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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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Running title: Antiplatelet and antioxidant effects of phenols isolated from alperujo.

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1 **Abstract**

2

3 Purpose: This study explored the *in vitro* anti-oxidant and anti-platelet activities of 4 hydroxytyrosol, hydroxytyrosol acetate, 3,4-dihydroxyphenylglycol, and two phenolic 5 olive extracts. These compounds and extracts were obtained from a new industrial 6 process to treated hydrothermally the alperujo (160 ºC/60 min), a by-product of olive oil 7 extraction.

8 Methods: The extracts and the purified compounds have been obtained 9 chromatographically using both ionic and adsorbent resins. The antioxidant activity has 10 been determined by measuring inhibition of human platelet aggregation and inhibition 11 of lipid peroxidation in liver microsomes of vitamin-E deficient rats.

12 Results: The positive effect of the extracts on the inhibition of platelets 13 aggregation is showed, being higher in the case of hydroxytyrosol acetate and by the 14 first time, its synergist effect with hydroxytyrosol has been proved. The phenolic extract 15 and the isolated phenols showed good results for inhibiting the lipid oxidation, mainly 16 the hydroxytyrosol acetate. A synergistic effect occurred when the hydroxytyrosol 17 acetate and the 3,4-dihydroxyphenylglycol were supplemented by hydroxytyrosol.

18 Conclusion: These results suggest these extract and compounds obtaining from a 19 novel industrial process could be natural alternatives for the prevention of diseases 20 related with cardiovascular disorder or oxidative damages.

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22

23

24 **KEYWORDS:** Alperujo, olive oil wastes, phenols, platelet function, lipid peroxidation,

25 vitamin E.

26 **1. Introduction**

27

28 Consumption of the Mediterranean diet, characterized by high consumption of
29 olive oil, fruits, vegetables, grains and legumes, reduces the incidence of major
30 cardiovascular events [1] and is associated with a lower risk of peripheral artery disease
31 [2]. The health benefits of the Mediterranean diet have been attributed to high
32 concentration of free radical-scavenging polyphenols such as flavonoids. Virgin olive
33 oil is rich in unsaponifiable minor components such as sterols, tocopherols, and
34 polyphenols. The polyphenols are natural antioxidants that not only contribute to the
35 stability of the oil, but also have anti-inflammatory and anti-atherosclerotic properties
36 [3]. Dietary polyphehols have been shown to inhibit LDL oxidation, scavenge
37 superoxide and other ROS and increase plasma antioxidant capacity [4]. Furthermore,
38 some dietary phenolic compounds, mainly polyphenols, have been shown to affect
39 human platelet function *in vitro* and *in vivo* [5, 6]. Platelets play a central role in the
40 formation of plaques within blood vessels, contributing to early inflammatory events
41 [5], so, the observed cardiovascular benefits attributed to olive oil may be linked to the
42 anti-platelet activity of olive oil polyphenols and thus to the suppression of platelet
43 activation.

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

47 systems [7]. The ability of dietary antioxidants to impair free radical-mediated oxidation
48 of proteins, lipids and DNA, which are implicated in the pathogenesis of many chronic
49 diseases [8], are believed to beneficially affect health.

50 After olive oil extraction, only a low percentage of the total phenolic compounds
51 present in the olive fruits are found in the virgin olive oil. The remaining phenolics (98-
52 99 %) end up in alperujo, a by-product from the modern two-phase processing
53 technique used in the olive oil production [9].

54 Nowadays the olive oil industry is starting to generate new by-products richer in
55 phenols by thermal process applications like hydroxytyrosol (HT), 3,4-
56 dihydroxyphenylglycol (DHPG), hydroxytyrosol acetate (HTA) or polymeric phenolic
57 fractions (PPF) [10]. The industrial use of a patented steam treatment (ST) [11] allows
58 the formation of liquid source that enables the extraction and the isolation of the most
59 important phenols present in virgin olive oil. The ST leads to obtain a natural liquid
60 source without suspended solid that is richer in phenols than the other liquid sources
61 obtained from olive oil wastes. This treatment was designed in base of the effects found
62 in the "Steam Explosion" system (SE) in which high temperatures and pressures (up to
63 240 ºC and 40 kg/cm^2) are needed for a few minutes followed by an explosive
64 depressuration. The new ST operates at lower temperatures up to 170 ºC and retention
65 time of one hour, avoiding the explosion and then, the technical complications; hence its
66 scale up has been successfully done.

67 In previous work [6] the anti-platelet effects of alperujo extract obtained after an
68 SE treatment was measured comparing with the effect of simple phenols like
69 hydroxytyrosol and 3,4-dihydroxyphenylglycol (DHPG), detecting by the first time the
70 synergic effect of these two simple phenols. The effects on animal model of the alperujo
71 extract treated by SE have been also studied [12]. Because the thermal conditions and

72 the concentration of phenols of both treatments are different, together with the industrial
73 use of the ST, the study of the *in vitro* activities of the phenols obtained from alperujo
74 treated by ST are necessary. In the present work, the phenolic extract obtained from the
75 alperujo treated industrially by ST has been tested, for the first time, to assess *in vitro*
76 their anti-platelet in human platelet screening tool and their antioxidant effects in animal
77 microsomes model.

78

79 **2. Materials and Methods**

80

81 **2.1 Test compounds**

82 Phenolic extracts (PE) were obtained from ST of alperujo at 160ºC for 60 min
83 [10]. Polymeric phenolic fractions (PPF) were isolated from the phenolic extracts by
84 chromatography fractionation using Amberlite® XAD [13]. Hydroxytyrosol (HT) and
85 3,4-dihydroxyphenylglycol (DHPG) were purified by an ionic resin column following
86 the process described by Fernández-Bolaños et al., (2002)[14] and Fernández-Bolaños
87 et al., (2008)[15], respectively. Hydroxytyrosol acetate (HTA) was isolated by an ionic
88 resin column following the process described by Rodríguez-Gutiérrez et al., (2011)[16].
89 Figure 1 shows the HPLC-profile of each of the test compounds used.

90

91 **2.2. Determination of the total phenolic content**

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

95

96 **2.3. HPLC-DAD.**

97 The different phenols were quantified using a Hewlett-Packard 1100 liquid
98 chromatography system with a C-18 column (Teknokroma Tracer Extrasil ODS-2, 250
99 mm × 4,6 mm i.d., 5 μm). The system was equipped with a diode array detector (DAD;
100 the wavelengths used for quantification were 254, 280, and 340 nm) and Rheodyne
101 injection valves (20 μL loop). The mobile phases were 0.01% trichloroacetic acid in
102 water and acetonitrile utilizing the following gradient over a total run time of 55 min:
103 95% A initially, 75% A at 30 min, 50% A at 45 min, 0% A at 47 min, 75% A at 50 min,
104 and 95% A at 52 min until the run was completed. Quantification was carried out by
105 integration of peaks at different wavelengths with reference to calibrations made using
106 external standards. The linearity of standards curve was expressed in terms of the
107 determination coefficients plots of the integrated peaks area versus concentration of the
108 same standard. These equations were obtained over a wide concentration range in
109 accordance with the levels of these compounds in the samples. The system was linear in
110 all cases ($r > 0.99$). Three replicates were carried out on the same day.
111
112 **2.4. Measurement of platelet aggregation**
113
114 *Blood sampling procedure*
115 Blood samples were obtained from 23 healthy volunteers from European countries
116 (9 males and 14 females and 25-60 years of age). Each volunteer signed consent form
117 before donating blood. Volunteers had abstained from anti-inflammatory drugs and food
118 supplements for at least two weeks prior to blood sampling and had a normal blood cell
119 count. Blood was obtained using siliconized 21 gauge butterfly needles into 10 mL S-
120 Monovette blood collection tubes containing 1 mL trisodium citrate as anticoagulant
121 (Sarstedt Ltd, Beaumont Leys, UK).

122

123 *In vitro platelet aggregation*

124 Agonist-induced platelet aggregation was measured in platelet rich plasma (PRP)
125 upon incubation with the test compounds using a PACKS 4 machine (platelet
126 aggregation chromogenic kinetic system) as described by us previously [6, 18]. Briefly,
127 blood from healthy volunteers was collected into sodium citrate 3.8% (9:1 v/v) and the
128 PRP was obtained according to standardized procedures. The plasma poor in platelets
129 (PPP) was used to adjust the PRP to a platelet count of 300±20 platelets /µL using the
130 sysmex haematology analyser (KX-21N, sysmex, Germany). Once adjusted, the PRP
131 was left to rest at 37°C. PRP was incubated at least ten times with different platelets
132 with 100 and 500 mg/L of PE; 10, 50 and 100 mg/L of HTA; 10, 50 and 100 mg/L of
133 PPF; 8+0.6, 43+3, 85+6 mg/L HTA + HT respectively; or PBS as a control, for 10
134 minutes at 37 ºC. To induce the platelet aggregation, collagen (final concentration 3 and
135 5 µg/mL) or thrombin receptor analogue peptide (TRAP) (final concentration 25µM)
136 were added. The platelet aggregation measurement was started 90 minutes after blood
137 sampling. After 10 minutes, the curve of platelets aggregation was obtained, and platelet
138 aggregation was expressed as percentage of maximal aggregation.

139

140 **2.5. Inhibition of lipid peroxidation in vitamin E-deficient microsomes.**

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

145 For this study, liver microsomes from vitamin E-deficient male weanling rats of
146 the Rowett Hooded Lister strain were used, as described by us previously [12]. Briefly,

147 rats that had been on a diet containing less than 0.5 mg/kg vitamin E (-VE) for 13 weeks
148 were anesthetized with ether and bled by cardiac puncture prior to removal of the liver.
149 This protocol was approved by the Ethical Review Committee of Animal Studies at the
150 Rowett Research Institute, and was conducted in compliance with the Animals
151 (Scientific Procedures) Act, 1986.

152 Microsomes were extracted from homogenised liver samples by washing with
153 0.154 M KCl and suspension in potassium phosphate buffer 0.05 M pH 7.4. The protein
154 concentration was determined by the Biuret method and adjusted to 10 mg/mL with
155 0.05 M potassium phosphate buffer pH 7.4. Vitamin E deficiency in rats was confirmed
156 by plasma, tissue and microsomal vitamin E concentrations, which were below the limit
157 of detection by HPLC. The effect of the test compounds on in vitro microsomal lipid
158 peroxidation in the microsomes was determined as described by the method of Duthie et
159 al., (1991)[19]. Briefly, ethanol solutions (20 μL) with the test compounds in a
160 concentration 25 times higher than required were incubated with microsomal
161 preparations (0.5 mL) for 30 min at room temperature. Solutions with α-tocopherol
162 were used as a control. For the control sample deficient in α-tocopherol, only 20 μL of
163 EtOH was added. 0.1 mL of microsome solution with compounds was mixed with 0.5
164 mL of 0.05mM ascorbic acid, 1 mL of a 2mMADP /6μM Fe^{2+} solution and 3.4 mL of
165 0.05M phosphate buffer pH 7.4. The mixture was incubated at 30 ºC and different
166 aliquots were taken at 0, 5, 10 and 20 min for determination of thiobarbituric acid-
167 reactive substances (TBARS) by HPLC. To this 1mL of 0.67% thiobarbituric
168 acid:acetic acid (1:1) and 2 mL of water were added and the solution was heated for 30
169 min at 100 ºC in order to let the colour develop (measured at 535 nm).

170 The amount of generated TBARS was quantified by HPLC (Allience) equipped
171 with a bomb (Waters 2695) and a fluorescence detector (Waters 2475). The column

172 used were Phenomenex Luna 5u C18 (2) 100 A, 150 x 4.60 mm, the mobile phase was
173 60 % of KH_2PO_4 50 mM pH 7.0 and 40% of MeOH at a flow of 0.8 mL/min for 12
174 minutes, in isocratic mode. Inhibition of lipid peroxidation was calculated as follows:

175
$$\%\,Inhibition = \left(\frac{AUC(-VE) - AUC(-VE + Comp)}{AUC(-VE)} \right) \cdot 100$$

176

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

185

186 **2.6. Statistical analysis**

187 STATGRAPHICS ® plus software was used for statistical analysis. Comparisons
188 amongst samples were made using one-way analysis of variance (ANOVA) and
189 the Least Significant Difference (LSD) method. A p-value less than 0.05 was
190 considered significant.

191

192 **3. Results**

193

194 **3.1 Inhibition of platelet aggregation**

195 In this study, the anti-platelet properties of two natural extracts (PE and PPF) and
196 a purified compound (HTA) obtained from a new industrial source have been screened.
197 Furthermore, the potential synergic effects between hydroxytytosol acetate and
198 hydroxytyrosol (HTA+HT) have been also studied by the first time.

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

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

220

221 **3.2. Inhibition of microsomal lipoxidation from vitamin E deficient rats.**

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

228 The phenolic extract (PE), HTA and the HT+DHPG mixture showed most
229 inhibition of lipid oxidation inhibition at the lowest test concentrations (Tables 2 and 3).
230 0.05 mM of HTA inhibited lipid peroxidation by 20.8% (Table 3). Interestingly this was
231 very similar to the highest concentration (0.4 mM) but much more effective than the
232 intermediate concentration (13.5% of inhibition). HT and DHPG decreased lipid
233 peroxidation of the microsomal preparation from vitamin E deficient rats in a time-
234 dependent manner, and the protection against peroxidation improved with increasing
235 concentrations (Figure 3).

236

237 **<u>4. Discussion</u>**

238 **4.1 Inhibition of platelet aggregation**

239 The anti-platelet property of the HTA and HT mixture was more effective than of
240 each component separately. So when HT was tested alone 6 mg/L of compound
241 inhibited platelet aggregation by 4 % using collagen-induced platelet aggregation and
242 any effect inhibitory was observed for 3 mg/L. Indeed, incubation with 43+3 mg/L
243 (219+19.5 μM) HTA and HT resulted in a significant ($p<0.05$) inhibition of 3 and 5
244 μg/L collagen and 25 μM TRAP-induced platelet aggregation by 30, 22 and 31%,

245 respectively (Figure 2). A similar effect was observed for 85+6 mg/L HTA + HT
246 (433+30.6 µM), inhibiting platelet aggregation by 85, 71 and 50%, respectively (Figure
247 2). González-Correa, et al., (2009)[21] observed that 26 and 48 µM HTA inhibited
248 collagen-induced platelet aggregation by 50%. This antiplatelet effect was stronger than
249 that of HT, and similar to that of acetylsalicylic acid (ASA), Hubbard, et al., (2003)[22]
250 considered different simple phenols and found that for p-coumaric, caffeic, ferulic and
251 sinapic acid a concentration of 478-816 µM was necessary to inhibit collagen-induced
252 platelet aggregation by 50%. Furthermore, concentrations between 10 and 100 µM of
253 gallic acid inhibited TRAP-induced platelet aggregation by 10-50 % [23]. Ostertag et al.
254 2011 [18] found that 100 µM of cathecol, resorcinol, pyrogallol and hippuric acid
255 inhibited in vitro collagen-induced platelet aggregation. A previous study in our group
256 using the same samples [6] showed that 40 mg/L of HT inhibited collagen-induced
257 platelet aggregation by 5%, whereas a mixture of HT and DHPG (40+5 mg/L
258 respectively) inhibited collagen-induced platelet aggregation by approximately 12 % in
259 a synergistic manner. In most of these studies, only relatively high concentrations of
260 phenolic compounds caused significant anti-platelet effects in an *in vitro* screening
261 model. Although these models can effectively measure the potential pharmacological
262 anti-platelet effects of a large range of bioactive compounds, the effective
263 concentrations may not necessarily be physiologically relevant from a dietary
264 perspective [6].

265 With regard to the phenolic extract, in a previous study [6] using an PE obtained
266 by SE, a similar concentration of 500 mg/L alperujo extract significantly inhibited
267 collagen- and TRAP-induced platelet aggregation by 25 and 16%, respectively. The
268 higher efficacy of PE in the current study could be due to a different phenol

269 composition because the lower severity used to treat the alperujo by the industrial ST
270 system.
271
272 **4.2. Inhibition of microsomal lipoxidation from vitamin E deficient rats.**
273 The HT+DHPG mixture produced a higher percentage inhibition (31%) for lower
274 test concentrations (0.25 mM+0.25mM), which was more powerful than the efficacy of
275 individual compounds (Table 2). Also increasing concentrations of PPF showed
276 improved inhibition of lipid peroxidation, up to 58 % inhibition for the highest
277 concentration (200 mg / L) (Table 3). The high efficacy of PE to act as an antioxidant
278 may be explained by the fact that the extract contains different compounds that work in
279 a synergistic fashion to inhibition lipid peroxidation, or it contains individual minor
280 components that have high capacity of inhibition of lipid peroxidation
281 In a detailed study, Duthie & Morrice, (2012)[7] found that different flavonoids
282 (i.e. quercetin, kaempferol, myricetin, galingin and fisetin) inhibited lipid peroxidation
283 by 68-88%, a similar range than the olive phenols studied in microsomes derived from
284 livers of rats deficient in vitamin E. Mitchell, et al., (1998)[24] observed similar results
285 for kaempherol, which of all phytoestrogens had the greatest ability to inhibit lipid
286 peroxidation in vitamin E-deficient microsomes with an IC_{50} of 160 μM. In
287 comparison, IC_{50} concentrations required to inhibit lipid peroxidation in liver
288 microsomes was 31 μM for α-tocopherol, and 124 μM for quercetin. The IC_{50} values
289 for the isoflavones, chalcones and coumestan were approximately 35, 22 and 16-fold
290 higher than that of α-tocopherol, respectively.
291
292 **<u>5. Conclusion</u>**
293

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. Beside, a synergist effect of HTA and HT has been found by the first time. The PE and the isolated phenols obtained may protect against platelet activation, platelet adhesion and have anti-oxidant 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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401
402
403
404
405 **Figure caption**.
406
407 **Figure 1:** HPLC-profile of different samples used in this work.
408
409 **Figure 2:** Inhibition of platelet aggregation by HTA, HTA+HT, PPF and PE using 3
410 µg/L collagen (A), 5 µg/L collagen (B) or 25 µM TRAP (C) as an agonist. Platelet
411 inhibition is expressed as the % decrease in the areas under the curve for platelet
412 aggregation measured by light-transmission aggregometry when compared with control
413 (PBS). * Significantly different from control ($p > 0.05$); (-) mean value.
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Figure 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:

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