidative damage.

Introduction

Consumption of the Mediterranean diet, characterized by high consumption of olive oil, fruits, vegetables, grains and legumes, reduces the incidence of major cardiovascular events [1] and is associated with a lower risk of peripheral artery disease [2]. The health benefits of the Mediterranean diet have been attributed to high concentration of free radical-scavenging polyphenols such as flavonoids. Virgin olive oil is rich in unsaponifiable minor components such as sterols, tocopherols and polyphenols. The polyphenols are natural antioxidants that not only contribute to the stability of the oil, but also have anti-inflammatory and anti-atherosclerotic properties [3]. Dietary polyphenols have been shown to inhibit LDL oxidation, scavenge superoxide and other ROS, and increase plasma antioxidant capacity [4]. Furthermore, some dietary phenolic compounds, mainly polyphenols, have been shown to affect human platelet function in vitro and in vivo [5, 6]. Platelets play a central role in the formation of plaques within blood vessels, contributing to early inflammatory events [5]; so, the observed cardiovascular benefits attributed to olive oil may be linked to the anti-platelet activity of olive oil polyphenols and thus to the suppression of platelet activation.

The ability of many flavonoids and phenols to inhibit peroxidation of hepatic microsomal preparations from vitamin E-deficient rats might indicate that these dietary compounds could have significant "vitamin E-like" antioxidant activity in biological systems [7]. The ability of dietary

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antioxidants to impair free radical-mediated oxidation of proteins, lipids and DNA, which are implicated in the pathogenesis of many chronic diseases [8], are believed to beneficially affect health.

After olive oil extraction, only a low percentage of the total phenolic compounds present in the olive fruits are found in the virgin olive oil. The remaining phenolics (98–99 %) end up in alperujo, a by-product from the modern two-phase processing technique used in the olive oil production [9].

Nowadays, the olive oil industry is starting to generate new by-products richer in phenols by thermal process applications such as hydroxytyrosol (HT), 3,4-dihydroxyphenylglycol (DHPG), hydroxytyrosol acetate (HTA), or polymeric phenolic fractions (PPF) [10]. The industrial use of a patented steam treatment (ST) [11] allows the formation of liquid source that enables the extraction and the isolation of the most important phenols present in virgin olive oil. The ST leads to obtain a natural liquid source without suspended solid that is richer in phenols than the other liquid sources obtained from olive oil wastes. This treatment was designed in base of the effects found in the "steam explosion" system (SE) in which high temperatures and pressures (up to 240 °C and 40 kg/cm^2) are needed for a few minutes followed by an explosive depressurization. The main effects were the high solubilization of phenols and sugars and the easy separation of its phases. The new ST operates at lower temperatures up to 170 °C and retention time of 1 h, avoiding the explosion and then, the technical complications and the high costs of operation, making easier its scale up that has been successfully done.

In previous work [6], the anti-platelet effects of alperujo extract obtained after an SE treatment were measured by comparing it with the effect of simple phenols such as hydroxytyrosol and 3,4-dihydroxyphenylglycol (DHPG), detecting for the first time the synergic effect of these two simple phenols. The effects on animal models of the alperujo extract treated by SE have been also studied [12]. Because the thermal conditions and the concentration of phenols of both treatments are different, together with the industrial use of the ST, the study of the in vitro activities of the phenols obtained from alperujo treated by ST are necessary. In the present work, the phenolic extract (PE) obtained from the alperujo treated industrially by ST has been tested, for the first time, to assess in vitro their anti-platelet in human platelet screening tool and their antioxidant effects in an animal microsomes model.

Materials and methods

Test compounds

Phenolic extracts (PE) were obtained from ST of alperujo at 160 °C for 60 min [10]. Polymeric phenolic fractions (PPF) were isolated from the PE by chromatography fractionation using Amberlite® XAD [13]. Hydroxytyrosol (HT) and 3,4-dihydroxyphenylglycol (DHPG) were purified by an ionic resin column [14, 15]. Hydroxytyrosol acetate (HTA) was isolated by an ionic resin column [16]. Figure 1 shows the HPLC profile of each of the test compounds used.

Determination of the total phenolic content

To complete the analysis of phenols, the total phenolic content of the test compounds was measured according to the Folin–Ciocalteu method [17] and expressed as grams of gallic acid equivalents per kilogram of extract.

HPLC-DAD

The different phenols were quantified using a Hewlett-Packard 1100 liquid chromatography system with a C-18 column (Teknokroma Tracer Extrasil ODS-2, 250 mm × 4.6 mm i.d., 5 μm). The system was equipped with a diode array detector (DAD; the wavelengths used for quantification were 254, 280 and 340 nm) and Rheodyne injection valves (20 μL loop). The mobile phases were 0.01 % trichloroacetic acid in water and acetonitrile utilizing the following gradient over a total run time of 55 min: 95 % A initially, 75 % A at 30 min, 50 % A at 45 min, 0 % A at 47 min, 75 % A at 50 min and 95 % A at 52 min until the run was completed. Quantification was completed by integration of peaks at different wavelengths with reference to calibrations made using external standards. The linearity of standard curves was expressed in terms of the determination coefficient plots of the integrated peak area versus concentration of the same standard. These equations were obtained over a wide concentration range in accordance with the levels of these compounds in the samples. The system was linear in all cases ($r > 0.99$). Three replicates were carried out on the same day.

Measurement of platelet aggregation

Blood sampling procedure

Blood samples were obtained from 23 healthy volunteers from European countries (9 males and 14 females and 25–60 years of age). Each volunteer signed consent form before donating blood. Volunteers had abstained from anti-inflammatory drugs and food supplements for at least 2 weeks prior to blood sampling and had a normal blood cell count. Blood was obtained using siliconized 21 gauge butterfly needles into 10-mL S-Monovette blood collection tubes containing 1 mL trisodium citrate as anticoagulant (Sarstedt Ltd, Beaumont Leys, UK).

Fig. 1 HPLC profile of different samples used in this work
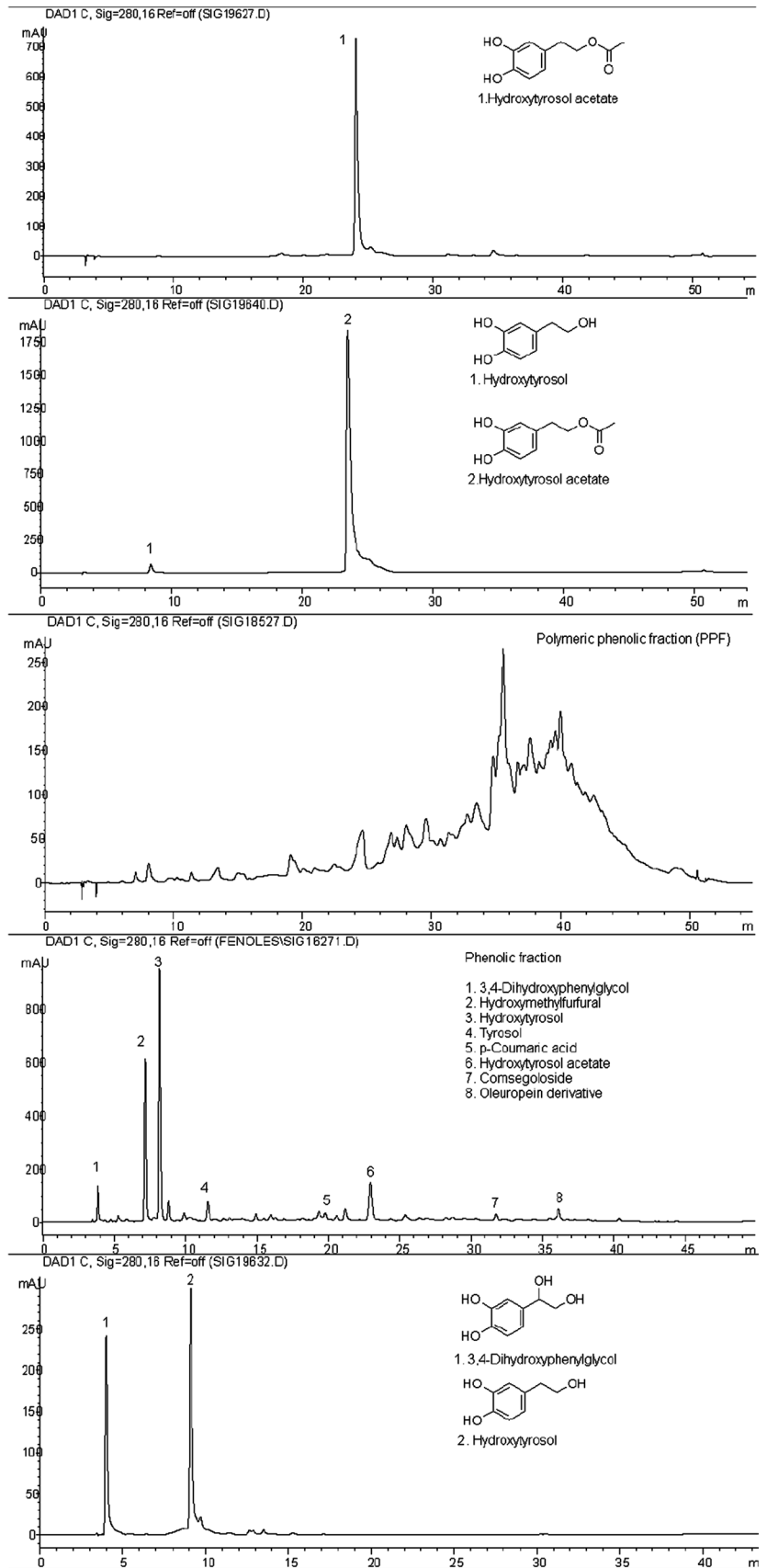
In vitro platelet aggregation

Agonist-induced platelet aggregation was measured in platelet-rich plasma (PRP) upon incubation with the test compounds using a PACKS 4 machine (platelet aggregation chromogenic kinetic system) as described by us previously [6, 18]. Briefly, blood from healthy volunteers was collected into sodium citrate 3.8 % (9:1 v/v), and the PRP was obtained according to standardized procedures. The plasma poor in platelets (PPP) was used to adjust the PRP to a platelet count of 300 ± 20 platelets/µL using the Sysmex haematology analyser (KX-21N, Sysmex, Germany). Once adjusted, the PRP was left to rest at 37 °C. PRP was incubated at least ten times with different platelets with 100 and 500 mg/L of PE; 10, 50 and 100 mg/L of HTA; 10, 50 and 100 mg/L of PPF; $8 + 0.6$, $43 + 3$, $85 + 6$ mg/L HTA + HT, respectively; or PBS as a control, for 10 min at 37 °C. To induce the platelet aggregation, collagen (final concentration 3 and 5 µg/mL) or thrombin receptor analogue peptide (TRAP) (final concentration 25 µM) was added. The platelet aggregation measurement was started 90 min after blood sampling. After 10 min, the curve of platelets aggregation was obtained, and platelet aggregation was expressed as percentage of maximal aggregation.

Inhibition of lipid peroxidation in vitamin E-deficient microsomes

Microsomal lipid peroxidation was assessed by measuring the reaction of malonaldehyde, a product of lipid oxidation, with thiobarbituric acid to produce thiobarbituric acid-reactive substances (TBARS). These are quantified by high-pressure liquid chromatography (HPLC). Each extract was tested in triplicate.

For this study, liver microsomes from vitamin E-deficient male weanling rats of the Rowett Hooded Lister strain were used, as described by us previously [12]. Briefly, rats that had been on a diet containing less than 0.5 mg/kg vitamin E (−VE) for 13 weeks were anesthetized with ether and bled by cardiac puncture prior to removal of the liver. This protocol was approved by the Ethical Review Committee of Animal Studies at the Rowett Research Institute and was conducted in compliance with the Animals (Scientific Procedures) Act, 1986.

Microsomes were extracted from homogenized liver samples by washing with 0.154 M KCl and suspension in potassium phosphate buffer 0.05 M pH 7.4. The protein concentration was determined by the Biuret method and adjusted to 10 mg/mL with 0.05 M potassium phosphate buffer pH 7.4. Vitamin E deficiency in rats was confirmed by plasma, tissue and microsomal vitamin E concentrations, which were below the limit of detection by HPLC. The effect of the test compounds on in vitro microsomal lipid peroxidation in the microsomes was determined as described by

the method of Duthie et al. [19]. Briefly, ethanol solutions (20 µL) with the test compounds in a concentration 25 times higher than required were incubated with microsomal preparations (0.5 mL) for 30 min at room temperature. Solutions with α-tocopherol were used as a control. For the control sample deficient in α-tocopherol, only 20 µL of EtOH was added. 0.1 mL of microsome solution with compounds was mixed with 0.5 mL of 0.05 mM ascorbic acid, 1 mL of a 2 mMADP/6 µM Fe^{2+} solution and 3.4 mL of 0.05 M phosphate buffer pH 7.4. The mixture was incubated at 30 °C and different aliquots were taken at 0, 5, 10 and 20 min for determination of thiobarbituric acid-reactive substances (TBARS) by HPLC. To this, 1 mL of 0.67 % thiobarbituric acid/acetic acid (1:1) and 2 mL of water were added and the solution was heated for 30 min at 100 °C in order to let the colour develop (measured at 535 nm).

The amount of generated TBARS was quantified by HPLC (Allience 2695) equipped with a fluorescence detector (Waters 2475). The column used was Phenomenex Luna 5u C18 (2) 100 A, 150×4.60 mm; the mobile phase was 60 % of KH_2PO_4 50 mM pH 7.0 and 40 % of MeOH at a flow of 0.8 mL/min for 12 min, in isocratic mode. Inhibition of lipid peroxidation was calculated as follows:

$$\% \text{ Inhibition} = \left(\frac{\text{AUC}(-\text{VE}) - \text{AUC}(-\text{VE} + \text{Comp})}{\text{AUC}(-\text{VE})} \right) \times 100$$

where AUC $(-\text{VE})$ = area under curve in microsomes from rats deficient in vitamin E and AUC $(-\text{VE} + \text{Comp})$ = area under curve in microsomes from rats deficient in vitamin E plus test compound. The ability to inhibit microsomal lipid peroxidation by each extract or compound (mixture) was compared with a negative control (no incubation) and two positive controls (effect in microsomes from rats with adequate diet of vitamin E (on a vitamin E-adequate diet 100 mg dα-tocopherol/kg) and effect in microsomes from rats on a vitamin E-deficient diet supplemented with 100 mg/kg diet of dα-tocopherol.

Statistical analysis

Statgraphics® plus software was used for statistical analysis. Comparisons amongst samples were made using one-way analysis of variance (ANOVA, equal variance test) and the least significant difference (LSD) method. A *p* value <0.05 was considered significant.

Results

Inhibition of platelet aggregation

In this study, the anti-platelet properties of two natural extracts (PE and PPF) and a purified compound (HTA)

Table 1 Subject characteristics

	Male	Female	Total
Number	9	14	23
Platelet count ($\times 10^9$/L)	221.65 ± 9.69[a]	204.58 ± 13.32	213.12 ± 11.50
Mean platelet volume (fL)	8.14 ± 0.26	8.61 ± 0.56	8.37 ± 0.15
Haematocrit (%)	38.72 ± 1.81	35.05 ± 0.78	36.89 ± 0.52
Haemoglobin (g/dL)	13.07 ± 0.89	11.74 ± 0.14	12.40 ± 0.38

[a] Values are mean ± SD

obtained from a new industrial source were screened. Furthermore, the potential synergic effects between hydroxytyrosol acetate and hydroxytyrosol (HTA + HT) have been also studied for the first time.

The characteristics of the volunteers from whom we obtained blood samples are shown in Table 1. Incubation of platelet-rich plasma from healthy volunteers with 100 mg/L of hydroxytyrosol acetate, which equates to approximately 510 μM, resulted in a significant ($p < 0.05$) inhibition of 3 and 5 μg/L collagen—and 25 μM TRAP—induced platelet aggregation by 38, 27 and 37 %, respectively (Fig. 2). Incubation with 50 mg/L HTA also resulted in a significant inhibition of 5 μg/L collagen and 25 μM TRAP-induced platelet aggregation by 7 and 22 %, respectively (Fig. 2).

In this study, two natural phenolic extracts, obtained from alperujo hydrothermally treated at 160 °C/60 min (PE) and a polymeric phenolic fraction (PPF) isolated from phenolic extract were also screened. The activity of this new phenolic polymeric fraction (PPF) isolated and characterized has been tested for the first time. This PPF was composed mainly of phenolic compounds with small amounts of carbohydrates, proteins and ash, and it was formed during the ethyl acetate extraction process from the autohydrolysis liquids of steam-treated alperujo [13]. Incubation with 100 mg/L PPF resulted in significant ($p < 0.05$) inhibition of 3 and 5 μg/L collagen and 25 μM TRAP-induced platelet aggregation by 23, 13 and 22 %, respectively. For the assays with PE, a concentration of total phenols of 240 g phenols/kg extract was found by the Folin–Ciocalteu method. The HPLC results suggested that 1 g of PE contained 109.3 mg HT, 10.3 mg PPF, 9.9 mg DHFG and 8.5 mg HTA. Only the highest test concentration of PE (i.e. 500 mg/L) significantly inhibited 3 and 5 μg/L collagen and 25 μM TRAP-induced platelet aggregation by 52, 40 and 19 %, respectively (Fig. 2).

Inhibition of microsomal lipoxidation from vitamin E-deficient rats

The antioxidant properties of two natural extracts (PE and PPF) and three purified compounds (HT, HTA and DHPG) were screened. Furthermore, two mixtures of compounds (HT + DHPG) and (HTA + HT) were evaluated. Previous studies showed that a decrease in membrane concentration of α-tocopherol increased the rates of TBARS formation in all tissues, but the effect was especially pronounced in adrenal mitochondria and microsomes [20].

The PE, HTA and the HT + DHPG mixture showed most inhibition of lipid oxidation at the lowest test concentrations (Tables 2, 3). 0.05 mM of HTA inhibited lipid peroxidation by 20.8 % (Table 3). Interestingly, this was very similar to the highest concentration (0.4 mM), but much more effective than the intermediate concentration (13.5 % of inhibition). HT and DHPG decreased lipid peroxidation of the microsomal preparation from vitamin E-deficient rats in a time-dependent manner, and the protection against peroxidation improved with increasing concentrations (Fig. 3).

Discussion

Inhibition of platelet aggregation

The anti-platelet property of the HTA and HT mixture was more effective than of each component separately. So when HT was tested alone, 6 mg/L of compound inhibited platelet aggregation by 4 % using collagen-induced platelet aggregation and no inhibitory effect was observed for 3 mg/L. Indeed, incubation with 43 + 3 mg/L (219 + 19.5 μM) HTA and HT resulted in a significant ($p < 0.05$) inhibition of 3 and 5 μg/L collagen and 25 μM TRAP-induced platelet aggregation by 30, 22 and 31 %, respectively (Fig. 2). A similar effect was observed for 85 + 6 mg/L HTA + HT (433 + 30.6 μM), inhibiting platelet aggregation by 85, 71 and 50 %, respectively (Fig. 2). González-Correa et al. [21] observed that 26 and 48 μM HTA inhibited collagen-induced platelet aggregation by 50 %. This anti-platelet effect was stronger than that of HT and similar to that of acetylsalicylic acid (ASA), Hubbard et al. [22] considered different simple phenols and found that for p-coumaric, caffeic, ferulic and sinapic acid, a concentration of 478–816 μM was necessary to inhibit collagen-induced platelet aggregation by 50 %. Furthermore, concentrations between 10 and 100 μM of gallic acid inhibited TRAP-induced platelet aggregation by 10–50 % [23]. Ostertag et al. [18] found that 100 μM of catechol, resorcinol, pyrogallol and hippuric acid inhibited in vitro collagen-induced platelet

Fig. 2 Inhibition of platelet aggregation by HTA, HTA + HT, PPF and PE using 3 μg/L collagen (**a**), 5 μg/L collagen (**b**) or 25 μM TRAP (**c**) as an agonist. Platelet inhibition is expressed as the % decrease in the areas under the *curve* for platelet aggregation measured by light-transmission aggregometry when compared with control (PBS). *Significantly different from control (*p* > 0.05); (*solid line*) mean value
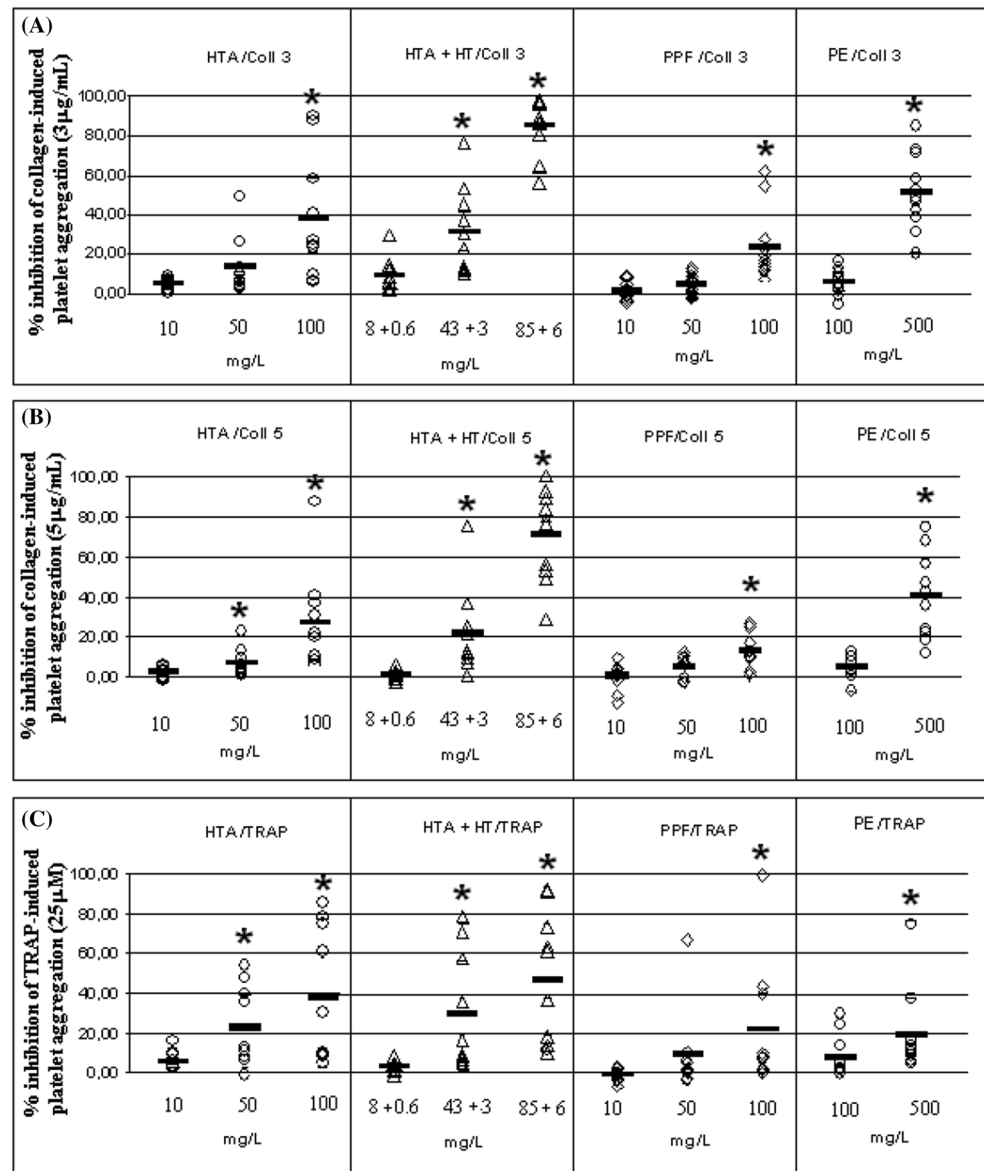
aggregation. A previous study in our group using the same samples [6] showed that 40 mg/L of HT inhibited collagen-induced platelet aggregation by 5 %, whereas a mixture of HT and DHPG (40 + 5 mg/L, respectively) inhibited collagen-induced platelet aggregation by approximately 12 % in a synergistic manner. In most of these studies, only relatively high concentrations of phenolic compounds caused significant anti-platelet effects in an in vitro screening model. Although these models can effectively measure the potential pharmacological anti-platelet effects of a large range of bioactive compounds, the effective concentrations may not necessarily be physiologically relevant from a dietary perspective [6].

With regard to the PE, in a previous study [6] using an PE obtained by SE, a similar concentration of 500 mg/L alperujo extract significantly inhibited collagen—and

TRAP—induced platelet aggregation by 25 and 16 %, respectively. The higher efficacy of PE in the current study could be due to a different phenol composition because the lower severity used to treat the alperujo by the industrial ST system.

Inhibition of microsomal lipoxidation from vitamin E-deficient rats

The HT + DHPG mixture produced a higher percentage inhibition (31 %) for lower test concentrations (0.25 + 0.25 mM), which was more powerful than the efficacy of individual compounds (Table 2). Also increasing concentrations of PPF showed improved inhibition of lipid peroxidation, up to 58 % inhibition for the highest concentration (200 mg/L) (Table 3). The high efficacy of

Table 2 Abilities of HT and DHPG to inhibit peroxidation of hepatic microsomal preparations from vitamin E-deficient rats

Compound	DHPG		HT	
	mg/L	% Inhibition	mg/L	% Inhibition
+VE		86.38 ± 7.9		81.39 ± 11.6
−VE + 0.05 mM	8.5	1.02 ± 0.1	8	−2.42 ± 0.6
−VE + 0.1 mM	17	4.89 ± 0.7	15	4.84 ± 0.9
−VE + 0.25 mM	43	10.74 ± 2.2	39	5.53 ± 1.1
−VE + 0.50 mM	85	11.8 ± 1.9	77	8.92 ± 3.4
−VE + 1 mM	170	13.83 ± 2.6	154	12.29 ± 1.3
−VE + 2 mM	340	24.61 ± 4.3	308	25.45 ± 5.7
Mixture	DHPG + HT			
	mg/L			% Inhibition
−VE + (0.25 + 0.25) mM	(43 + 39)			31.26 ± 6.0*
−VE + (0.50 + 0.50) mM	(85 + 77)			9.34 ± 1.2*
−VE + (0.75 + 0.75) mM	(128 + 116)			18.07 ± 3.2*

* Significant difference ($p < 0.05$) between DHPG + HT and the individual sum of DHPG and HT

Table 3 Abilities of different phenols and PE to inhibit peroxidation of hepatic microsomal preparations from vitamin E-deficient rats

Compound	HTA		α-Tocopherol	
	mg/L	% Inhibition	mg/L	% Inhibition
−VE + 0.05 mM	9.8	20.76 ± 4.82	23.7	47.6 ± 4.05
−VE + 0.2 mM	39.2	13.45 ± 1.27	94.6	69.8 ± 2.36
−VE + 0.4 mM	78.4	21.06 ± 0.79	189.2	72.9 ± 1.65
Extracts	PPF		PE	
	mg/L	% Inhibition	mg/L	% Inhibition
−VE + extract	10	15.4 ± 3.73	39	62.6 ± 1.51
−VE + extract	100	16.5 ± 4.69	58	45.4 ± 0.66
−VE + extract	200	57.9 ± 4.30	77	50.7 ± 0.33
Mixture	HTA + HT			
	mg/L			% Inhibition
−VE + (0.1 + 0.01) mM	(19.6 + 1.5)			23.8 ± 3.17
−VE + (0.4 + 0.04) mM	(78.4 + 6.2)			23.72 ± 0.70
−VE + (0.8 + 0.08) mM	(157 + 12.4)			37.72 ± 1.62

PE to act as an antioxidant may be explained by the fact that the extract contains different compounds that work in a synergistic fashion to inhibit lipid peroxidation, or it contains individual minor components that have high capacity of inhibition of lipid peroxidation.

In a detailed study, Duthie and Morrice [7] found that different flavonoids (i.e. quercetin, kaempferol, myricetin, galingin and fisetin) inhibited lipid peroxidation by 68–88 %, a similar range to the olive phenols studied in microsomes derived from livers of rats deficient in vitamin E. Mitchell et al. [24] observed similar results for kaempferol, which of all phytoestrogens had the greatest ability to inhibit lipid peroxidation in vitamin E-deficient microsomes with an IC_{50} of 160 μM. In comparison, IC_{50} concentrations required to inhibit lipid peroxidation in liver microsomes was 31 μM for α-tocopherol and 124 μM for quercetin. The IC_{50} values for the isoflavones, chalcones and coumestan were approximately 35-, 22- and 16-fold higher than that of α-tocopherol, respectively.

Conclusion

The results of this study show that the olive extracts obtained from a new industrial liquid source produced by ST application to alperujo have higher activities that the PE previously reported using the SE as a treatment. Besides, a synergist effect of HTA and HT has been found for the first

Fig. 3 Pre-incubation of hepatic microsomal preparations from vitamin E-deficient rats with a HT and DHPG on production of thiobarbituric reactive substances (TBARS) following initiation of peroxidation with Fe/ADP
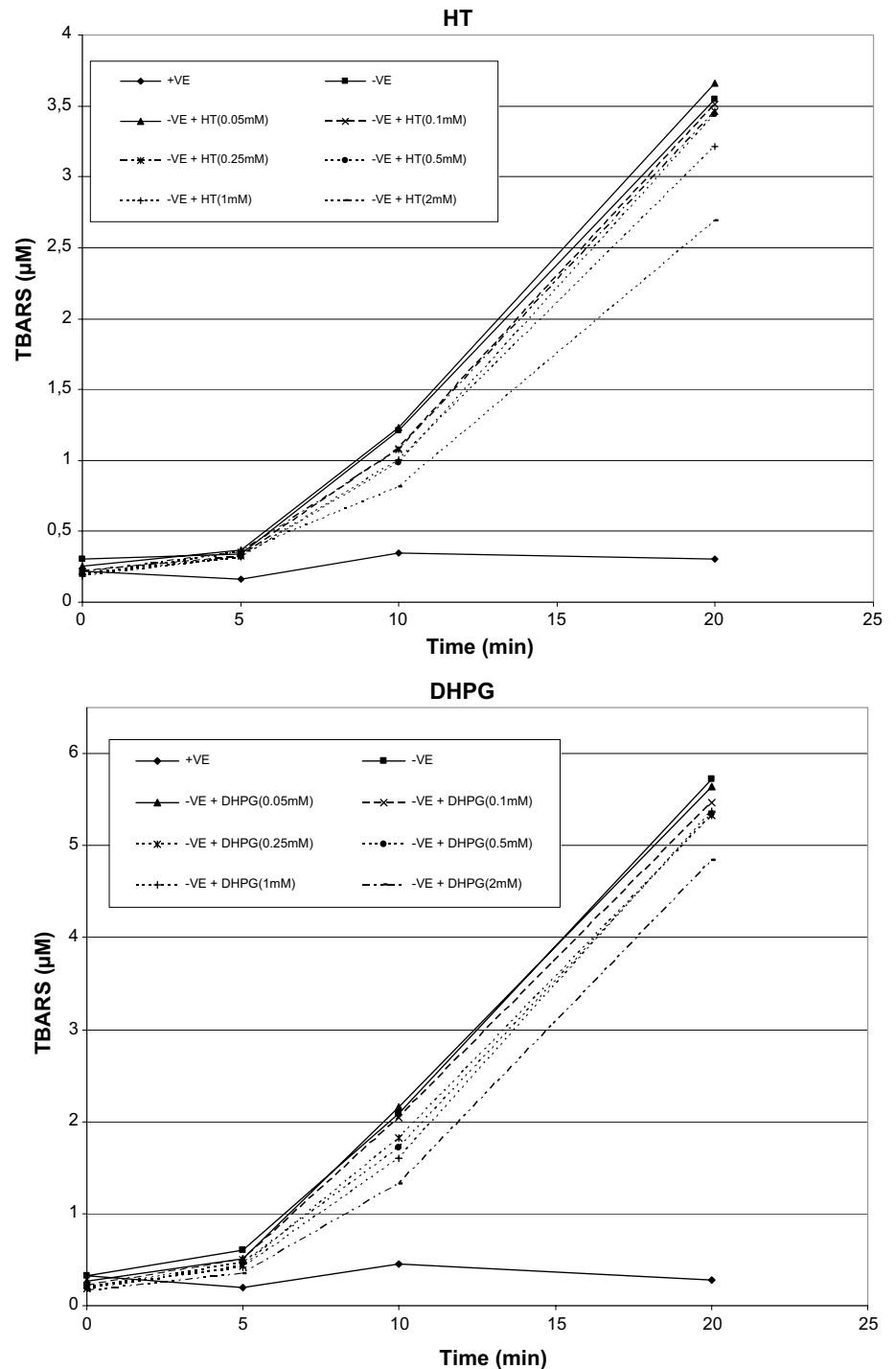
time. The PE and the isolated phenols obtained may protect against platelet activation and platelet adhesion and have antioxidant properties. The extraction of these compounds can be accomplished in a sustainable manner through effective use of the industrial source from the olive oil by-product manufacturing process, offering a unique opportunity to produce functional ingredients that alone, or in effective combinations, could be added to foods to enhance their health properties.

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