Research Article

Effects of olive oil polyphenols on erythrocyte oxidative damage

Fátima Paiva-Martins[1, 2], João Fernandes[2], Susana Rocha[3, 4], Henrique Nascimento[3, 4], Rui Vitorino[5], Francisco Amado[5], Fernanda Borges[6], Luís Belo[3, 4] and Alice Santos-Silva[3, 4]

[1] Centro de Investigação em Química (CIQ), Faculdade de Ciências, Universidade do Porto, Porto, Portugal
[2] Departamento de Química, Faculdade de Ciências, Universidade do Porto, Porto, Portugal
[3] Serviço de Bioquímica, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal
[4] Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto, Porto, Portugal
[5] Departamento de Química, Universidade de Aveiro, Aveiro, Portugal
[6] UQFM, Serviço de Química Orgânica, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal

Many studies have investigated the protective effects of oleuropein and hydroxytyrosol against cell injury, but few have investigated the protective effects of oleuropein aglycones 3,4-dihydroxyphenylethanol-elenolic acid (3,4-DHPEA-EA) and 3,4-dihydroxyphenylethanol-elenolic acid dialdehyde (3,4-DHPEA-EDA). The present work studied and compared the capacity of these four compounds, found at high concentrations in olive oil, to protect red blood cells (RBCs) from oxidative injury. The *in vitro* oxidative stress of RBCs was induced by the water-soluble radical initiator 2,2′-azo-bis(2-amidinopropane) dihydrochloride. RBC changes were evaluated either by optical microscopy or by the amount of hemolysis. All compounds were shown to significantly protect RBCs from oxidative damage in a dose-dependent manner. The order of activity at 20 μM was: 3,4-DHPEA-EDA > hydroxytyrosol > oleuropein > 3,4-DHPEA-EA. Even at 3 μM, 3,4-DHPEA-EDA and hydroxytyrosol still had an important protective activity. However, deleterious morphological RBC changes were much more evident in the presence of hydroxytyrosol than with 3,4-DHPEA-EDA. For the first time it was demonstrated that 3,4-DHPEA-EDA, one of most important olive oil polyphenols, may play a noteworthy protective role against ROS-induced oxidative injury in human cells since lower doses of this compound were needed to protect RBCs *in vitro* from oxidative mediated hemolysis.

Keywords: Erythrocytes / Hydroxytyrosol / *Olea europaea* / Olive oil / Polyphenols

Received: July 3, 2008; revised: August 22, 2008; accepted: September 15, 2008

1 Introduction

A large body of epidemiological studies shows that the incidence of coronary heart disease and of certain cancers in the Mediterranean countries is low, suggesting a crucial protective role of the diet in this southern European area where virgin olive oil is the principal source of fat [1]. The hypothesis of an antioxidant/atherosclerosis relationship led to experimental studies on the potential protective role of olive oil phenols against coronary heart disease. *In vitro* studies and a few *in vivo* studies suggested that the high concentration of phenolic compounds in extra virgin olive oil may contribute to the healthy nature of this diet but controversial results have been obtained in several randomized, crossover, controlled studies [2 – 8].

In recent years, there has been much interest in antioxidants that retard oxidative modification of low-density lipoproteins (LDL), which is believed to be a key step in the development of atherosclerosis. The stability of LDL isolated from animals and humans fed with virgin olive oil is increased, and this increased stability is attributable to the phenolic compounds present in the oil [3, 7, 9 – 12]. Nowadays, olive oil is marketed as being healthier than other vegetable oils but a pertinent question is whether this claim is valid for all virgin olive oils, or whether some have better nutritional value than others depending on their phenolic

Correspondence: Dr. Fátima Paiva-Martins, Departamento de Química, Faculdade de Ciências, Universidade do Porto, Rua do Campo Alegre 687, 4169-007 Porto, Portugal
E-mail: mpmartin@fc.up.pt
Fax: +351-22-6082959

Abbreviations: **AAPH**, 2,2′-azo-bis(2-amidinopropane) dihydrochloride; **3,4-DHPEA-EA**, 3,4-dihydroxyphenylethanol-elenolic acid; **3,4-DHPEA-EDA**, 3,4-dihydroxyphenylethanol-elenolic acid dialdehyde; **MBH**, membrane-bound hemoglobin; **RBC**, red blood cell

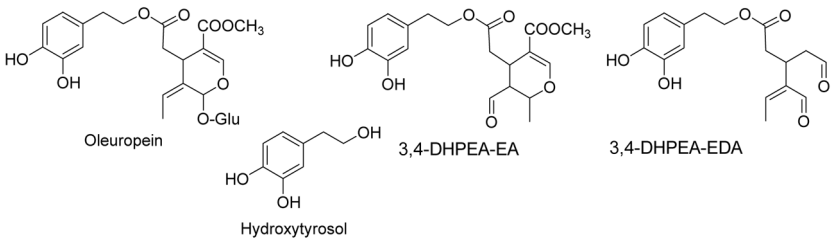

Figure 1. Structures of olive oil phenolics.

composition. Current research findings suggest that olive oil consumption could reduce oxidative damage due to its richness in oleic acid and due to its minor components, particularly the phenolic compounds. However, which components have a major role on this protection, is still unknown.

The red blood cell (RBC), anucleated and without cytoplasmatic organelles, has poor repair and biosynthetic mechanisms, suffering and accumulating oxidative lesions whenever oxidative stress develops. Moreover, RBCs are particularly exposed to endogenous oxidative damage because of their specific role as oxygen carriers. As the most abundant blood cell, RBCs also play an important role in the oxidative status of the whole blood constituents, in particular of the lipoproteins. Nevertheless, RBCs are equipped with several antioxidants, *i.e.*, antioxidant enzymes, glutathione, tocopherol and ascorbate. If reactive oxygen species (ROS), *i.e.*, H_2O_2 and O_2^-, are overproduced outside or within the erythrocyte, or if the endogenous antioxidant defenses are impaired, an "oxidative stress" condition will develop, inducing oxidative damage to erythrocyte constituents, *i.e.*, those on membrane and hemoglobin, which may ultimately leads to hemolysis. A wide variety of drugs and xenobiotics that can undergo oxidation-reduction reactions have been found to cause RBC destruction. Interaction between the xenobiotic and hemoglobin is very important in the process, which is usually characterized by hemoglobin oxidation to methemoglobin and formation of radical intermediates. When hemoglobin is denatured, it links to the membrane at the cytoplasmic domain of band 3 protein, inducing its aggregation and the linkage of natural anti-band 3 antibodies and complement fixation on the erythrocyte surface, marking the cell for removal by the macrophages of the reticuloendothelial system. Human RBCs are, therefore, a metabolically simplified model system, useful in the evaluation of antioxidant properties of several compounds, *e.g.*, olive oil polyphenols.

Hydroperoxyl radicals (HOO.), from the aqueous phase, are important for initiating lipid peroxidation and protein damage in membranes [13]. 2,2′-Azo-bis(2-amidinopropane) dihydrochloride (AAPH) is a water-soluble azo compound extensively used as a free radical initiator for biological studies. It can generate radicals, at a constant rate, in the aqueous phase through its thermo degradation at 37°C [13].

Hydroxytyrosol, oleuropein and its aglycones 3,4-dihydroxyphenylethanol-elenolic acid (3,4-DHPEA-EA) and 3,4-dihydroxyphenylethanol-elenolic acid dialdehyde (3,4-DHPEA-EDA) (Fig. 1) are phenols present in particularly high concentration in extra virgin olive oil, accounting for up to 55% of the total phenolic fraction [14 – 16]. In some olive oils, 3,4-DHPEA-EDA may even represent 50% of the phenolic fraction [15, 16]. Although many studies have investigated the antioxidant properties of oleuropein and hydroxytyrosol [17], as well as their protective effects against cell injury and their bioavailability, there have been only few reports of the protective effects of oleuropein aglycones.

The aim of this work was to study and compare the capacity of hydroxytyrosol, oleuropein, 3,4-DHPEA-EA and 3,4-DHPEA-EDA, to protect RBCs from AAPH-induced oxidative injury, by evaluating the capacity of these compounds to inhibit hemolysis and oxidative damage to the RBC membrane.

2 Materials and methods

2.1 Phenolic compounds

Hydroxytyrosol was synthesized from 3,4-dihydroxyphenylacetic acid (Sigma-Aldrich Quimica-S.A., Madrid, Spain) according to the procedure of Baraldi *et al*. [18]. Oleuropein was extracted from olive leaves according to the procedure of Gariboldi *et al*. [19]. The aglycone 3,4-DHPEA-EA was obtained from oleuropein by enzymatic reaction using β-glycosidase (Fluka, Buchs, Switzerland), according to the procedure of Limirioli *et al*. [20]. 3,4-DHPEA-EDA was obtained from olive leaves as described by Paiva-Martins and Gordon [21].

2.2 Preparation of RBC suspensions

Blood was obtained from healthy, non-smoker volunteers (two women and two men aged 23 – 50 years) by venipuncture, and collected into tubes containing ethylenediaminetetraacetic acid (EDTA), as an anticoagulant. Samples were immediately centrifuged at 400 × *g* for 10 min; plasma and buffy coat were carefully removed and discarded. RBCs were washed three times with PBS (125 mM NaCl and 10 mM sodium phosphate buffer, pH 7.4) at 4°C and, finally, resuspended in PBS, to obtain RBC suspensions at 10% or 2% hematocrit. RBC suspensions were used in the day they were prepared.

To evaluate the capacity of the olive oil phenolic compounds to protect RBCs from oxidative injury induced by AAPH several *in vitro* studies were performed: RBC lysis, morphology, membrane-bound hemoglobin (MBH) and membrane protein profile.

2.3 AAPH-induced hemolysis and morphological changes

RBC suspensions were prepared at 2% hematocrit, and the assays were performed using AAPH at final concentration of 60 μM. In all sets of experiments ($n = 4$), a negative control (RBCs in PBS) was used, as well as phenolic compound controls (RBCs in PBS, with each phenolic compound). Controls and sample tests were run in duplicate. Incubations of RBC suspensions were carried out at 37°C for 4 h, under gentle shaking, in the presence of each individual compound or in the presence of the phenolic compound plus AAPH. Phenolic compounds were incubated for 15 min with RBC before addition of AAPH and they were tested at concentrations of 20, 40 and 80 μM. Only hydroxytyrosol and 3,4-DHPEA-EDA were tested for lower concentrations (3, 6 and 10 μM) as these compounds still showed a protective effect at 20 μM.

Hemolysis was determined spectrophotometrically, according to Ko *et al.* [22]. After the incubation period, an aliquot of the RBC suspension was diluted with 20 volumes of saline and centrifuged (4000 rpm for 10 min). The absorption (A) of the supernatant was read at 540 nm. The absorption (B), corresponding to a complete hemolysis, was acquired after centrifugation of a RBC suspension that was previously treated with 20 volumes of ice-cold distilled water. The percentage of hemolysis was then calculated ($A/B \times 100$).

To study the morphological changes of RBC suspensions by optical microscopy, aliquots (50 μL) were taken from samples containing 40 μM phenolic compounds, with and without AAPH, and controls at the end of the incubations. The samples were mounted on a slide with a cover slip. By using the same volume of the RBC suspension, it was possible to roughly compare the number of RBC per microscopic field with the RBC lysis quantified previously by spectrophotometry.

2.4 AAPH-induced erythrocyte membrane changes

To study the effect of the phenolic compounds to protect RBCs from AAPH-induced oxidative injury, we chose the more suitable initiator concentration of this compound. We then evaluated the changes induced in MBH and in membrane protein profile, using increasing concentrations of AAPH (3, 8, 16, 40, 60 and 120 μM). RBC suspensions (10% hematocrit) were incubated at 37°C for 4 h, under gentle shaking. Afterwards, RBCs were washed in a saline solution and immediately lysed according to the procedure of Dodge *et al.* [23]. Membranes were washed in Dodge buffer; the protease inhibitor phenylmethylsulfonyl fluoride was added to the first two washes (final concentration of 0.1 mM). The protein concentration of the RBC membrane suspensions was determined by Bradford's method [24]. It should be noted that in these studies RBC suspensions at 10% hematocrit were used to obtain a significant volume of RBC membranes.

MBH was spectrophotometrically measured, after membrane protein dissociation with Triton X-100 (5% in Dodge buffer), at 415 nm; the absorbance at this wavelength was corrected by subtracting the absorbance of the background at 700 nm; this value and membrane protein concentration were then used to calculate the %MBH.

Membranes of RBCs were treated with a solubilization buffer, heat denatured and submitted to electrophoresis (8 μg protein/lane). SDS-PAGE was carried out on a discontinuous system, using a 5 – 15% linear acrylamide gradient gel and a 3.5 – 17% exponential acrylamide gradient gel, according to Laemmli and Fairbanks methods, respectively [25, 26]. The proteins were stained with Coomassie brilliant blue, and scanned (Darkroom CN UV/wl, *Bio-CaptMW version 99*, Vilber Lourmat, France).

The concentration of 50 μM AAPH was found to be suitable for the membrane protein assays, as important modifications in RBC membrane proteins were produced. Therefore, the evaluation of the capacity of the phenolic compounds to inhibit oxidative changes was performed under these experimental conditions.

2.5 Protective effect of phenolic compounds against AAPH-induced RBC membrane changes

In all set of experiments ($n = 4$) controls (RBCs in PBS and RBCs in PBS plus AAPH) were run in duplicate. A fivefold concentration of RBC suspension (10% hematocrit) was needed to obtain RBC membranes to perform the assays. Phenolic compounds at 200 μM (final concentration) were added to controls and tests. The assay conditions were those as described above for the AAPH assays.

To clarify the nature of the hemoglobin linked to RBC membranes and the concentration of oxy-hemoglobin in hemolysates [27], spectral scans (450 – 650 nm) were performed [28].

2.6 MS analysis of RBC membrane proteins

A protein band of high molecular mass (>220 kDa) and a protein band of approximately 16 – 20 kDa observed in SDS-PAGE gels from the interaction of 3,4-DHPEA-EDA with RBCs were excised from the gels stained with Coomassie brilliant blue. The gel pieces were washed three times with 25 mM NH_4HCO_3/50% ACN, once with ACN

and dried in a SpeedVac (Thermo Savant); 25 μL 10 μg/mL sequence grade modified porcine trypsin (Promega) in 25 mM NH_4HCO_3 was added to the dried gel pieces and the samples were incubated overnight at 37°C. Extraction of tryptic peptides was performed by addition of a 10% formic acid (FA)/50% ACN solution (three times); the extract was then lyophilized in a SpeedVac. Tryptic peptides were resuspended in 10 μL 50% ACN/0.1% FA solution. The samples were mixed (1:1) with a matrix consisting of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% ACN/0.1% FA. Aliquots of samples (0.5 μL) were spotted onto the MALDI sample target plate. Peptide mass spectra were obtained using a MALDI-TOF/TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems) in the positive ion reflector mode, in the mass range 800–4500 Da with ~1500 laser shots. For each sample spot, a data-dependent acquisition method was created to select the most intense peaks, excluding those from the matrix, trypsin autolysis, or acrylamide peaks, for subsequent MS/MS data acquisition. Trypsin autolysis peaks were used for internal calibration of the mass spectra, allowing a routine mass accuracy of better than 50 ppm. Spectra were processed and analyzed by the Global Protein Server Workstation (Applied Biosystems), which uses internal MASCOT (Matrix Science Ltd) software for searching the peptide mass fingerprints and MS/MS data. Searches were performed against the NCBI non-redundant protein database.

2.7 Statistical analysis

The results obtained for the four independent hemolysis experiments (blood obtained each time from a different donor), performed in duplicate, are expressed as means ± SE. Statistical differences between groups of experiments with different antioxidant compounds were analyzed by two-way analysis of variance with post-hoc testing using Tukey's test. A p value lower than 0.05 was accepted as being statistically significant.

3 Results and discussion

In recent years, increasing evidence has supported the hypothesis that a number of nutrients or non-nutrient dietary components, designated "antioxidants", might have a beneficial role regarding the course of chronic degenerative diseases. In particular, it has been claimed that olive oil polyphenolic components may play a major role on the protective effects against oxidative damage. However, little research has been addressed to the study of the antioxidant profile of the most significant phenolic compounds found in olive oil, the oleuropein aglycones 3,4-DHPEA-EA and 3,4-DHPEA-EDA, particulary in biological systems. Consequently, in this work the antioxidant properties of the

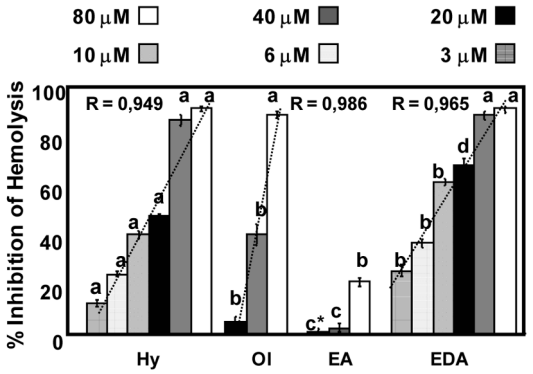

Figure 2. Percentage of inhibition of lysis of RBCs at 2% hematocrit incubated for 4 h with 60 mM AAPH and phenolic compounds. Mean (error bars represent standard error) of four determinations for each duplicate. Hy: hydroxytyrosol; Ol: oleuropein; EA: 3,4-DHPEA-EA; EDA: 3,4-DHPEA-EDA. Different letters within a concentration indicate samples that were significantly different ($p < 0.05$). * Sample not different from control.

olive oil polyphenols oleuropein, hydroxytyrosol, 3,4-DHPEA-EA and 3,4-DHPEA-EDA were assessed using human RBC under AAPH-induced oxidative stress. This biological model has been extensively studied both as a source of free radicals and as a target for oxidative damage. To achieve this objective, the following parameters were evaluated: RBC lysis, morphology, MBH and membrane protein profile.

RBC lysis experiments showed that all compounds significantly protected RBCs from oxidative AAPH-induced hemolysis at a concentration of 80 μM (Fig. 2). At lower concentrations (20–40 μM), oleuropein, hydroxytyrosol and 3,4-DHPEA-EDA still protected RBCs from oxidative hemolysis in a dose-dependent manner. Nevertheless, oleuropein protection effect was modest at 20 μM. The ranking activity order at 20 μM was: 3,4-DHPEA-EDA > hydroxytyrosol > oleuropein. At this concentration, 3,4-DHPEA-EA did not show any protection. At the lowest concentration tested (3 μM), 3,4-DHPEA-EDA and hydroxytyrosol still had an important protective activity (Fig. 2). The data obtained for hydroxytyrosol agree with those acquired in a similar system by Manna *et al.* [17]. In this study, hydroxytyrosol also protected RBC from oxidative injury in a dose-dependent manner.

The RBCs morphology before and after exposure to AAPH in the absence and presence of 40 μM olive oil polyphenols is illustrated in Fig. 3. At this concentration, olive phenolics, except 3,4-DHPEA-EA, were still able to protect RBC from hemolysis induced by AAPH. However, differences in morphology could already be observed. Our data suggest that the presence of polyphenols changed the RBC morphology prior to the addition of AAPH (Figs. 3A and C). Hydroxytyrosol and oleuropein increased the number of echinocytes produced by incubation, while 3,4-DHPEA-

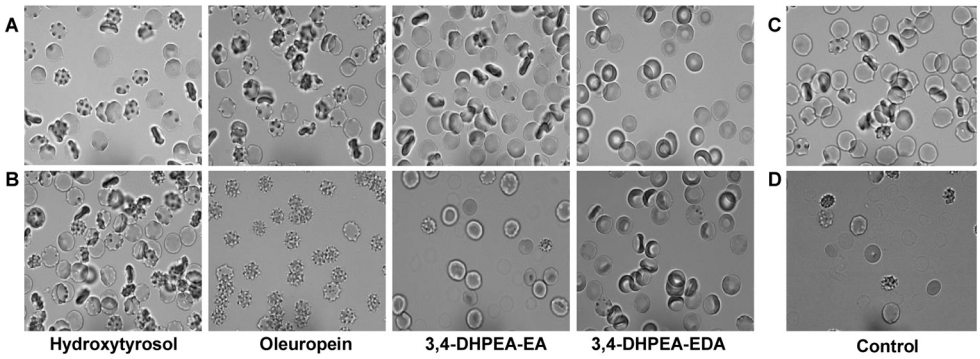

Figure 3. Optical microscopic evaluation of the erythrocyte morphology. (A) RBCs plus phenolic compounds at 40 μM. (B) RBCs plus phenolic compounds at 40 μM and AAPH after 4 h of incubation (original magnification 6400). (C) Control just with RBCs. (D) Control with RBCs plus AAPH.

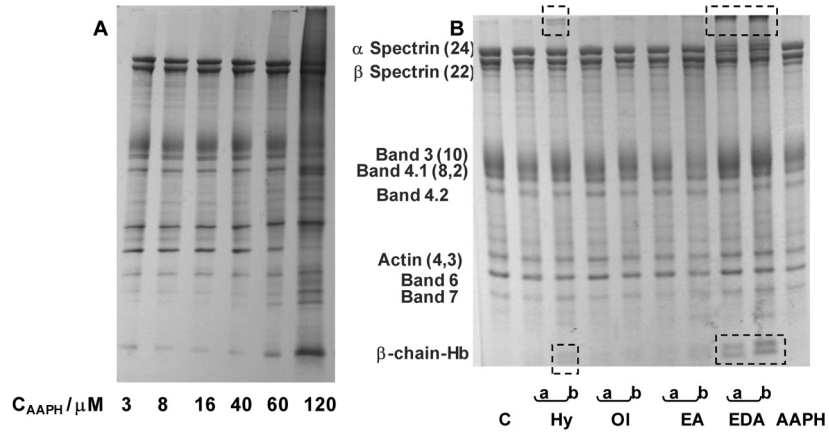

Figure 4. (A) SDS-PAGE (linear gradient gel) of human RBC membrane proteins incubated for 4 h with different AAPH concentrations (3 – 120 μM). (B) SDS-PAGE (exponential gradient gel) of human RBC membrane proteins incubated with AAPH, for 3 h. C: control; AAPH: 2,2'-azo-bis(2-amidinopropane) dihydrochloride; Hy: hydroxytyrosol; Ol: oleuropein; EA: 3,4-DHPEA-EA; EDA: 3,4-DHPEA-EDA; a: RBCs incubated only with the phenolic compound; b: RBCs incubated with the phenolic compound and AAPH.

EA and especially 3,4-DHPEA-EDA lowered them in the samples. In the presence of AAPH (Figs. 3B and D), the number of echinocytes was increased with oleuropein and hydroxytyrosol, in the latter case to a lesser extent. In the presence of 3,4-DHPEA-EDA, the addition of AAPH did not significantly change the morphology of RBCs. Using the same volume of the RBC suspensions, it was also possible to observe that the cellular density in the 3,4-DHPEA-EA was lower and this observation was in accordance with the hemolysis study. In fact, 3,4-DHPEA-EA was not able to prevent hemolysis of most RBCs at this concentration.

To study the capacity of the phenolic compounds to inhibit RBC membrane oxidative changes (Fig. 4), a concentration of 50 μM of AAPH was selected, as it was found capable of producing significant modifications in RBC membrane proteins. At 40 μM slight oxidative changes were observed, whereas at 60 μM they were too strong (Fig. 4A).

The protein membrane analysis performed in the absence of AAPH, showed interactions between 3,4-DHPEA-EDA and RBC membrane proteins as suggested by the appearance of a new protein band (Fig. 4B, in the upper right dot square) identified by MS as being α-spectrin plus band 3 protein (Table 1). This band was also observed in the assays performed with 3,4-DHPEA-EDA in the presence of AAPH. In the assays with hydroxytyrosol plus AAPH a similar band was observed (Fig. 4B, in the upper left dot square). A further examination of the SDS-PAGE gels corresponding to the assays performed with these two phenolic compounds also showed two additional bands (Fig. 4B, in the lower dot squares) identified as hemoglobin beta chain and alpha chain (Table 1) by MS. All proteins were identified with 100% confidence.

Concerning MBH, an increase in its value in the assays performed with AAPH (Table 2) was observed; MBH was higher in the hydroxytyrosol and 3,4-DHPEA-EDA assays. Hemoglobin, when denatured, links to the RBC membrane; however, it is important to highlight that the MBH value was already elevated for the assay with 3,4-DHPEA-EDA in the absence of AAPH. All these observations are in agreement with the results obtained in SDS-PAGE gels and in MS analysis, suggesting the occurrence of interactions of compounds with RBC membrane proteins.

The increment of MBH in the presence of 3,4-DHPEA-EDA (even in the absence of AAPH) prompted us to investigate the nature of hemoglobin in the hemolysate. When performing spectral scans (450 – 650 nm) of lysed RBC suspensions [27] in the presence of phenolic compounds (lyses after 3-h incubation, without AAPH), we did not observe any change in the oxy-hemoglobin peaks (540 and 578 nm; Fig. 5A) or in its concentration [28] (data not shown), as compared with the control assay (RBCs without phenolic

Table 1. Identification of band 3, spectrin α chain, hemoglobin β chain and hemoglobin α chain by MALDI-TOF/TOF MS and MS/MS spectra. Sequence coverage was 8%, 2%, 48% and 21% of full length, which allowed identification with 100% confidence

(P02730) Band 3 anion transport protein

Calculated Mass	Observed Mass	Δ Mass (Da)	Start Seq Pos	End Seq Pos	Sequence
2204.1458	2204,221	0,076	139	155	FIFEDQIRPQDREELLR
2326.2612	2326,366	0,105	117	138	GTVLLDLQETSLAGVANQLLDR
2286.228	2286,281	0,053	264	283	FLFVLLGPEAPHIDYTQLGR
1490.8002	1490,861	0,060	234	246	ADFLEQPVLGFVR

(P02549) Spectrin alpha chain, erythrocyte

Calculated Mass	Observed Mass	Δ Mass (Da)	Start Seq Pos	End Seq Pos	Sequence
1622.9153	1622,981	0,066	138	150	HLWDLLLELTLEK
2117.1277	2117,215	0,087	2110	2128	ALGVPSSPYTWLTVEVLER
2253.1663	2253,268	0,102	2232	2250	YSTIGLAQQWDQLYQLGLR

(P69905) Hemoglobin alpha chain

Calculated Mass	Observed Mass	Δ Mass (Da)	Start Seq Pos	End Seq Pos	Sequence
1529,7343	1529,7844	0,0501	17	31	VGAHAGEYGAEALER
1833,8918	1833,9213	0,0295	41	56	TYFPHFDLSHGSAQVK
1274,7256	1274,8035	0,0779	31	40	LLVVYPWTQR

(P68871) Hemoglobin beta chain

Calculated Mass	Observed Mass	Δ Mass (Da)	Start Seq Pos	End Seq Pos	Sequence
2074,9426	2074,9509	0,0083	41	59	FFESFGDLSTPDAVMGNPK
1274,7256	1274,8092	0,0836	31	40	LLVVYPWTQR
1314,6648	1314,7335	0,0687	18	30	VNVDEVGGEALGR
1378,7002	1378,7692	0,069	121	132	EFTPPVQAAYQK
1669,8907	1669,9412	0,0505	67	82	VLGAFSDGLAHLDNLK
1797,9857	1798,0123	0,0266	66	82	KVLGAFSDGLAHLDNLK
2058,9478	2058,9575	0,0097	41	59	FFESFGDLSTPDAVMGNPK

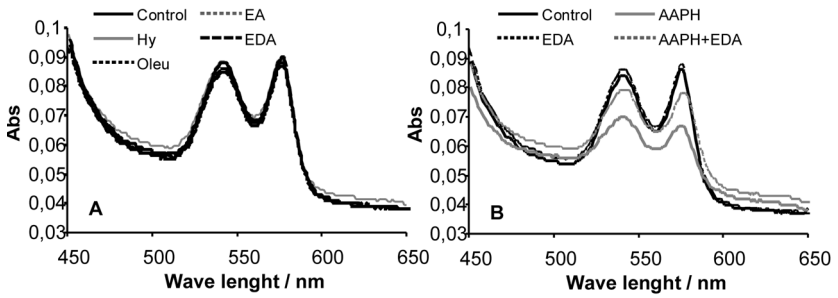

Figure 5. Spectral scans (450 – 650 nm). (A) Lysed RBC suspensions, obtained after 3 h of incubation in the presence of phenolic compounds. (B) Lysed RBC suspensions, obtained after 3 h of incubation in the presence of 3,4-DHPEA-EDA with and without AAPH.

compounds). Furthermore, the oxy-hemoglobin peaks decreased in the presence of AAPH, but this was almost completely reversed by the addition of 3,4-DHPEA-EDA (Fig. 5B).

Combining the results of hemolysis with those of RBC membrane protein analysis, one may say that the superior capacity of 3,4-DHPEA-EDA to prevent AAPH-induced hemolysis may be associated with the observed interaction of this phenolic compound with RBC membrane proteins.

In summary, all compounds confer antioxidant protection to RBC oxidative hemolysis, especially the oleuropein aglycone derivative 3,4-DHPEA-EDA, which operates at very low concentrations. In this experimental system, a significant protective effect was observed in RBCs treated with 3 μM 3,4-DHPEA-EDA and hydroxytyrosol in the presence of AAPH.

These results, together with literature data, reinforce the hypothesis that olive oil polyphenols can exert their beneficial action also *in vivo*. Even though oleuropein aglycones concentrations attained *in vivo* by the dietary intake of olive oil have not been evaluated, recent papers have pointed out that hydroxytyrosol, oleuropein and one unidentified oleur-

Table 2. Percentage of RBC MBH after incubation of RBCs with phenolic compounds (200 μM), in the presence and absence of AAPH (50 μM)[a)]

	% MBH				
	Hy	Ol	EA	EDA	B
Without AAPH	5.2[a] (± 2.5)	5.1[a] (± 3.0)	6.5[a] (± 0.9)	30.7[b] (± 3.5)	3.9[a] (± 1.4)
With AAPH	9.8[a] (± 1.3)	5.4[b] (± 1.5)	7.8[ab] (± 1.9)	41.9[c] (± 9.0)	7.1[ab] (± 1.4)

a) Mean of four experiments ran in duplicate ± SE. Hy: hydroxytyrosol; Ol: oleuropein; EA: 3,4-DHPEA-EA; EDA: 3,4-DHPEA-EDA. Different letters within a row indicate samples that were significantly different ($p < 0.05$).

opein aglycone have human blood concentrations ranging from 1 to 18 μM [9–11]. The structural similarity of 3,4-DHPEA-EDA to oleuropein (it is an oleuropein aglycone) and its higher lipophicity (Log $P_{(3,4\text{-DHPEA-EDA})}$ = 1,02; log $P_{(Oleuropein)}$ = 0,13) [29] suggest that this compound may also be absorbed into the bloodstream. In fact, according to the "Lipinski rule of 5" [30], oleuropein aglycones are likely to be absorbed. Moreover, both 3,4-DHPEA-EDA and 3,4-DHPEA-EA showed good stability after 48 h at acidic pH values (pH 3.5 and 5.5) [31], and did not change significantly in acidified water up to 4 h [32]. Likewise, the concentration of hydroxytyrosol in a phenolic extract was only slightly increased during a 4-h gastric simulation study [32]. These studies suggest that these compounds may survive the acidic conditions of stomach and be available for absorption.

4 Concluding remarks

Oleuropein aglycones have often been regarded as simple hydroxytyrosol derivatives [8], with no more characteristics than those given by the cathecol group. However, these compounds have, at a minimum, a particular lipophicity, which is quite different from oleuropein or hydroxytyrosol [29].

For the first time, this work shows that one of the oleuropein aglycones, the 3,4-DHPEA-EDA, can interact with RBC proteins and protect these cells from oxidative hemolysis initiated by peroxyl radicals using a mechanism different from the simple radical scavenging activity. The exact mechanism of the effect is not yet known. One can assume that the ability to protect the RBCs from hemolysis is related not only to a radical scavenging activity but also to its ability to interact directly with cell membranes (in the outer or inner membrane surface), inducing modifications of the protein profile.

Since 3,4-DHPEA-EDA is usually the phenolic compound found in olive oil in higher concentration, and RBC are the most important cell involved in the oxidative blood status, further studies on these understudied molecules are needed to understand the mechanisms involved in olive oil cardiovascular protection. The results represent the first evidence that 3,4-DHPEA-EDA may play a much more important protective role against ROS-induced oxidative injury in human cells than hydroxytyrosol or oleuropein, and may be of great importance regarding the protective effect of virgin olive oil. The data gathered in this study may help to increase the knowledge of the polyphenols of olive oil performance in human cells.

The authors have declared no conflict of interest.

5 References

[1] Visioli, F., Galli, C., Olive oil phenols and their potential effects on human health. *J. Agric. Food Chem*. 1998, *46*, 4292 – 4296.

[2] Wiseman, S., Mathot, J. N. N. J., Fouw, N. J., Tijburg, L. B. M., Dietary non-tocopherol antioxidants present in extra virgin olive oil increase the resistance of low density lipoproteins to oxidation in rabbits. *Atherosclerosis* 1996, *120*, 15 – 23.

[3] Fitó, M., Covas, M. I., Lamuela-Raventós, R. M., Vila, J., *et al.*, Protective effect of olive oil and its phenolic compounds against low density lipoprotein oxidation. *Lipids* 2000, *35*, 633 – 638.

[4] Leenen, R., Roodenburg, A. J. C., Vissers, M. N., Schuubiers, J. A. E. *et al*., Supplementation of plasma with olive oil phenols and extracts: Influence on LDL oxidation. *J. Agric. Food Chem*. 2002, *50*, 1290 – 1297.

[5] Fitó, M., Cladellas, M., de la Torre, R., Martí, J., *et al*., Antioxidant effect of virgin olive oil in patients with stable coronary heart disease: A randomised, crossover, controlled, clinical trial. *Atherosclerosis* 2005, *181*, 149 – 158.

[6] Visioli, F., Caruso, D., Grande, S., Bosisio, R., *et al.*, Virgin olive oil study (VOLOS): Vasoprotective potential of extra virgin olive oil in mildly dyslipidemic patients. *Eur. J. Nutr.* 2005, *44*, 121 – 127.

[7] Covas, M. I., de la Torre, K., Farré-Albaladejo, M., Kaikkonen, J., *et al.,* Postprandial LDL phenolic content and LDL oxidation is modulated by olive oil phenolic compound in humans. *Free Radic. Biol. Med*. 2006, *40*, 608 – 616.

[8] Bogani, P., Galli, C., Villa, M., Visoli, F., Postprandial antiinflammatory and antioxidant effects of extra virgin olive oil. *Atherosclerosis* 2007, *190*, 181 – 186.

[9] Visioli, F., Bellomo, G., Montedoro, G., Galli, C., Low density lipoprotein oxidation is inhibited in vitro by olive oil constituents. *Atherosclerosis* 1995, *117*, 25 – 32.

[10] Coni, E., Benedetto, R., Pasquale, M., Masella, R., *et al*., Protective effect of oleuropein, an olive oil biophenol, on low density lipoprotein oxidizability in rabbits. *Lipids* 2000, *35*, 45 – 53.

[11] Covas, M. I., Fito, M., Lamuela-Raventos, R. M., Sebastia, N., *et al*., Virgin olive oil phenolic compounds: Binding to human low density lipoprotein (LDL) and effect on LDL oxidation. *Int. J. Clin. Pharmacol. Res*. 2000, *20*, 49 – 54.

[12] Fito', M., De La Torre, R., Covas, M. I., Olive oil and oxidative stress. *Mol. Nutr. Food Res*. 2007, *51*, 1215 – 1224.

[13] Terao, J., Piskula, M., Yao, Q., Protective effect of epicatechin, epicatechin gallate, and quercetin on lipid peroxidation in phospholipid bilayers. *Arch. Biochem. Biophys.* 1994, *308*, 278 – 284.

[14] García, A., Brenes, M., Martínez, F., Alba, J., *et al*., High-performance liquid chromatography evaluation of phenols in virgin olive oil during extraction at laboratory and industrial scale. *J. Am. Oil Chem. Soc*. 2001, *78*, 625 – 629.

[15] Tovar, M. J., Moltiva, M. J., Romero, M. P., Changes in the phenolic composition of virgin olive oil from young trees (*Olea europaea* L. cv. Arbequina) grown under linear irrigation strategies. *J. Agric. Food Chem*. 2001, *49*, 5502 – 5508.

[16] Brenes, M., García, A., García, P., Garrido, A., Acid hydrolysis of secoiridoid aglycons during storage of virgin olive oil. *J. Agric. Food Chem*. 2001, *49*, 5609 – 5614.

[17] Manna, C., Galletti, P., Cucciolla, V., Montedoro, G., Azppia, V., Olive oil hydroxytyrosol protects human erythrocytes against oxidative damages. *J. Nutr. Biochem.* 1999, *10*, 159 – 165.

[18] Baraldi, P. G., Simoni, D., Manfredini, S., Menziani, E., Preparation of 3,4-dihydroxy-1-benzeneethanol: A reinvestigation. *Liebigs Ann. Chem.* 1983, 684 – 686.

[19] Gariboldi, P., Jommi, G., Verotta, L., Secoiridoids from *Olea europaea. Phytochemistry* 1986, *25*, 865 – 869.

[20] Limiroli, R., Consonni, R., Ottolina, G., Marsilo, V., *et al*., ^{1}H NMR and ^{13}C NMR characterization of new oleuropein aglycones. *J. Chem. Soc. Perkin Trans.* 1995, *1*, 1519 – 1523.

[21] Paiva-Martins, F., Gordon, M. H., Isolation and characterization of the antioxidant component 3,4-dihydroxyphenylethyl 4-formyl-3-formylmethyl-4-hexenoate from olive (*Olea europaea*). *J. Agric. Food Chem.* 2001, *49*, 4214 – 4219.

[22] Ko, F. N., Hsiao, G., Kuo, Y. H., Protection of oxidative hemolysis by demethyldiisoeugenol in normal and β-thalassemic red blood cells. *Free Radic. Biol. Med.* 1997, *22*, 215 – 222.

[23] Dodge, J. T., Mitchell, C., Hanahan, D. J., The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Arch. Biochem. Biophys.* 1963, *100*, 119 – 130.

[24] Bradford, M. M., A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of the protein dye binding. *Anal. Biochem.* 1976, *72*, 248 – 254.

[25] Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of the bacteriophage T. *Nature* 1970, *227*, 680 – 685.

[26] Fairbanks, G., Steck, T. L., Wallach, D. F. H., Electrophoresis of the major polypeptides of the human erythocyte membrane. *Biochemistry* 1971, *10*, 2606 – 2616.

[27] Blakney, G. B., Dinwoodie, A. J., A spectrophotometric scanning technique for the rapid determination of plasma hemoglobin. *Clin. Biochem.* 1975, *8*, 96 – 102.

[28] Lewis, S. M., Roper, D., in: Lewis, S. M., Bain, B. J., Bates, I. (Eds.), *Dacie and Lewis Practical Haematology*, Churchill Livingstone, New York 2002, pp. 149 – 198.

[29] Paiva-Martins, F., Gordon, M. H., Gameiro, P., Activity and location of olive oil phenolic antioxidants in liposomes. *Chem. Phys. Lipids* 2003, *124*, 26 – 34.

[30] Lipinski, C. A., Lombardo, F., Dominy, B. W., Feeney, P. J., Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Deliv. Rev.* 1997, *23*, 3 – 25.

[31] Paiva-Martins, F., Gordon, M. H., Interactions of ferric ions with olive oil phenolic compounds. *J. Agric. Food Chem* 2005, *53*, 2704 – 2709.

[32] Romero, C., Medina, E., Vargas, J., Brenes, M., De Castro, A., *In vitro* activity of olive oil polyphenols against *Helicobacter pylori*. *J. Agric. Food Chem.* 2007, *55*, 680 – 686.

