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ORIGINAL CONTRIBUTION

Anti-platelet effects of olive oil extract: in vitro functional and proteomic studies

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Abstract
Purpose Platelets play a key role in haemostasis and wound healing, contributing to formation of vascular plugs. They are also involved in formation of atherosclerosic plaques. Some traditional diets, like the Mediterranean diet, are associated with a lower risk of cardiovascular disease. Components in these diets may have anti-platelet functions contributing to their health benefits.
Methods We studied the effects of alperujo extract, an olive oil production waste product containing the majority of polyphenols found in olive fruits, through measurement of effects on platelet aggregation and activation in isolated human platelets, and through identification of changes in the platelet proteome.
Results Alperujo extract (40 mg/L) significantly decreased in vitro ADP- ($p = 0.002$) and TRAP- ($p = 0.02$) induced platelet activation as measured by the flow cytometry using the antibody for p-selectin (CD62p), but it did not affect the conformation of the fibrinogen receptor as measured by flow cytometry using the antibodies for anti-fibrinogen, CD42a and CD42b. Alperujo extract (100 mg/L) inhibited both collagen- and TRAP-induced platelet aggregation by 5% ($p < 0.05$), and a combination of hydroxytyrosol and 3,4-dihydroxyphenylglycol were, at least partly, responsible for this effect. Proteomic analysis identified nine proteins that were differentially regulated by the alperujo extract upon ADP-induced platelet aggregation. These proteins represent important mechanisms that may underlie the anti-platelet effects of this extract: regulation of platelet structure and aggregation, coagulation and apoptosis, and signalling by integrin αIIb/β3.
Conclusions Alperujo extract may protect against platelet activation, platelet adhesion and possibly have anti-inflammatory properties.

Keywords Platelet function · Mediterranean diet · Alperujo extract · Proteomics

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Introduction

The "Mediterranean Diet", which is high in olive oil, fruits, vegetables, grains and legumes, is associated with a lower rate of coronary heart disease [1, 2], as well as a reduction in all-cause mortality [3, 4]. Olive oil is the main source of fat in this type of diet [5, 6]. A mechanism by which consumption of the Mediterranean diet might protect against coronary heart disease is by improving the lipoprotein profile [7] lowering of blood pressure, and improving insulin sensitivity both in healthy and in type 2 diabetic patients [8]. In addition, virgin olive oil (especially the first-pressed "extra virgin" type) contains a wide range of important minor anti-oxidant compounds, such as polyphenols, that contribute to the stability of the oil and may have anti-inflammatory and anti-atherosclerotic properties [9, 10]. Consumption of polyphenols has been

associated with prevention from and treatment of atherosclerosis [11].

Specific components of the phenolic fraction of olive oil may also inhibit platelet function and eicosanoid formation. Supplementation of patients with uncomplicated type 1 diabetes with olive oil waste water providing 25 and 12.5 or 6.6 mg of hydroxytyrosol significantly decreased serum thromboxane B_2 (TxB_2) production after blood clotting [12, 13]. These in vivo results are in agreement with an in vitro study where pre-incubation of platelet-rich plasma (PRP) with hydroxytyrosol for at least 10 min resulted in maximal inhibition of aggregation induced by ADP or collagen (IC_{50} were 23 and 67 μM, respectively), and TxB_2 production by collagen or thrombin stimulation. In addition, this treatment inhibited the production of TxB_2 and 12-hydroxyeicosatetraenoic acid (12-HETE) produced during blood clotting in serum [14]. However, whilst hydroxytyrosol inhibits platelet aggregation in vitro [14], nothing is known about the effects of hydroxytyrosol on platelet activation, and moreover, no information is available on the effects of other polyphenols from olives and olive oil on both platelet aggregation and activation.

In this study, we investigated the potential anti-platelet effects of an alperujo extract, a waste product of the olive oil extraction process, representing a mix of the most important phenols present in the olive fruit [15], as well as two major phenolic compounds present in this extract: hydroxytyrosol and 3,4-dihydroxyphenylglycol (DHPG) (Fig. 1). Hydroxytyrosol and DHPG are present in alperujo as free phenolic monomers or as a conjugated phenolic oleuropein, acteoside (verbascoside) or glucoside molecule [16], and both compounds have significant anti-oxidant properties [17, 18]. We evaluated the anti-platelet properties in vitro as the ability of the alperujo extract, hydroxytyrosol and 3,4-dihydroxyphenylglycol (DHPG) to (1) inhibit collagen- or TRAP-induced platelet aggregation; (2) decrease expression of P-selectin or the activated fibrinogen receptor as a platelet activation marker on the platelet surface or (3) change the ADP-stimulated platelet proteome.

Materials and methods

Ethical approval for the use of human blood samples for the in vitro studies described below was obtained from the North of Scotland Research Ethics Committee.

Preparation of phenolic compounds

All extracts were produced at the Instituto de la Grasa, Seville, Spain. Alperujo, a waste product of the olive oil production process, was obtained by crushing olives to a fine paste, which was subsequently pumped into a two-phase decanter generating a liquid phase (oil) and a semi-solid waste (alperujo) [19]. Alperujo is formed from the olive pulp, the pit and the vegetable water, containing a high percentage of moisture (65–70%) and low percentage

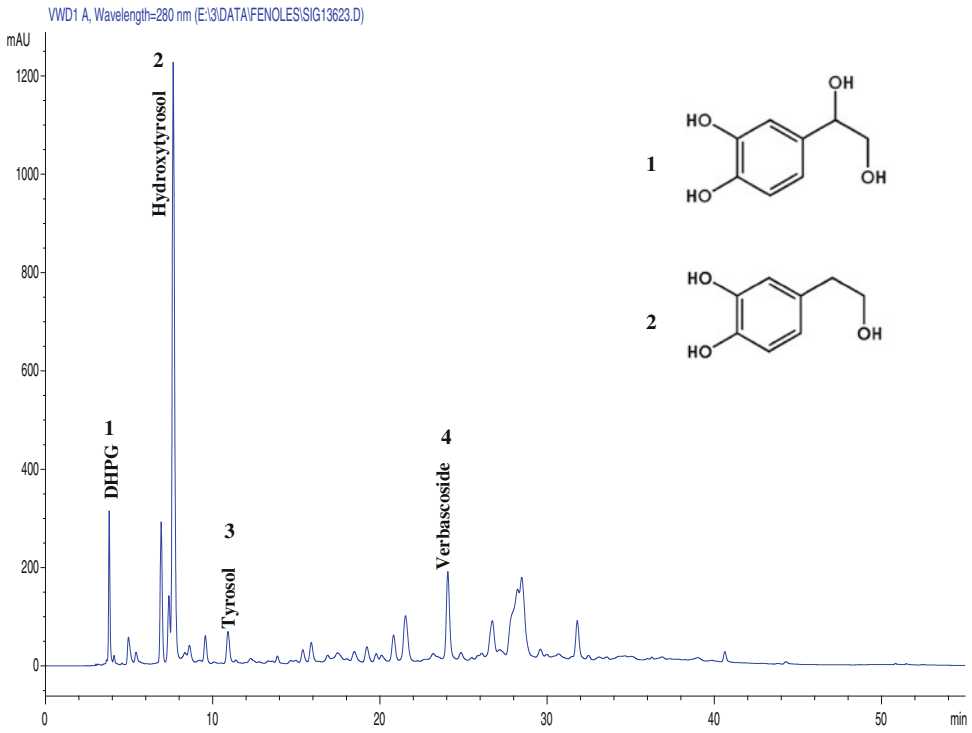

Fig. 1 HPLC-profile of alperujo extract including its main phenolic components. *1* 3,4-Dihydroxyphenylglycol) (representing 1% of dry weight); *2* hydroxytyrosol (representing 8% of dry weight); *3* tyrosol (representing 0.6% of dry weight); and *4* verbascoside (representing 1.1% of dry weight). The figure includes the chemical structures of hydroxytyrosol (*1*) and 3,4-dihydroxyphenylglycol (*2*)

of fat (2.5–3.5%). It also contains all sugars and other soluble substances from the olive [19]. Alperujo extract was produced by organic solvent extraction from alperujo pre-treated under hydrothermal conditions, as described by us previously [15]. Briefly, alperujo samples were centrifuged, and the liquid fraction was extracted with ethyl acetate and dried under vacuum at 35 °C [15]. Hydroxytyrosol was produced from alperujo extract by a patented industrial system [20]. 3,4-Dihydroxyphenylglycol (DHPG) purification from alperujo extract was performed by a new system that has been patented [21]. The amounts of hydroxytyrosol and DHPG in the alperujo extract were determined by HPLC on a Hewlett-Packard Series 1100 liquid chromatograph system with an UV–visible detector and a Rheodyne injection valve (20 μL-loop). A spherisorb ODS-2 column (250 × 4.6 mm i.d.; particle size 5 μm) (Tecnokroma, Barcelona, Spain) was used at 25 °C. Elution was performed as described by us previously [22]. Briefly, a mobile phase of trifluoracetic acid in water (pH = 2.5) and acetonitrile, with a gradient from 5 to 25% of acetonitrile in 30 min, were used at 1.0 mL/min. Chromatograms were recorded at 280 nm (Fig. 1). The substances were dissolved to the required concentration in PBS and buffered to pH 7.4 before use.

In vitro platelet aggregation

Blood from fasting healthy male and female volunteers with normal platelet function was collected in sodium citrate and centrifuged at 200*g* for 10 min at room temperature. The 10- to 15-min incubation time was chosen based on previous work with plant bioactive compounds [23–25]. PRP was removed, and the remaining sample was spun at 2,000*g* for 10 min to isolate platelet-poor plasma (PPP). PPP was used to adjust PRP to a platelet count of 300 × 10^6 platelets/mL as counted with the Sysmex haematology analyser (KX-21N, Sysmex, Germany). This PRP was used for the measurement of platelet aggregation before and after incubation with different concentrations of alperujo extract, hydroxytyrosol, DHPG or 0.9% of saline as a control for 10 min at 37 °C. Platelet aggregation was then induced by adding collagen (final concentration 2–5 μg/mL) or thrombin receptor analogue peptide (TRAP, final concentration 25 μM) as agonists. The platelet aggregation measurement was performed 90 min after blood sampling using the PACKS 4 platelet aggregation chromogenic kinetic system.

Platelet activation

Blood from healthy male and female volunteers with normal platelet function was collected in sodium citrate and diluted 1:10 with HEPES-Mg buffer, pH 7.4 (450 μL) and was incubated with alperujo extract (40 mg/L) or with the same volume of saline for 10 min. Aliquots of these mixtures were then incubated with or without ADP (final concentration, 10 μmol/L), or TRAP (final concentration, 25 μmol/L), or phorbol 12-myristate 13-acetate (PMA; final concentration, 1 μmol/L) for 10 min at room temperature. PMA was used as the positive control marker on the flow cytometer. The peridinin chlorophyll protein (PerCP)-labelled monoclonal anti-CD61 (BD Bioscience), allophycocyanin (APC)-labelled monoclonal antibody anti-CD62P (BD Phamingen) and the fluorescein isothiocyanate (FITC)–labelled monoclonal antibody anti-fibrinogen (DakoCytomation) (all 0.002 μg/μL blood), and FITC-, PerCP- and APC-labelled mouse immunoglobulin G antibodies (BD Biosciences; 0.004 μg/uL) were used as isotype controls. After 20 min of incubation in the dark at room temperature, all samples were quenched with 2 mL of cold PBS and counted using the FACSCalibur (Becton–Dickinson) with CellQuest software (version 2: BD Biosciences). Activated platelets were defined as the percentage of CD61-positive events co-expressing with one of the other two antibodies (CD62P and anti-fibrinogen) binding.

Platelet proteomics

Preparation of treated platelets

Blood from healthy male and female volunteers with normal platelet function was collected in sodium citrate and centrifuged at 200*g* for 15 min at 37 °C. The top 2/3rd of the PRP was removed, and PGI_2 was added (final concentration of ~50 ng/mL). Four millilitres of PRP was layered onto an albumin cushion of ~0.5 mL of 50% bovine serum albumin (fatty acid free, pH adjusted to 7.4) and warmed to 37 °C. Samples were spun at 1,600*g* for 15 min at room temperature. The platelet layers were taken off and re-suspended in warmed Tyrodes calcium-free buffer (containing 50 ng/mL PGI_2) to obtain a platelet count of ~300 × 10^6 platelets/mL. Upon full degradation of the PGI_2, half of the platelet suspensions ($n = 5$) were incubated with alperujo extract (40 mg/L), and the other half of the platelet suspensions ($n = 5$) were incubated with saline at room temperature for 15 min. Then, ADP was added (final concentration of 10 μmol/L) for 10 min at 37 °C in order to measure the effect of alperujo extract on the ADP-activated platelet proteome. ADP was used as a mild agonist to enable comparison with the outcomes of the platelet activation assays and to prevent platelets from clotting during the incubation time. Samples were then centrifuged at 8,700*g* for 10 min at 4 °C, and supernatants were removed. The remaining platelet pellet was washed with cold Tyrodes buffer, and samples were again centrifuged at 8,700*g* for 10 min at 4 °C, after which the Tyrodes buffer was removed.

Protein isolation from platelets

An extraction buffer (50 mM Tris, 100 mM KCL, 20% glycerol pH 7.1, add 0.1% Triton) was added to the platelet pellets, and samples were sonicated for 2 × 10 s on ice. Samples were then centrifuged at 8,700*g* for 10 min at 4 °C, after which the supernatant was carefully removed for protein analysis. Cytosolic platelet protein concentrations were measured by the bicinchoninic acid (BCA) protein assay (Sigma).

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was performed as described by us previously [26–30]. Briefly, 1 gel was run per incubation (total of $n = 10$ gels) using BioRad immobilized pH gradient (IPG) 24 cm strips (pH 4–7) for separation of the proteins in the first dimension. Gels were stained with Flamingo stain (BioRad) according to the manufacturer's instructions. 2D-electrophoresis gels were analysed using PDQuest software (BioRad). Only protein spots that were present in all replicates were used for subsequent analysis. Spots with densities that significantly differed between treatment groups were excised from the SDS–PAGE gels using the BioRad ExQuest spot cutter, alkylated and trypsinized using a standard protocol on a MassPrep Station (Micromass), and analysed by electrospray LC mass spectrometric methods as described [26–30]. The total ion current (TIC) data were submitted for database searching using the MASCOT search engine (Matrix Science) using the MSDB database with the following search criteria: allowance of 0 or 1 missed cleavages; peptide mass tolerance of ±1 Da; fragment mass tolerance of ±0.8 Da, trypsin as digestion enzyme; carbamidomethyl modification of cysteine; methionine oxidation as partial modification; and charged state as MH+.

Statistical analysis

Data are presented as means ± SD. A two-sided *t*-test of two independent samples was carried out on protein spot density data (Genstat/E10.0, VSN International Ltd) with a *p*-value of $p < 0.05$ considered significant.

Results

Effects of olive oil phenolics on ex vivo platelet aggregation

Incubation of platelet-rich plasma from healthy volunteers with 100, 200 and 500 mg/L of alperujo extract (which is equivalent to approximately 52, 104 and 260 µmol/L of hydroxytyrosol and approximately 6, 12 and 30 µmol/L DHPG) resulted in significant ($p < 0.05$) inhibition of collagen-induced platelet aggregation of 5, 7 and 28%, respectively, and in TRAP-induced platelet aggregation of 5, 8 and 16%, respectively (Fig. 2). HPLC analysis of the alperujo extract revealed that 8% of dry weight consisted of hydroxytyrosol and 1% of dry weight consisted of DHPG (Fig. 1). Incubation with concentrations of 8, 16 and 40 mg/L hydroxytyrosol did not result in significant inhibition of collagen- or TRAP-induced platelet aggregation. Incubation with concentrations of 1, 2 and 5 mg/L DHPG only resulted in significant ($p < 0.05$) inhibition of collagen-induced platelet aggregation of 3% for the highest concentration, and no inhibition of TRAP-induced platelet aggregation. Incubation with a mixture of 40 mg/L hydroxytyrosol and 5 mg/L DHPG resulted in a significant ($p < 0.05$) inhibition of both collagen- and TRAP-induced platelet aggregation of 12 and 16%, respectively (Fig. 2), indicating that a combination of hydroxytyrosol and DHPG was, at least partly, responsible for the anti-platelet effects of alperujo extract.

Effects of alperujo extract on ex vivo platelet activation

For the platelet activation studies, we used lower concentrations of the alperujo extract as measurement of platelet surface activating markers often represents a more sensitive method than measuring the whole platelet aggregation [31]. Alperujuo extract, at a concentration of 40 mg/L (which is equivalent to approximately 20.8 µmol/L of hydroxytyrosol and approximately 2.4 µmol/L DHPG), caused a significant decrease in ADP- and TRAP-induced platelet activation as measured by the antibody for CD62p using flow cytometry (Table 1).

Effects of alperujo extract on the platelet proteome

Using 2D gel electrophoresis, we found 9 proteins upon incubation with alperujo extract in platelets stimulated with ADP, compared with control (Table 2).

Discussion

Alperujo extract from olives is a new by-product from the modern two-phase processing technique used in olive oil production. Upon olive oil extraction, only a low percentage of the total phenolic compounds present in the olive fruits end up in the virgin olive oil; the majority of phenolic compounds (about 98%) remain in the liquid and solid olive-mill waste. Therefore, alperujo represents an extremely rich source of phenolic compounds [15], which potentially could have anti-platelet effects, comparable

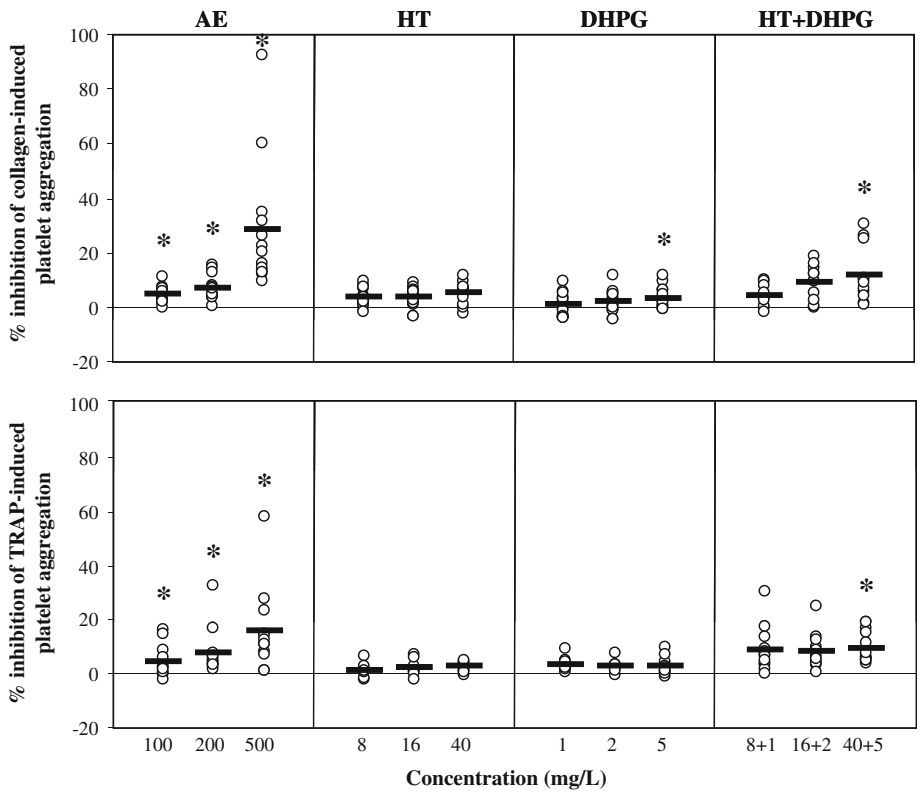

Fig. 2 Inhibition of platelet aggregation by alperujo extract (*AE*), hydroxytyrosol (*HT*), 3,4-dihydroxyphenylglycol (*DHPG*) or a combination of hydroxytyrosol and DHPG using collagen or TRAP as an agonist. Platelet inhibition is expressed as the % decrease in the areas under the curve for platelet aggregation measured by light-transmission aggregometry when compared with control (saline). *Significantly different from control ($p < 0.05$); (—) mean value

Table 1 Effect of alperujo extract (40 mg/L) on ADP- and TRAP-induced platelet activation, measured as the percentage of activated platelets expressing CD62p or fibrinogen receptor

Agonist	Antibody	Activated platelets (%)		Inhibition (%)	*p* value
		Alperujo extract	Saline control		
ADP	CD62p	36.7 ± 14.1	44.6 ± 12.7	17.8	0.002
	Anti-fibrinogen	52.0 ± 14.6	59.2 ± 10.2	12.1	0.145
TRAP	CD62p	70.6 ± 13.3	76.9 ± 9.8	8.2	0.018
	Anti-fibrinogen	44.3 ± 14.1	48.6 ± 18.5	8.9	0.278

Values represent mean ± SD, $n = 7$ incubations per measurement

with those observed in the literature [32, 33]. We found that alperujo extract inhibited both human platelet activation and aggregation. At a concentration of 40 mg/L, alperujo extract significantly inhibited both ADP- and TRAP-induced platelet activation, measured as the percentage of activated platelets expressing P-selectin, which is released to the platelet surface during activation to mediate the rolling of the blood cells on the surface of the endothelium and the initial recruitment of leucocytes to the site of injury during inflammation [34]. At this concentration, the inhibition of ADP- and TRAP-induced platelet activation measured as the percentage of platelets expressing the activated fibrinogen receptor by alperujo extract did not reach statistical significance (Table 1). At a concentration of 100 mg/L, alperujo extract significantly inhibited both collagen- and TRAP-induced platelet aggregation (Fig. 2). Inhibition of collagen- and TRAP-induced platelet aggregation was even more pronounced when exposing the platelets to alperujo extract at a concentration of 500 mg/L (28 and 16%, respectively). Our HPLC results suggested that 500 mg/L alperjuo extract contained approximately 40 mg/L hydroxytyrosol and 5 mg/L of DHPG, which on their own did not inhibit collagen- or TRAP-induced platelet aggregation, apart from DHPG in the highest concentration (Fig. 2). However, a mixture of 40 mg/L of hydroxytyrosol and 5 mg/L of DHPG did significantly inhibit collagen-induced platelet aggregation by 12% and TRAP-induced platelet aggregation by 16%. This suggests that an interaction between hydroxytyrosol and DHPG could be, for a large part,

Table 2 Measures of confidence for protein identification and characterization by MS/MS analysis of platelet proteins which were significantly increased or decreased upon incubation with alperujo and ADP in activated platelets

SSP	Protein	Accession	Mr exp	Mr theor	Protein score	Matched peptides	Peptide sequence	Peptide charge	Fold change
2522	Actin, cytoplasmic 2	P63261	53.4	42.1	408	7	AGFAGDDAPR	+2	1.53
							GYSFTTTAER	+2	
							EITALAPSTMK	+2	
							EITALAPSTMK + Ox (M)	+2	
							DSYVGDEAQSK	+2	
							QEYDESGPSIVHR	+3	
							LCYVALDFEQEMATAASSSSLEK	+3	
1211	Rho GDP-dissociation inhibitor 1	P52565	26.5	23.1	428	8	YIQHTYR	+2	1.30
							SIQEIQELDK	+2	
							TDYMVGSYGPR	+2	
							TDYMVGSYGPR + Oxidation (M)	+2	
							IDKTDYMVGSYGPR	+3	
							IDKTDYMVGSYGPR	+2	
							AEEYEFLTPVEEAPK	+2	
							SIQEIQELDKDDESLR	+2	
1316	Annexin A5	P08758	32.6	36	1094	13	FITIFGTR	+2	1.87
							LYDAYELK	+2	
							GAGTDDHTLIR	+2	
							MLVVLLQANR + Ox (M)	+2	
							NFATSLYSMIK	+2	
							NFATSLYSMIK + Ox (M)	+2	
							GTVTDFPGFDER	+2	
							DLLDDLKSELTGK	+2	
							ETSGNLEQLLLAVVK	+2	
							GLGTDEESILTLLTSR	+2	
							SIPAYLAETLYYAMK	+2	
							YMTISGFQIEETIDR	+2	
							DPDAGIDEAQVEQDAQALFQAGELK	+3	
1317	Annexin A5	P08758	32.6	36	803	10	LYDAYELK	+2	0.58
							SEIDLFNIR	+2	
							GAGTDDHTLIR	+2	
							MLVVLLQANR + Ox (M)	+2	
							NFATSLYSMIK + Ox (M)	+2	
							GTVTDFPGFDER	+2	
							DLLDDLKSELTGK	+2	
							GLGTDEESILTLLTSR	+2	
							YMTISGFQIEETIDR	+2	
							QVYEEEYGSSLEDDVVGDTSGYYQR	+3	
2804	Platelet membrane glycoprotein IIb	P08514	149.2	114.5	184	6	K.IVLLDVPVR.A	+2	0.92
							R.FGSAIAPLGDLDR.D	+2	
							R.AEGGQCPSLLFDLR.D	+2	
							K.TPVGSCFLAQPESGR.R	+2	
							R.DGYNDIAVAAPYGGPSGR.G	+2	
							K.ENETRVVLCELGNPMK.K	+3	

Table 2 continued

SSP	Protein	Accession	Mr exp	Mr theor	Protein score	Matched peptides	Peptide sequence	Peptide charge	Fold change
1321	Fibrinogen gamma chain precursor	P02679	37.0	50.1	312	5	VELEDWNGR	+2	1.30
							TSTADYAMFK	+2	
							DNCCILDER	+2	
							YEASILTHDSSIR	+3	
							YLQEIYNSNNQK	+2	
2514	Protein disulphide isomerase-related protein 5 (ERp5)	Q15084	54.2	46.5	620	10	NSYLEVLLK	+2	1.14
							GESPVDYDGGR	+2	
							GFPTIKIFQK	+2	
							DVIELTDDSFDK	+2	
							NLEPEWAAAASEVK	+2	
							KDVIELTDDSFDK	+2	
							KDVIELTDDSFDK	+3	
							LYSSSDDVIELTPSNFNR	+2	
							VGAVDADKHHSLGGQYGVQGFPTIK	+4	
							TCEEHQLCVVAVLPHILDTGAAGR	+4	
2314	Serine/threonine protein phosphatase	Q5U0I7	36.5	36.2	249	4	YGNANVWK	+2	1.77
							QLNENQVR	+2	
							ELDQWVEQLNECK	+2	
							GAGYTFGQDISETFNHANGLTLVSR	+3	
1218	Syntaxin-7	O15400	29.0	29.8	318	5	QQYTNQLAK	+2	1.29
							VSGSFPEDSSK	+2	
							ITQCSVEIQR	+2	
							EFGSLPTTPSEQR	+2	
							LVAEFTTSLTNFQK	+2	

responsible for the anti-platelet effects of alperujo. In addition, other compounds contained in the alperujo extract could also be implicated in the effects observed. For example, 1.1% of the dry weight of alperujo extract was verbascoside, a conjugated glucoside of hydroxytyrosol and caffeic acid (Fig. 1). However, verbascoside is easily hydrolysed into hydroxytyrosol and a caffeoyl group, with hydroxytyrosol believed to be responsible for the activities of verbascoside [35]. HPLC of the alperujo extract showed the presence of a range of more than 15 compounds that, considering their elution time and absorbance characteristics, are likely to have phenolic structures (Fig. 1). The strong effects observed on collagen-mediated aggregation in other studies support this suggestion, since phenolics can affect collagen-induced platelet TxA_2 formation [32, 33]. Proteomics of platelets incubated with alperujo extract, compared with control, elucidated three important mechanisms that may underlie the anti-platelet effects of this extract: regulation of platelet structure and aggregation, coagulation and apoptosis, and signalling by integrin $\alpha_{IIb}/\beta 3$.

Platelet structure and platelet aggregation

Alperujo extract caused a significant 1.5-fold increase in platelet protein γ-actin levels (Table 2). The actin-based cytoskeleton maintains the discoid shape of the human platelet using a cytoplasmic scaffolding composed of rigidly cross-linked actin filaments. During activation of platelets, however, the cytoskeleton also can get quickly taken apart and reassembled in a process influenced by platelet proteins and signalling pathways, which initiate cell shape changes mediated by the actin filament system of cells [36]. Increased protein levels of γ-actin upon treatment with alperujo extract may promote cytoskeletal stability of the resting platelet and reduce the incidence of shape-change in response to external stimuli. Alperujo extract also increased protein levels of Rho GDP-dissociation inhibitor 1, a negative regulator of Rho family GTPases [37]. Rho family GTPases regulate the formation of actin cytoskeletal structures and play important roles in cytoskeletal reorganization [38]. Rho is, however, also involved in the regulation of thrombin-induced platelet aggregation, since treatment of

platelets with Rho GDP-dissociation inhibitor 1 blocked the aggregation [39]. Increased protein levels of Rho GDP-dissociation inhibitor 1 may therefore, at least partly, explain the effects seen on inhibition of platelet aggregation by alperujo extract in this study.

Coagulation and apoptosis

Alperjuo extract differentially regulated two isoforms of annexin A5 protein with one variant showing increased protein levels, i.e. SSP 1316—the isoform with the lowest pI, and one variant showing decreased protein levels, i.e. SSP 1317—the isoform with the highest pI (Table 2; Fig. 3). Annexin A5, or vascular anticoagulant-alpha, is an anticoagulant protein that acts as an indirect inhibitor of the thromboplastin-specific complex, which is involved in the blood coagulation cascade, and it has been proposed to play a role in the prevention of atherothrombosis [40]. Human annexin A5 binds, in a Ca^{2+}-dependant manner, to phosphatidylserine with high affinity, where it is thought to be anti-thrombotic through mechanical shielding of phospholipids, thereby reducing their availability for coagulation reactions [41]. Post-translational modification of annexin A5 includes the attachment of a single phosphate group or of a complex molecule through a phosphate group. Thus, the annexin isoform with the lowest pI may represent the phosphoprotein variant (Fig. 3). Protein levels of this variant were significantly increased upon treatment with alperujo, possibly leading to an anti-coagulant effect. The binding of annexin A5 to phosphatidylserine is also used as a clinical marker to detect apoptosis [42, 43]. Indeed, recently, it was shown that resveratrol, a phytoalexin produced naturally by several plants, simultaneously inhibited platelet aggregation and stimulated platelet apoptosis. The authors argued that stimulation of platelet apoptosis by resveratrol may promote platelet destruction and prevent pathological clotting in certain patient groups [44]. Phenolic compounds from alperujo extract may affect pathways of platelet apoptosis in a similar way.

Signalling via integrin $\alpha_{IIb}/\beta 3$ (fibrinogen receptor)

Protein levels of integrin α_{IIb} were lower upon treatment with alperujo extract (Table 2, Fig. 3), and this could impair the formation of the full fibrinogen receptor, integrin $\alpha_{IIb}/\beta 3$. During platelet activation, conversion of integrin $\alpha_{IIb}/\beta 3$ to a high affinity conformation initiates platelet–platelet interaction through binding of fibrinogen and von Willibrand factor. This step leads to rapid platelet aggregation that physically plugs ruptured endothelial cell surface. This is an essential and common step in full platelet aggregation in response to all stimuli [45]. In our study, we observed lower protein levels of integrin α_{IIb} despite concomitant increased protein levels of the fibrinogen γ precursor upon treatment with alperujo extract. Fibrinogen is a soluble glycoprotein, consisting of a hexamer containing two sets of three different chains (α, β and γ), linked to each other by disulphide bonds. The main γ-chain form is γA, but a fraction of fibrinogen contains a differentially spliced γ chain called γ', which presents itself in plasma mainly as a heterodimer with the common γA chain as $\gamma A/\gamma'$ fibrinogen. Platelets can take up fibrinogen from plasma, but are also capable of synthesizing this compound through a translation process. Interestingly, fibrinogen stored in the platelet α-granules completely consists of $\gamma A/\gamma A$ fibrinogen. The carboxy-terminal end of the γA chain is involved in binding to platelet integrin α_{IIb}-$\beta 3$, but the γ' chain lacks the binding site required for platelet adhesion and therefore reduces thrombin-induced platelet aggregation [46]. Our LC–MS/MS analyses did not enable us to distinguish between the γA or γ' chain. Increased protein levels of the latter variant could imply an anti-platelet effect that would underpin the effects of alperujo extract on platelet aggregation and activation.

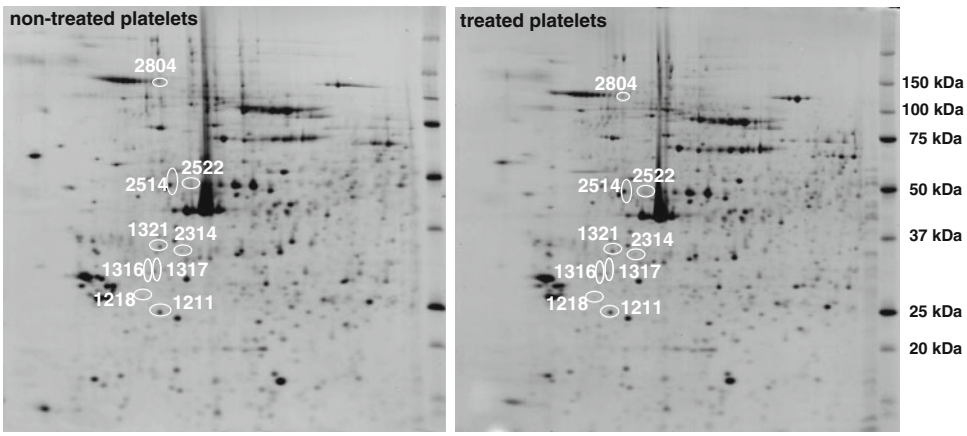

Fig. 3 Representative two-dimensional gel electrophoresis gel indicating the proteins (shown as SSP numbers on the gels) that showed differential regulation as identified by LC–MS/MS and described in "Materials and methods"

Protein levels of endoplasmic reticulum protein 5 (or ERp5) were also significantly increased by alperujo extract. ERp5, a thiolesterase that is present mainly on platelet intracellular membranes, is rapidly recruited to the platelet cell surface and associates with the $\beta 3$ subunit of integrin $\alpha_{IIb}/\beta 3$ upon platelet activation. Indeed, inhibition of 'cell-surface' ERp5 using inhibitory antibodies decreased integrin activation and granular secretion, albeit not sufficient to completely inhibit platelet aggregatory responses [47]. It is currently unclear how and whether increased protein levels of ERp5 could contribute to anti-platelet effects of alperujo since it is not known whether our proteomics approach could differentiate between intracellular or cell-surface protein levels.

In conclusion, the phenolic compounds in alperujo extract may protect against platelet activation, platelet adhesion and possibly have anti-inflammatory properties, as evidenced by the lower percentage of activated platelets expressing P-selectin. However, these effects were instigated by relative high concentrations of phenolic compounds that will be difficult to obtain in vivo from olive oil consumption only. The beneficial effects could be accomplished by regulation of pathways involved in organization of platelet structure and aggregation, coagulation and apoptosis, and signalling by integrin $\alpha IIb/\beta 3$. This study provides additional evidence that specific phenolic compounds from a Mediterranean diet may improve platelet function. The extraction of these compounds, perhaps for future human consumption purposes, can be accomplished in a sustainable manner through effective use of a by-product from olive oil manufacturing process.

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