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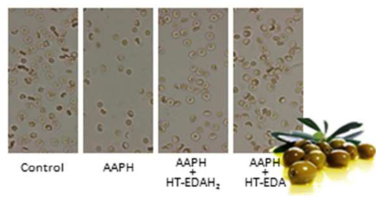

The olive oil polyphenol HT-EDA metabolite, HT-EDAH2, protects human erythrocytes from induced oxidative damage.

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Effects of the olive oil phenol metabolite 3,4-DHPEA-EDAH$_2$ on human erythrocyte oxidative damage

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Red blood cells (RBCs), as anucleated cells, have poor repair and biosynthetic mechanisms, suffering and accumulating oxidative lesions whenever oxidative stress develops. RBCs are particularly exposed to endogenous oxidative damage because of their specific role as oxygen carriers. However, as the most abundant blood cells, RBCs also play an important role in the oxidative status of the whole blood constituents. In previous studies of our group, the most important polyphenolic compounds found in virgin olive oil, 3,4-dihydroxyphenylethanol-elenolic acid (3,4-DHPEA-EA) and 3,4-dihydroxyphenylethanol-elenolic acid dialdehyde (3,4-DHPEA-EDA), were shown to significantly protect RBCs from oxidative damage initiated by AAPH and H_2O_2, with the most active compound being 3,4-DHPEA-EDA. However, the in vivo protective effects of these phenols are dependent on their bioavailability. It has been demonstrated that 3,4-DHPEA-EDA is absorbed by intestinal cells and is then metabolized, yielding a reduced metabolite, 3,4-DHPEA-EDAH$_2$. In order to assess the importance of VOO phenolic compounds metabolites for the overall in vivo protective activity, the capacity of this phase I metabolite to protect RBCs in the presence of the radical initiators AAPH or H_2O_2 was evaluated in the presence and absence of the naturally occurring antioxidant ascorbic acid. The metabolite was shown to protect RBCs from haemolysis induced by both initiators, in a dose dependent way, after 2 h and 4 h of incubation. The protective effect was however lower than that of the parental compound. The analysis of the membrane proteins of erythrocytes showed that the metabolite can interact with these biological structures.

1. Introduction

Several studies have shown a lower incidence of cardiovascular mortality and morbidity among populations following the Mediterranean diet, in which the main source of fat is virgin olive oil (VOO). Many of these beneficial effects seem to be mediated in part by phenols, namely hydroxytyrosol, oleuropein and its derivatives.[1] The discovery of antioxidant activities (*in vitro*, *ex vivo* and *in vivo* animal models) for the most important olive oil phenolic derivatives has stimulated intense research on their presence in olive oils and on their bioavailability, metabolism and disposition in humans. In previous studies of our group,[2,3] the most important polyphenolic compounds found in virgin olive oil, the 3,4-dihydroxyphenylethanol-elenolic acid (3,4-DHPEA-EA, HT) and 3,4-dihydroxyphenylethanol-elenolic acid dialdehyde (3,4-DHPEA-EDA, HT-EDA) (Fig. 1), were shown to significantly protect RBCs from oxidative damage initiated by AAPH and H_2O_2, with the most active compound being 3,4-DHPEA-EDA. This compound is usually the most important phenolic compound found in olive oil and in some olive oils, 3,4-DHPEA-EDA may even represent 50% of the phenolic fraction.[1, 4-7] However, the *in vivo* protective effect of this phenol is also dependent on their bioavailability. It has been demonstrated[4] that 3,4-DHPEA-EDA is absorbed by intestinal cells and then metabolized, yielding a glucuronide of a reduced form, the 3,4-DHPEA-EDAH$_2$ (HT-EDAH$_2$), as the major small-intestinal metabolite entering the portal blood (Fig. 1). Aldo-keto reductase enzymes are widely distributed in mammals and include human aldose reductase and human small intestine aldose reductase.[8] These enzymes are capable of catalysing the reduction of a variety of carbonyl-containing compounds, and are responsible for the reduction of the conjugated carbonyl group of retinal to retinol in the human small intestine,[9] as well as the reduction of various other molecules, including carbohydrates, aliphatic and aromatic aldehydes, and steroids. Previous studies suggest that during transfer across enterocytes, aldose reductase reduces the conjugated carbonyl group of 3,4-DHPEA-EDA, yielding a reduced metabolite consistent with the MS fragmentation pattern of 3,4-DHPEA-EAH$_2$, which is

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further glucuronated. A major issue is therefore to what extent the beneficial effects reportedly associated with this secoiridoid are attributed to metabolites rather than to the parent molecule.[8]

Another aspect attracting attention is the role of phase II conjugates as temporary "deposits" of more active molecules.[10-14] Some studies propose that glucuronides may act not only as detoxified metabolites but also as bioactive agents, being precursors of more hydrophobic aglycones. Accordingly, aglycones may be assumed to emerge in the target site by the action of glucuronidases under oxidative stress. The cardiovascular system and central nervous system seem to be the major targets of phenol glucuronides circulating in the human blood.[12-14] In fact, recent results are suggesting that during inflammation glucuronidase is released from stimulated neutrophils or from certain injured cells and then deglucuronidation of phenols occurs.[13-14] Therefore, in order to assess the importance of VOO phenolic compounds metabolites for the overall in vivo protective activity, the capacity of the phase I metabolite 3,4-DHPEA-EDAH$_2$ to protect red blood cells (RBCs) against the radical initiators AAPH or H_2O_2 was evaluated, in the presence and absence of the naturally occurring antioxidant ascorbic acid. RBCs are a good model for studying antioxidant related effects since these cells are particularly susceptible to endogenous and exogenous oxidative damage because of their specific role as oxygen carriers and their poor biosynthetic capacity and limited repair mechanisms.[15-17] Moreover, these cells, the most abundant cells in blood, are closely related to vascular tonus regulation[18,19] and have an important function as mobile free radical scavengers[20] and, therefore, any functional abnormality in these cells will have major health consequences.

2. Materials and methods

2.1. Phenolic compounds.

The olive oil component 3,4-DHPEA-EDA was obtained from olive leaves, according to the procedure of Paiva-Martins and Gordon.[21] The 3,4-DHPEA-EDAH$_2$ metabolite was synthesized from 3,4-DHPEA-EDA by selective reduction of the conjugated carbonyl with $NaBH_4$ in the presence of $ErCl_3$ (Fig. 1).[22] In a 50 ml round flask 0.444 g (1.4 mmol) of 3,4-DHPEA-EDA were dissolved in 7 mL of a 0.2 M methanol $CeCl_3$.6 H_2O solution. The flask was put in an ice bath under a stream of Argon. After addition of 0.090 g (2.4 mmol/1.7 eq) of $NaBH_4$ the flask was left in the dark under stirring and argon for 2 h. Then 10 ml of HCl 1 M was added and the mixture extracted with ethyl ether and ethyl acetate. The combined organic phases were transferred into the Erlenmeyer flask and dried with anhydrous sodium sulfate. After solvent evaporation, the product was purified by column chromatography (Silica Gel 50 g) eluted with Et_2O/MeOH (35:1) (η = 25%). A mixture of 2 geometric isomeres (A and B, 0,6:0,4) were obtained. Structural assignment of 3,4-DHPEA-EDAH$_2$ (Fig. 1) isomers was performed by 1H and 13C, DEPT, bidimensional HSQC and COSY NMR spectra and ESI-MS.

^{1}H NMR (CD_3COCD_3, 400 MHz): δ 7.78 (s, br, 2H, H-19 + H-18); 6.80 (d, 1H, J = 8.0 Hz, H-14); 6.75 (d, 1H, J = 2.0 Hz, H-17); 6.6 (dd, 1H, ^3J = 8.0 Hz, ^4J = 2 Hz, H-13); 5.38 (q, 1H, J = 7.0 Hz, H-5A); 5.34 (q, 1H, J = 7.0 Hz, H-5B); 4.66 (dd, 1H, $J_{1,2a}$ = 4.0 Hz, $J_{1,2b}$ = 8.0 Hz, H-1B); 4.64 (dd, 1H, $J_{1,2a}$ = 4.0 Hz, $J_{1,2b}$ = 2.0 Hz, H-1A); 4.33 (d, 1H, $J_{7a,7b}$ = 12 Hz, H-7a); 4.25-4.00 (m, 2H, H-9); 3.60 (d, 1H, H-7b); 3.20-3.25 (m, 1H, H-3); 3.05 (s, br, 1H, H-20); 2.75 (t, 2H, H-10); 2.74 (dd, 1H, H-8aA); 2.64 (dd, 1H, H-8aA); 2.53 (dd, 1H, $J_{8a,3}$ = 8.0 Hz, $J_{8a,8b}$ = 16.0 Hz, H-8aB); 2.45 (dd, 1H, $J_{8b,3}$ = 8.0 Hz, H-8aB); 1.78 (m, 1H, H-2a); 1.58 (m, 1H, H-2b); 1.57 (d, 1H, J = 2.0 Hz, H-6A); 1.54 (d, 1H, J = 2.0 Hz, H-6B). ^{13}C NMR (CD_3COCD_3, 100 MHz): δ 172.84 (A); 172.28 (B); 145.79; 144.43 (A); 144.41 (B); 137.00 (B); 136.55 (A); 130.59 (A); 130.51 (B); 121.13 (A); 121.01; 120.56 (B); 116.77; 116.00; 99.76 (B); 99.38 (A); 67.11 (B); 66.09 (A); 62.43; 39.40 (A); 35.15 (B); 35.12 (A); 35.08 (B); 35.04 (A); 30.79 (B); 30.09 (A); 12.73 (B); 12.39 (A). ESI-MS (negative mode): 321, 185, 110.

2.2. Preparation of RBC suspensions and induction of oxidative stress.

Blood was obtained from healthy volunteers by venipuncture and collected into tubes containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant. Samples were then centrifuged at 400 X g for 10 minutes; the supernatant (plasma) and buffy coat were carefully removed by aspiration and discarded. RBCs were washed three times with phosphate buffered saline (PBS; 125 mM NaCl and 10 mM sodium phosphate buffer, pH 7.4) and finally resuspended in PBS to obtain the desired 10% and 2% hematocrits.

To perform the in vitro RBC studies under oxidative stress conditions, H_2O_2 and AAPH solutions were prepared. The assays were performed with H_2O_2 at a final concentration of 7.5 mM (2% hematocrit) and with AAPH at final concentration of 60 mM (2% hematocrit). These studies were carried out at 37°C for four hours under gentle shaking of the RBC suspensions.

2.3. Assay system for evaluation of RBC hemolysis.

To assay the capacity of 4-DHPEA-EDA and its metabolite 3,4-DHPEA-EDAH$_2$ to protect RBCs from oxidative-induced injury, the cells were pre-treated at 37°C for 15 minutes in the presence of the chosen concentration of the polyphenols with or without ascorbic acid (Sigma-Aldrich Quimica-S.A. Madrid, Spain) at physiological concentration (proportional to the hematocrit used, 3 μM), and then H_2O_2 or AAPH was added. Four independent assays (n = 4) were performed for each tested antioxidant.

The rate amount of haemolysis was determined spectrophotometrically according to Ko *et al.*[23] In all sets of experiments, a negative control (RBCs in saline) was used, and all sample tests were run in duplicate. After four hours of incubation, an aliquot of the RBC suspensions was taken out, diluted with 20 volumes of saline and centrifuged, (400 *g* for 10 min). The absorption (A) of the supernatant was read at 540 nm. To yield the absorption (B) of a complete hemolysis, the

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RBC suspension was treated with 20 volumes of ice cold distilled water and, after centrifugation, the absorption was measured at the same wave length. The percentage of hemolysis was then calculated: (A/B) ×100.

2.4. Evaluation of RBC morphology.

At the end of the haemolysis assays, before the centrifugation, aliquots of RBC suspensions were taken out, placed in glass films and evaluated by optical microscopy observation. To get an approximated idea (visual observation) of the amount of RBC lysis (decrease in RBC count and detection of RBC ghosts), an exact volume of the RBC suspension (20 µl) was taken to the slide for observation.

2.5. Protective effect of phenolic compounds against AAPH and H_2O_2-induced erythrocyte membrane changes.

RBC suspensions at 10% hematocrit were used. RBC suspensions were incubated at 37 °C for three hours, under gentle shaking. Afterwards, RBCs were washed in a saline solution and immediately lysed by hypotonic lysis according to Dodge *et al*.[24] The obtained membranes were washed in Dodge buffer, adding in the first two washes phenylmethylsulphonyl fluoride as a protease inhibitor with a final concentration of 0.1 mM. The protein concentration of the RBC membrane suspensions was determined by the Bradford method.[25]

MBH was measured spectrophotometrically after protein dissociation of membrane components 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 was then used to calculate the % MBH.

Membranes of RBCs were treated with a solubilisation buffer, heat denatured and submitted to electrophoresis (8 µg of protein/lane). The electrophoresis were carried out on a discontinuous system of polyacrylamide in the presence of sodium dodecylsulfate (SDS-PAGE), using a 5-15% linear acrylamide gradient gel according to Laemmli method.[26] The proteins were stained with Coomassie brilliant blue, and finally the gel was scanned (Darkroom CN UV/wl, BioCaptMW version 99, Vilber Lourmat, France).

In all sets of experiments (n = 4), controls (RBCs in PBS and RBCs in PBS with H2O2 or AAPH) were used. Controls and tests, using 10-80 µM (final concentration) for phenolic compounds, were run in duplicate. The assay conditions were those described above for the assays (7.5 mM H_2O_2 or 60 mM AAPH, 4 hours of incubation at 37 ^oC under gentle shaking).

2.6. Statistical analysis.

The results obtained for the four independent haemolysis experiments, performed in duplicate, are expressed as means ± SEM. Statistical differences between groups of experiments with different antioxidant compounds were analysed by two-way analysis of variance with post-hoc testing using Tukey's test. A p value lower than 0.05 was accepted as statistically significant.

3. Results and discussion

Increasing evidence has supported the hypothesis that antioxidants might have a beneficial role regarding the course of chronic diseases. In particular, it has been claimed that olive oil polyphenols may play a major role on the protective effects of olive oil consumption against oxidative damage. However, no research has been addressed to the study of the antioxidant profile of the metabolites of the most significant olive oil phenolic compound, 3,4-DHPEA-EDA, in biological systems. In this work we addressed that issue by studying the protective properties of one of the most important metabolites of this compound, the 3,4-DHPEA-EDAH$_2$, upon human RBC under AAPH and H_2O_2 induced oxidative stress. This biological model has been extensively studied as a target for oxidative damage.

The 3,4-DHPEA-EDAH$_2$ metabolite was therefore synthesized from 3,4-DHPEA-EDA by selective reduction of the conjugated carbonyl with $NaBH_4$ in the presence of $ErCl_3$ (Figure 1).

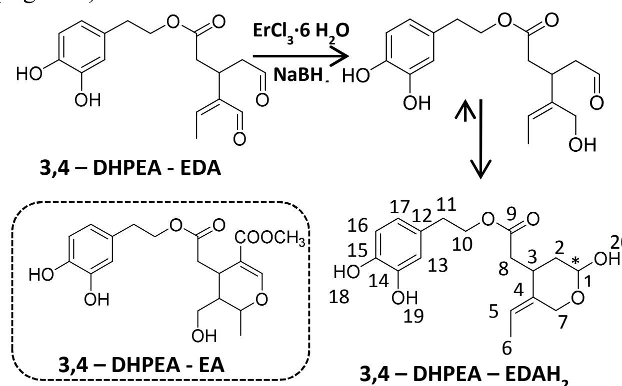

Fig. 1 – Synthesis of 3,4-DHPEA-EDAH$_2$ and chemical structure of 3,4-DHPEA-EA.

NMR spectra analysis of the obtained compound, however, did not show the expected signals. In fact, the ^{1}H NMR signal for the remaining carbonyl group also disappeared, but spectra was not consistent with the reduction of the two carbonyls. In fact, NMR data showed the presence of a similar ring to the one observed for oleuropein aglycone. Therefore, after reduction of one carbonyl a hemiacetalic ring is formed (Fig. 1). The obtained ISI-MS spectra were also consistent with this structure and similar to the one obtained previously for the reduced metabolite.[8]

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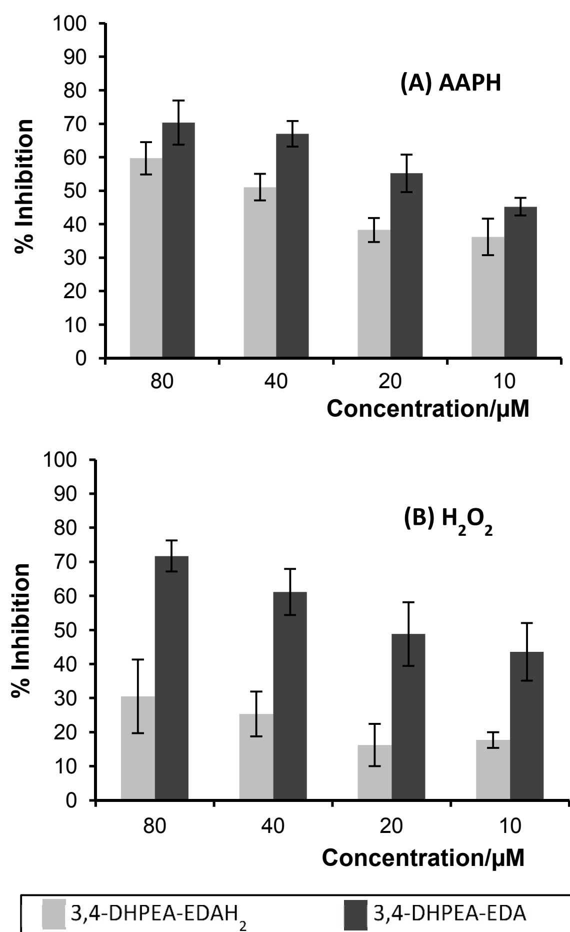

Fig. 2 - Percentage of inhibition of RBC lysis incubated for 4 h with AAPH (A) or H_2O_2 (B) in the presence of 3,4-DHPEA-EDA or 3,4-DHPEA-EDAH$_2$. Mean (error bars represent standard error) of 4 determinations for each duplicate.

Both compounds, 3,4-DHPEA-EDA and its metabolite 3,4-DHPEA-EDAH2, were shown to significantly protect RBCs from oxidative hemolysis at concentrations of 10-80 μM, being the order of activity: 3,4-DHPEA-EDA > 3,4-DHPEA-EDAH2 (Fig. 2). Nevertheless, the metabolite was more effective against the water-soluble radical initiator AAPH (Fig. 2A) than against the physiological radical initiator H_2O_2 (Fig. 2B), being much better in protecting RBC from oxidative injury when the free radicals were generated outside the cell than when free radicals were generated on both sides or within the membrane. Moreover, in the presence of H_2O_2, the metabolite showed only a limited protection that was independent of the concentration used, probably because of its more rigid structure that may compromise the transport across membranes. This result is in accordance with the results also observed for the 3,4-DHPEA-EA that also bear a ring in its structure (Fig. 1). In fact, this compound has a higher radical scavenging activity than 3,4-DHPEA-EDA[27] but showed a lower efficiency in protecting RBCs from AAPH and H_2O_2 induced oxidative injury.[2,3]

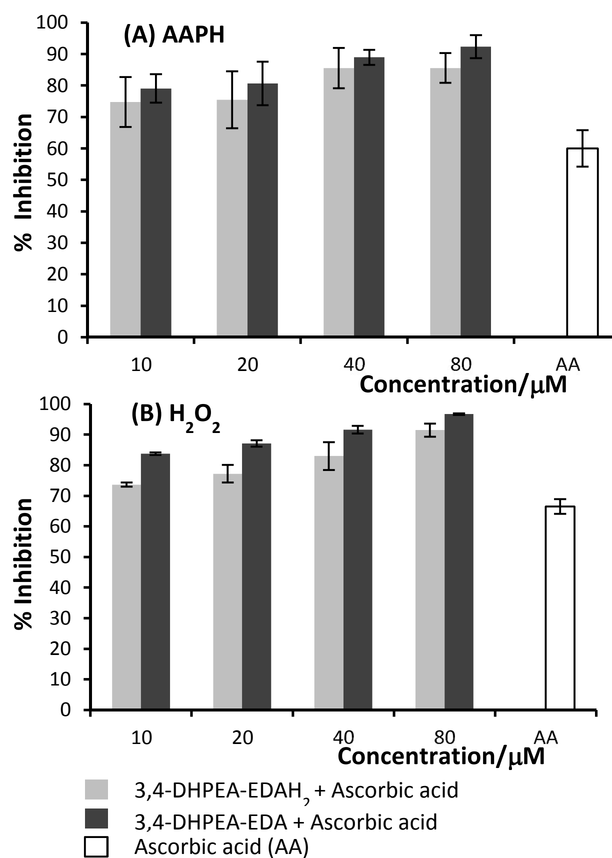

Fig. 3- Percentage of inhibition of RBC lysis incubated for 4 hours with AAPH (A) or H_2O_2 (B) in the presence of 3,4-DHPEA-EDA and 3,4-DHPEA-EDAH$_2$ and ascorbic acid (AA). Mean (error bars represent standard error) of 4

Table 1 - Percentage of RBC MBH after incubation of RBCs with phenolic compounds, in the presence and absence ofAAPH or H_2O_2.

	%MBH[a] (10^{-3})						
		3,4-DHPEA-EDA			3,4-DHPEA-EDAH$_2$		
Concentration / mM	0	10	40	80	10	40	80
AAPH	31.4	26.9	46.4	48.0	23.7	28.4	30.5
H_2O_2	31.4	14.9	33.8	46.2	22.7	23.3	33.8

[a] Mean of four experimental runs performed in quadruplicate (SE<3). Different letters within a row indicate samples that were significantly different ($p < 0.05$).

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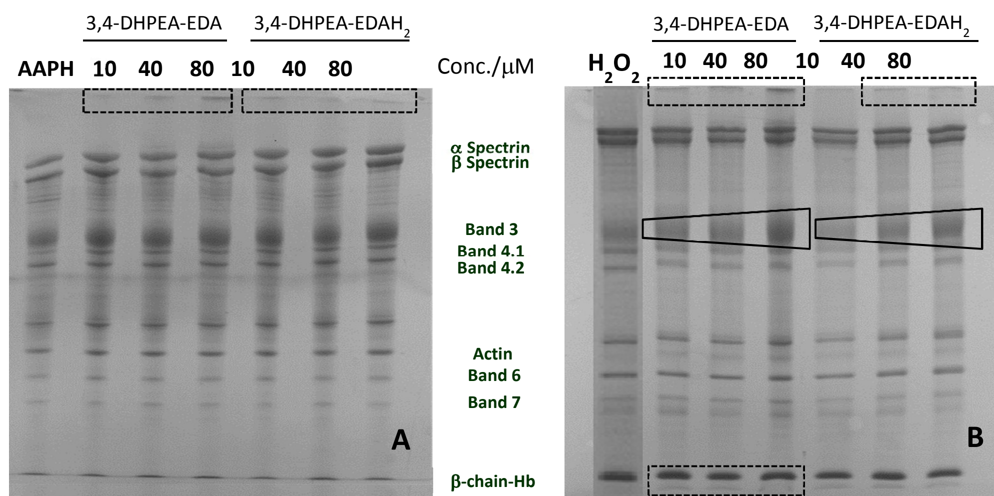

Fig. 4 - SDS polyacrylamide-gel electrophoresis (linear gradient gel) of human RBC membrane proteins incubated with AAPH (A) and H_2O_2 (B) for 4 hours. Cells incubated with the 3,4-DHPEA-EDAH$_2$ and with 3,4-DHPEA-EDA. The gel was stained with Coomassie blue.

determinations for each duplicate.

In the presence of the naturally occurring antioxidant ascorbic acid, both compounds produced further protection when compared with the protection given by the ascorbic acid alone, even at the lowest concentration against both radical initiators (Fig. 3).

The protein membrane analysis performed in the absence of AAPH or H_2O_2 showed interactions between 3,4-DHPEA-EDA and 3,4-DHPEA-EDAH$_2$ and the RBC membrane proteins as suggested by the appearance of a new protein band (data not shown) identified by MS previously as being α-spectrin plus band 3 protein.[2] In the presence of radical initiators, protein analysis showed interactions of 3,4-DHPEA-EDA and 3,4-DHPEA-EDAH$_2$ with RBC membrane proteins, with this interaction being more important for the former (Fig. 4, in the dotted line boxes). According to the haemolysis results, this interaction of both compounds with spectrin, band 3 protein and also with Hb seems to improve the stability of RBCs to haemolysis in accordance with our previous results.[2,3]
Haemoglobin when denatured links to the RBC membrane via the cytoplasmic domain of band 3 protein, inducing its clustering and the linkage of anti-band 3 antibodies that mark the cell for death. In our study, we observed an increase in MBH in the assays performed with H_2O_2 and with AAPH (Table 1), but the amount of MBH was lower in the presence of antioxidants at 10 μM as compared with the control (Table 1). At the higher compound concentrations, however, higher values were obtained in contrast with what was expected if these compounds had been protective. The higher value of MBH in the presence of 3,4-DHPEA-EDA (even in the absence of the oxidant) was already observed in previous works from our group,[2,3] which led us to investigate the nature of haemoglobin that is linked to the RBC membrane.[2] By performing spectral scans (450-650 nm) of lysed RBC suspensions in the presence of 3,4-DHPEA-EDA (lysis performed after 3 h of incubation, without oxidant), we demonstrated that most of the haemoglobin linked to the RBC membrane induced by this compound was not in the oxidized form.[2] In this work we also observed the same behaviour for the metabolite 3,4-DHPEA-EDAH$_2$. In fact, when performing spectral scans (450-650 nm) of lysed RBC suspensions in the presence of metabolite (lyses after 4 h of incubation without AAPH), we did not observe any change in the oxy-haemoglobin peaks (540 and 578 nm) nor in its concentration (data not shown), as compared with the control. Furthermore, the oxy-haemoglobin disappeared in the presence of AAPH, but this was partially reversed by the addition of the metabolite (Fig. 5).

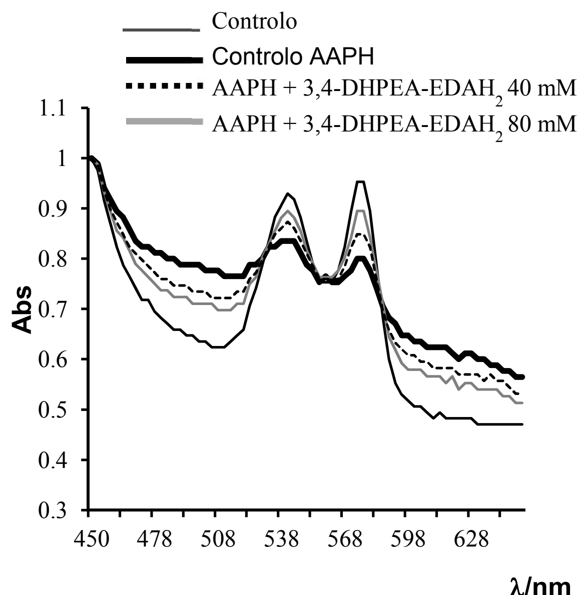

Fig. 5 – Oxi-haemoglobin spectral scans (450-650 nm) of lysed RBC suspensions, obtained after 3 h of incubation in the presence of 3,4-DHPEA-EDAH$_2$ and AAPH.

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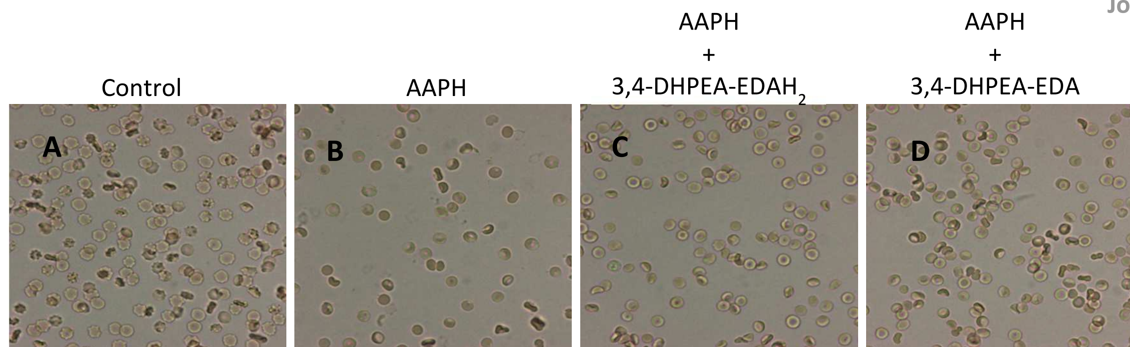

Figure 6 - Optical microscopic evaluation of the erythrocyte morphology. A – in the absence of AAPH; B – In the presence of AAPH after 4 h of incubation; C – in the presence of AAPH and 3,4-DHPEA-EDAH$_2$ at a concentration of 40 µM; D – in the presence of AAPH and 3,4-DHPEA-EDA at a concentration of 40 µM (original magnification x 400).

In the optical microscopic evaluation of the erythrocyte morphology (Fig. 6), by using the same volume of the RBC suspensions, it was also possible to observe that the cellular density in the 3,4-DHPEA-EDAH$_2$ samples was lower than that in the 3,4-DHPEA-EDA samples at the same concentration but still much higher than observed in the samples containing only AAPH, showing that RBCs with the addition of 3,4-DHPEA-EDAH$_2$ were still protected against morphologic injury caused by AAPH. This observation was in accordance with the hemolysis study.

4. Conclusions

In conclusion, 3,4-DHPEA-EDA and its phase I metabolite seem to confer antioxidant protection even at low concentrations, either alone or in the presence of ascorbic acid. Since 3,4-DHPEA-EDA is normally the major compound found in virgin olive oil and it can be found in a concentration of up to 780 mg/kg[5-7] in the oil, this compound and its metabolites are of great importance regarding the protective effect of virgin olive oil.

An intake of up to 20 mg per day of 3,4-DHPEA-EDA may be achieved by the consumption of extra virgin olive oils, which would still lead to a relatively low plasma concentration of it and its metabolites (up to 5 µM),[8,28] however, if consumed regularly it might achieve health effects. Once more, an important interaction is observed between both compounds with the RBC membrane, which may keep these molecules in the blood, reaching a higher net concentration than expected with a daily consumption of extra virgin olive oil. This phenomenon may explain why clinical trials show that short-term consumption of olive oil in humans (50 mL/day) can change several oxidative stress markers,[29,30] although the concentrations of phenols are lower than those required to show biological activity *in vitro*. Therefore, it is possible that the regular low lifetime intake of extra virgin olive oil results in an overall protective effect. Moreover, food components should not be regarded as drugs that are habitually employed for limited timeframes. In contrast, food with its macro- and microcomponents are ingested throughout a lifetime, during which even modest effects may become noteworthy.

Notes and references

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