Postprint of European Journal of Nutrition 2015, Volume 54, [Issue 8](#), pp 1287-1295

doi: 10.1007/s00394-014-0807-8

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

Fátima Rubio-Senent^a, Baukje de Roos^b, Garry Duthie^b, Juan Fernández-Bolaños^a, Guillermo Rodríguez-Gutiérrez^{a*}.

^a Food Phytochemistry Department, Instituto de la Grasa (CSIC), Avda. Padre García Tejero 4, Apartado 41012 Sevilla, Spain.

^b Rowett Institute of Nutrition & Health, University of Aberdeen, Greenburn Road, Bucksburn, Aberdeen AB21 9SB, UK

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

*Corresponding author. Tel.: +34 354692516; fax: +34 954691262

E-mail address: guirogu@cica.es (G. Rodríguez-Gutiérrez)

Abstract

Purpose: This study explored the *in vitro* anti-oxidant 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 treated hydrothermally the alperujo (160 °C/60 min), a by-product of olive oil extraction.

Methods: The extracts and the purified compounds have been obtained chromatographically using both ionic and adsorbent resins. The antioxidant activity has been 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 platelets aggregation is showed, being higher in the case of hydroxytyrosol acetate and by the first time, its synergist effect with hydroxytyrosol has been proved. The phenolic extract and the isolated phenols showed good results for inhibiting the lipid oxidation, mainly the hydroxytyrosol acetate. A synergistic effect occurred when the hydroxytyrosol acetate and the 3,4-dihydroxyphenylglycol were supplemented by hydroxytyrosol.

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

KEYWORDS: Alperujo, olive oil wastes, phenols, platelet function, lipid peroxidation, vitamin E.

1. 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 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 like 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/cm²) are needed for a few minutes followed by an explosive depressuration. The new ST operates at lower temperatures up to 170 °C and retention time of one hour, avoiding the explosion and then, the technical complications; hence its scale up has been successfully done.

In previous work [6] the anti-platelet effects of alperujo extract obtained after an SE treatment was measured comparing with the effect of simple phenols like hydroxytyrosol and 3,4-dihydroxyphenylglycol (DHPG), detecting by the first time the synergic effect of these two simple phenols. The effects on animal model 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 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 animal microsomes model.

2. Materials and Methods

2.1 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 phenolic extracts by chromatography fractionation using Amberlite® XAD [13]. Hydroxytyrosol (HT) and 3,4-dihydroxyphenylglycol (DHPG) were purified by an ionic resin column following the process described by Fernández-Bolaños et al., (2002)[14] and Fernández-Bolaños et al., (2008)[15], respectively. Hydroxytyrosol acetate (HTA) was isolated by an ionic resin column following the process described by Rodríguez-Gutiérrez et al., (2011)[16]. Figure 1 shows the HPLC-profile of each of the test compounds used.

2.2. Determination of the total phenolic content

To complete the simple 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.

2.3. 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 carried out by integration of peaks at different wavelengths with reference to calibrations made using external standards. The linearity of standards curve was expressed in terms of the determination coefficients plots of the integrated peaks 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.

2.4. 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 two 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).

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 / μ L 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 minutes at 37 °C. To induce the platelet aggregation, collagen (final concentration 3 and 5 μ g/mL) or thrombin receptor analogue peptide (TRAP) (final concentration 25 μ M) were added. The platelet aggregation measurement was started 90 minutes after blood sampling. After 10 minutes, the curve of platelets aggregation was obtained, and platelet aggregation was expressed as percentage of maximal aggregation.

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

Microsomal lipid peroxidation was assessed by measuring the reaction of malonadehyde, 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 were 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 homogenised 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., (1991)[19]. Briefly, ethanol solutions (20 μ L) 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 μ L of EtOH was added. 0.1 mL of microsome solution with compounds was mixed with 0.5 mL of 0.05mM ascorbic acid, 1 mL of a 2mMADP /6 μ M Fe²⁺ solution and 3.4 mL of 0.05M 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 1mL 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) equipped with a bomb (Waters 2695) and a fluorescence detector (Waters 2475). The column

used were Phenomenex Luna 5u C18 (2) 100 A, 150 x 4.60 mm, the mobile phase was 60 % of KH₂PO₄ 50 mM pH 7.0 and 40% of MeOH at a flow of 0.8 mL/min for 12 minutes, in isocratic mode. Inhibition of lipid peroxidation was calculated as follows:

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

where AUC (-VE) = area under curve in microsomes from rats deficient in vitamin E and AUC (-VE + 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 d α -tocopherol/kg) and effect in microsomes from rats on a vitamin E-deficient diet supplemented with 100 mg/kg diet of d α -tocopherol.

2.6. Statistical analysis

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

3. Results

3.1 Inhibition of platelet aggregation

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

The characteristics of the volunteers from which 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 μ M, resulted in a significant ($p < 0.05$) inhibition of 3 and 5 μ g/L collagen- and 25 μ M TRAP- induced platelet aggregation by 38, 27 and 37%, respectively (Figure 2). Incubation with 50 mg/L HTA also resulted in a significant inhibition of 5 μ g/L collagen and 25 μ M TRAP-induced platelet aggregation by 7 and 22%, respectively (Figure 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 have been also screened. The activity of this new phenolic polymeric fraction (PPF) isolated and characterized has been tested for first time. This PPF was composed mainly of phenolic compounds with small amounts of carbohydrates, proteins and ash, and it was formed during 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 μ g/L collagen and 25 μ M TRAP-induced platelet aggregation by 23, 13 and 22%, respectively. For the assays with PE a concentration of 240 g phenols/kg extract has been found by the Folin-Calteciau method. The HPLC results suggested that 1 gram of phenolic extract 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 μ g/L collagen and 25 μ M TRAP-induced platelet aggregation by 52, 40 and 19%, respectively (Figure 2).

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

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

The phenolic extract (PE), HTA and the HT+DHPG mixture showed most inhibition of lipid oxidation at the lowest test concentrations (Tables 2 and 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 (Figure 3).

4. Discussion

4.1 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 any effect inhibitory was observed for 3 mg/L. Indeed, incubation with 43+3 mg/L (219+19.5 μ M) HTA and HT resulted in a significant ($p<0.05$) inhibition of 3 and 5 μ g/L collagen and 25 μ M TRAP-induced platelet aggregation by 30, 22 and 31%,

respectively (Figure 2). A similar effect was observed for 85+6 mg/L HTA + HT (433+30.6 μ M), inhibiting platelet aggregation by 85, 71 and 50%, respectively (Figure 2). González-Correa, et al., (2009)[21] observed that 26 and 48 μ M HTA inhibited collagen-induced platelet aggregation by 50%. This antiplatelet effect was stronger than that of HT, and similar to that of acetylsalicylic acid (ASA), Hubbard, et al., (2003)[22] considered different simple phenols and found that for p-coumaric, caffeic, ferulic and sinapic acid a concentration of 478-816 μ M was necessary to inhibit collagen-induced platelet aggregation by 50%. Furthermore, concentrations between 10 and 100 μ M of gallic acid inhibited TRAP-induced platelet aggregation by 10-50 % [23]. Ostertag et al. 2011 [18] found that 100 μ M of catechol, resorcinol, pyrogallol and hippuric acid inhibited in vitro collagen-induced platelet 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 phenolic extract, 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.

4.2. 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 mM+0.25mM), 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 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 inhibition lipid peroxidation, or it contains individual minor components that have high capacity of inhibition of lipid peroxidation

In a detailed study, Duthie & Morrice, (2012)[7] found that different flavonoids (i.e. quercetin, kaempferol, myricetin, galingin and fisetin) inhibited lipid peroxidation by 68-88%, a similar range than the olive phenols studied in microsomes derived from livers of rats deficient in vitamin E. Mitchell, et al., (1998)[24] observed similar results for kaempherol, which of all phytoestrogens had the greatest ability to inhibit lipid peroxidation in vitamin E-deficient microsomes with an IC₅₀ of 160 µM. In comparison, IC₅₀ concentrations required to inhibit lipid peroxidation in liver microsomes was 31 µM for α-tocopherol, and 124 µM for quercetin. The IC₅₀ values for the isoflavones, chalcones and coumestan were approximately 35, 22 and 16-fold higher than that of α-tocopherol, respectively.

5. 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 than the PE previously reported using the SE as a treatment. Besides, a synergistic 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.

Acknowledgements

The authors wish to express their gratitude to the Ministerio de Economía y Competitividad of Spain and co-funded by European Social Fund (ESF) (Project AGL2013-48291-R). for providing financial support. Dr. Rodríguez-Gutiérrez (RYC-2012-10456 contract) wishes to thank to the “Ramón y Cajal” Program from the Spanish Ministry of Economy and Competitiveness, and Ms Rubio-Senent (JAE-Pre104) to the Spanish National Research Council and co-funded by European Social Fund (CSIC-ESF) for providing financial support.

6. References.

- 318 (1) Estruch R, Ros E, Salas-Salvado J, Covas MI, Corella D, Aros F, Gómez-Gracia
319 E, Ruíz-Gutiérrez V, et al (2013) Prevention of Cardiovascular Disease with a
320 Mediterranean Diet. *New Engl J Med* 368: 1279-1290
- 321 (2) Ruiz-Canela M, Estruch R, Corella D, Salas-Salvado J, Martínez-González MA
322 (2014) Association of Mediterranean diet with peripheral artery disease: The
323 PREDIMED randomized trial. *J Amer Med Assoc* 311: 415-417
- 324 (3) González-Santiago M, Martín-Bautista E, Carrero JJ, Fonolla J, Baro L,
325 Bartolomé MV, Gil-Loyzaga P, López-Huertas E (2006) One-month
326 administration of hidroxitirosol, a phenolic antioxidant present in olive oil, to
327 hyperlipemic rabbits improve blood lipid profile, antioxidant status and reduces
328 atherosclerosis development. *Atherosclerosis* 188: 35-42
- 329 (4) Visioli F, Poli A, Galli C (2002) Antioxidant and other biological activities of
330 phenols from olives and olive oil. *Med Res Rev* 22: 65-75
- 331 (5) Ostertag LM, O'Kennedy N, Kroon PA, Duthie GG, de Roos B (2010) Impact of
332 dietary polyphenols on human platelet function - A critical review of controlled
333 dietary intervention studies. *Mol Nutr Food Res* 54: 60-81
- 334 (6) De Roos B, Zhang X, Rodríguez-Gutiérrez G, Wood S, Rucklidge GJ, Reid MD,
335 Duncan GJ, Cantlay LL, Duthie GG, O'Kennedy N (2011) Anti-platelet effects
336 of olive oil extract: in vitro functional and proteomic studies. *Eur J Nutr* 50: 553-
337 562
- 338 (7) Duthie GG and Morrice P (2012) Antioxidant Capacity of flavonoids in hepatic
339 microsomes is not reflected by antioxidant effects in vivo. *Oxid Med Cell*
340 *Longev* 165127
- 341 (8) Aruoma OI (1998) Free radicals, oxidative stress, and antioxidants in human
342 health and disease. *J Amer Oil Chem Soc* 75: 199-212

- (9) Fernández-Bolaños J, Rodríguez G, Gómez E, Guillén R, Jiménez A, Heredia A, Rodríguez R (2004) Total recovery of the waste of two-phase olive oil processing: isolation of added-value compounds. *J Agric Food Chem* 52: 5849-5855
- (10) Rubio-Senent F, Rodríguez-Gutiérrez G, Lama-Munoz A, Fernández-Bolaños J (2012) New phenolic compounds hydrothermally extracted from the olive oil by-product alperujo and their antioxidative activities. *J Agric Food Chem* 60: 1175-1186
- (11) Fernández-Bolaños J, Rodríguez G, Lama-Muñoz A, Sánchez P (2011) Dispositivo y procedimiento para el tratamiento de los subproductos de la obtención del aceite de oliva. Patent Request No. PCT/ES2011/070583
- (12) Rodríguez-Gutiérrez G, Duthie GG, Wood S, Morrice P, Nicol F, Reid M, Cantlay LL, Kelder T, Horgan GW, Fernández-Bolaños J, de Roos B (2012) Alperujo extract, hydroxytyrosol, and 3,4-dihydroxyphenylglycol are bioavailable and have antioxidant properties in vitamin E-deficient rats: a proteomics and network analysis approach. *Mol Nutr Food Res* 56: 1137-47
- (13) Rubio-Senent F, Rodríguez-Gutiérrez G, Lama-Muñoz A, Fernández-Bolaños J (2013) Chemical characterization and properties of a polymeric phenolic fraction obtained from olive oil waste. *Food Res. Int* 54: 2122–2129
- (14) Fernández-Bolaños J, Rodríguez G, Rodríguez R, Heredia A, Guillén R, Jiménez A (2002) Production in large quantities of highly purified hydroxytyrosol from liquid-solid waste of two-phase olive oil processing or “Alperujo”. *J Agric Food Chem* 50: 6804-6811

- 366 (15) Fernández-Bolaños J, Rodríguez G, Lama A, Rodríguez-Arcos R, Jiménez A,
367 Guillén R (2008) Purification of 3,4-dihydroxyphenylglycol (DHPG) from
368 vegetable products. Patent Request No 200803630
- 369 (16) Fernández-Bolaños J, Rodríguez-Gutiérrez G, Lama-Muñoz A, Fernández-
370 Bolaños JM, Maya-Castilla I, Rubio Senent F, López López O, Marset Castro A
371 (2013) Method for obtaining Hydroxytyrosol extract, mixture of Hydroxytyrosol
372 and 3,4-dihydroxyphenylglycol extract, and Hydroxytyrosyl acetate extract from
373 by-products of the olive tree and the purification of thereof. International patent
374 N: WO 2013/007850A1
- 375 (17) Singleton VL, Rossi JA Jr (1965) Colorimetry of total phenolics with
376 phosphomolybdic-phosphotungstic acid reagents. *Amer J Enol Vitic* 16:
377 144–158
- 378 (18) Ostertag LM, O’Kennedy N, Horgan GW, Kroon PA, Duthie GG., de Roos B
379 (2011) In vitro anti-platelet effects of simple plant-derived phenolic compounds
380 are only found at high, non-physiological concentrations. *Mol Nutr Food Res*
381 55: 1-13
- 382 (19) Duthie GG, González BM, Morrice PC, Arthur JR (1991) Inhibitory effects of
383 isomers of tocopherol on lipid-peroxidation of microsomes from vitamin E-
384 deficient rats. *Free Rad Res Commun* 15: 35-40
- 385 (20) Burczynski JM, Southard SJ, Hayes JR, Longhurst PA, Colby HD (2001)
386 Changes in mitochondrial and microsomal lipid peroxidation and fatty acid
387 profiles in adrenal glands, testes, and livers from α -tocopherol-deficient rats.
388 *Free Rad Biol Med* 30: 1029-1035
- 389 (21) González-Correa JA, López-Villodres JA, Asensi R, Espartero JL, Rodríguez-
390 Gutiérrez G, de la Cruz JP (2009) Virgen olive oil polyphenol hidroxitirosol

acetate inhibits in vitro platelet aggregation in human whole blood: comparison with hidroxitirosol and acetylsalicylic acid. Brit J Nutr 101: 1157-1164

(22) Hubbard GP, Wolfram S, Lovegrove JA, Gibbins JM (2003) The role of polyphenols compounds in the diet as inhibitors of platelet function. P Nutr Soc 62: 469-478

(23) Lill G, Voit S, Schror K, Weber AA (2003) Complex effect of different green tea catechins on human platelet. FEBS Lett 546: 265-270

(24) Mitchell JH, Gardner PT, McPhail DB, Morrice PC, Collins AR, Duthie GG (1998) Antioxidant efficacy of phytoestrogens in chemical and biological model systems. Arch Biochem Biophys 360: 1, 142-148

Figure caption.

Figure 1: HPLC-profile of different samples used in this work.

Figure 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 ($p > 0.05$); (-) mean value.

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:

Conflict of Interest Disclosure Form

It is the policy of the *Psychopharmacology* to ensure balance, independence, objectivity, and scientific rigor in the Journal. All authors are expected to disclose to the readers any real or apparent conflict(s) of interest that may have a direct bearing on the subject matter of the article. This pertains to relationships with pharmaceutical companies, biomedical device manufacturers or other corporations whose products or services may be related to the subject matter of the article or who have sponsored the study.

The intent of the policy is not to prevent authors with a potential conflict of interest from publication. It is merely intended that any potential conflict should be identified openly so that the readers may form their own judgements about the article with the full disclosure of the facts. It is for the readers to determine whether the authors' outside interest may reflect a possible bias in either the exposition or the conclusions presented.

The corresponding author will complete and submit this form, along with his or her submission, to the responsible Editor on behalf of all authors listed below.

Article Title Inhibitory and synergistic effect of natural olive oil extracts on human platelets aggregation and lipid peroxidation of microsomes from vitamin-E deficient rats

Authors Vitamin-E deficient rats

Please note that a conflict of interest statement is published with each paper.

I certify that there is no actual or potential conflict of interest in relation to this article. If any conflict exists, please define hereafter:

Conflict (if none, "None" or describe financial interest/arrangement with one or more organizations that could be perceived as a real or apparent conflict of interest in the context of the subject of this article):

No conflict of interest

Name

Signature

Date

Guliano Rodriguez Ghisler
20/8/2014

Please scan the completed and signed form and then submit it as an electronic file along with your manuscript. No manuscripts will be accepted for review without this form.