Grape epicatechin conjugates prevent erythrocyte membrane protein oxidation

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Abstract

Epicatechin conjugates obtained from grape have shown antioxidant activity in various systems. However, how these conjugates exert their antioxidant benefits has not been widely studied. We assessed the activity of epicatechin and epicatechin conjugates on erythrocyte membrane in presence and absence of a peroxyl radical initiator, to increase our understanding of their mechanisms. Thus, we studied cell membrane fluidity by fluorescence anisotropy measurements, morphology of erythrocytes by scanning electron microscopy and, finally, red cell membrane proteins by SDS-PAGE electrophoresis. Our data showed that incubation of red cells in presence of epicatechin derivatives altered membrane fluidity and erythrocyte morphology, but not the membrane protein pattern. The presence in the medium of the peroxyl radical initiator AAPH resulted in membrane disruptions at all levels analysed, causing changes in membrane fluidity, cell morphology and protein degradation. The presence of antioxidants avoided protein oxidation, indicating that the interaction of epicatechin conjugates with the lipid bilayer might reduce the accessibility of AAPH to membranes, which could explain in part the inhibitory ability of these compounds against haemolysis induced by peroxidative insult.

Keywords: erythrocytes, membrane fluidity, cell morphology, band-3, AAPH, epicatechin, membrane protein

Introduction

Polyphenols are products of the secondary metabolism of plants and constitute one of the most numerous and widely distributed groups of natural antioxidants in the plant kingdom. Out of a wide range of natural compounds, polyphenols function as antioxidants by virtue of their hydrogen-donating properties (1,2). Epicatechins are monomeric members of the flavanol family of polyphenols, components of green tea and red grapes, with powerful antioxidant properties *in vitro* (3). It has been demonstrated that flavanols such as epicatechin, epigallocatechin and their gallate esters scavenge both aqueous and lipophilic radicals and act as chain-breaking antioxidants (4).

Cysteinyl-epicatechin (Cys-Ec) and cysteinyl-epicatechin gallate (Cys-EcG) were obtained by depolymerization of grape polymeric flavanols in the presence of cysteine in our lab (5). We demonstrated their antioxidant activity in various systems and models, such as, for example, the prevention of haemolysis induced by hydrogen peroxide (H_2O_2) (5,6) and 2,2'-Azobis(amidinopropane) dihydrochloride (AAPH) (7). However, the mechanism by which they perform their protective effect is still under discussion. Free radical attack decreases membrane fluidity by modifying lipids *via* lipid peroxidation, which may significantly alter membrane properties and possibly disrupt the function of membrane-associated proteins (8). Membrane-active flavonoids are believed to cause antioxidant activity by rigidifying membranes cooperatively, with an effect on reactive oxygen species. Along these lines, Sato et al. (9) modelled haemolysis induced by free radicals by competitive reaction between lipid

peroxidation and protein oxidation, including the redistribution of oxidized band-3 proteins to form haemolytic holes.

The aim of this study was to assess the effect of epicatechin and epicatechin conjugates on erythrocyte membrane in presence and absence of AAPH, a peroxyl radical initiator, because we are interested in their possible applications in the fields of food preservation and skin protection Thus, we studied cell membrane fluidity by fluorescence anisotropy measurements using 1,6-diphenyl-1,3,5-hexatriene (DPH) and (1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,4-hexatriene p-toluenesulfonate) (TMA-DPH) as fluorescent probes. We also studied by scanning electron microscopy (SEM) the morphology of erythrocytes when incubated in the presence of antioxidants alone and with AAPH. Finally, red cell membrane proteins were evaluated by SDS-PAGE electrophoresis.

Material and Methods

Chemicals. Conjugates were prepared by acidic depolymerisation of proanthocyanidins obtained from grape (*Vitis Vinifera*) pomace and from hazel (*Hamamelis virginiana*) bark, essentially as described in Torres and Bobet (2001) (5) and Lozano et al. (2006) (10). We aimed to generate bio-based antioxidants with modified physicochemical and biological properties. The following compounds were studied: (-)-epicatechin (**1**), 4β-(*S*-cysteinyl)epicatechin (**2**) and 4β-(*S*-cysteinyl)epicatechin 3-*O*-gallate (**3**) (Figure 1). 2,2'-Azobis(amidinopropane) dihydrochloride (AAPH) was purchased from Sigma (St. Louis, MO). Fluorescent probes DPH (1,6-diphenyl-1,3,5-hexatriene) and TMA-DPH (1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,4-hexatriene p-

toluenesulfonate) were purchased from Molecular Probes (Eugene, OR, USA). Acrylamide 40%, bisacrylamide 2%, Tetramethylethylenediamine (TEMED), ammonium persulphate, β-mercaptoethanol and blue bromophenol used for SDS-PAGE were supplied by GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Finally, Precision plus Unstained Standard was purchased at BioRad (Spain).

Blood Samples and Preparation of Red Blood Cells. Blood samples were obtained from healthy donors by venipuncture (Tissue and Blood Bank of Hospital Vall d'Hebron, Barcelona, Spain), following the ethical guidelines of the hospital, and were collected in citrated tubes. Red blood cells (RBCs) were separated from the plasma and buffy coat by centrifugation at 1,000 *g* for 10 min. The erythrocyte layer was washed three times in phosphate-buffered isotonic saline (PBS) containing 22.2 mM Na_2HPO_4, 5.6 mM KH_2PO_4, 123.3 mM NaCl, and 10.0 mM glucose in distilled water (pH 7.4). The cells were then suspended in an isotonic saline solution at a density of $8x10^9$ cells/ml.

Erythrocyte treatments. Aliquots of 250 µl of the red cell suspension were treated with AAPH (100 mM, 90 minutes) in constant agitation at 37ºC. Samples were also studied in presence of 75 µM of Ec and cysteinyl conjugates in presence and absence of the oxidant product. Untreated controls were included in all experiments to monitor spontaneous haemolysis. To avoid potential interferences attributed to tonicity fluctuations, this variable was previously monitored when treatments were added and subsequently corrected with the solvent, if necessary.

Fluorescence emission anisotropy measurements. To determine cell membrane fluidity, DPH and TMA-DPH fluorescent probes were selected. To carry out the steady-state fluorescence anisotropy measurements of the probes in treated and untreated red blood cells, the erythrocyte suspensions (hematocrit of 0.01%) in PBS were labelled with the fluorescent dyes (final concentration in samples 10^{-6} M) at room temperature for 1 hour in dark conditions. Steady-state anisotropy measurements were carried out with an AB-2 spectrofluorometer SLM-Aminco using polarizers in the L configuration in a quartz cuvette under constant stirring at room temperature. Samples were lit with linearly (vertically or horizontally) polarized monochromatic light (λ_{ex} = 365 nm); and the fluorescence intensities (λ_{em} = 425 nm), emitted parallel or perpendicular to the direction of the excitation beam (slit-widths: 8 nm), were recorded. Fluorescence anisotropy (*r*) was calculated automatically by software provided with the instrument, according to:

$$r = (I_{vv} – I_{vh}G) / (I_{vv} + 2I_{vh}G),$$

where I_{vv} and I_{vh} represent the components of the light intensity emitted in parallel and in perpendicular, respectively, to the direction of the vertically polarized excitation light. A factor $G = I_{hv} / I_{hh}$ was used to correct the inequality of the detection beam to horizontally and vertically polarized emission (11).

Scanning electron microscopy (SEM). At the end of incubation, samples without previous wash were immersed in glutaraldehyde 5% in 0.1 mol/l phosphate buffer (pH 7.4, 4°C) for 1 hour and, after centrifuging, a solution of glutaraldehyde 2.5% (PBS, pH 7.4 0.1 M) was added for another hour. Then samples were washed in 0.1 mol/l phosphate buffer and post-fixed in OsO_4 (1%

in 0.1 M PBS, 4°C 1 h). Then cells were dehydrated in a graded series of ethanol. Finally, the samples were transferred into isoamyl acetate for critical point drying with liquid CO_2 and coated with gold. Specimens were examined in a Hitachi 2300 electron microscope, operating at 15 kV. Samples were processed and examined at the Serveis Cientificotècnics of the Universitat de Barcelona.

Erythrocyte ghost preparation. Human erythrocyte ghost membranes were prepared after treatments, following the procedure of Fairbanks et al. (12). The packed erythrocytes were haemolysed by hypotonic lysis and the pellet obtained by centrifugation subsequent to haemolysis was resuspended and washed several times until white ghost membranes were obtained.

SDS-PAGE. Membrane protein oxidation was evaluated by polyacrylamide electrophoresis (SDS-PAGE), following Fairbanks et al. (1971) (12). After treatment, the protein content of the erythrocyte ghost samples was measured, using a commercial kit (Bio-Rad) and BSA as a protein standard. 15 µg of extracted proteins were electrophoresed in parallel into a 7.5% SDS-polyacrylamide gel under reducing conditions. The proteins were viewed with Coomassie Blue staining. Densitometric analysis was performed using software developed in our laboratory. Actin (band 5) was used as the “internal” standard for quantitative calculations.

Statistical analysis*.* All experiments were run at least three times. Anisotropy fluorescence values were expressed as the mean ± standard error (SEM) of at least 3 independent experiments. Data were analyzed by one-way analysis of variance (ANOVA), followed by Dunnett’s *post hoc* test for multiple comparisons

between compounds in relation to the untreated and oxidant controls, all using the SPSS software (SPSS Inc., Chicago, IL, USA). Differences were considered significant for $p < 0.05$.

Results and discussion

There is increasing public awareness of the fact that natural resources are limited and of the need to rationalize their exploitation (5). Therefore, sustainability must be economically viable, apart from being environmentally advantageous. An interesting approach is the recovery of high added-value chemicals from residues and byproducts which still contain a variety of biologically active species. In this sense, grape pomace (skin, seeds, and stems) obtained after pressing in the wine industry, is a rich source of catechins, namely monomeric and oligomeric flavan-3-ols (proanthocyanidins), and glycosylated flavonols. These products may be used as starting materials for the preparation of novel compounds with antioxidant properties. Our laboratory adopted the strategy to obtain biobased antioxidant compounds by depolymerizing polymeric flavanols in the presence of cysteine (13). The new conjugates appeared to be promising products since they were more potent than their underivatized counterparts and they include ionic groups, which may be used to modulate their action within different physicochemical and biological environments (6). Therefore, agricultural byproducts evidence the suitability of using raw materials for the production of novel antioxidative compounds of possible relevance in biological, pharmacological, and nutritional fields.

In the present study, we investigated the interactions of epicatechin and epicatechin conjugates with erythrocyte membrane in order to clarify the

mechanisms of their antioxidant activity, and consequently strengthen the use of agricultural by-products as source of antioxidant material.

Because of their susceptibility to peroxidation, erythrocytes are used as a model to assess oxidative damage in biomembranes. Erythrocytes are considered a prime target for free radical attack due to the presence of high contents of polyunsaturated fatty acid in their membrane and their oxygen transport, which are potent promoters of reactive oxygen species (ROS). Exposure of erythrocytes to oxidative conditions results in successive free radical-mediated reactions that ultimately lead to cell lysis (14).

One of the important parameters relating to the structure and functional state of the cell membrane is membrane fluidity (11). Membrane-active flavonoids are believed to show antioxidant activity by rigidifying membranes (15). To determine whether membrane fluidity was modified by epicatechin and its conjugates in presence or absence of the oxidant agent, the fluorescent probes DPH and TMA-DPH were incorporated into the membranes of erythrocytes. DPH is a hydrophobic molecule that is incorporated into the region near the centre of the bilayer. Differences in the fluorescence polarization of this probe may reflect a direct effect on the motion of the lipid molecules in the core region of the bilayer (16). The TMA-DPH molecules are believed to accumulate and remain almost exclusively in the outer leaflet of the cell membrane, since their polar heads (trimethylammonium groups) are anchored at the lipid-water interface, while hydrocarbon moieties enter the lipid part of the membrane. Therefore, fluidity assessed by steady-state fluorescence with both probes

187 reveals the arrangement and mobility of membrane components in different
188 regions of the bilayer (17).

189 Figure 2 shows the anisotropy values for both probes. An increase in the
190 anisotropy parameter (r) of a probe is indicative of a decrease in the fluidity of
191 the membrane. The baseline fluorescence for TMA-DPH and DPH was 0.308 ±
192 0.012 and 0.306 ± 0.016, respectively. Epicatechin and its conjugates reduced
193 fluidity in the outer and inner parts of membranes, as shown by the increase in
194 anisotropy values for both probes. An exception should be noted in the case of
195 Cys-Ec, in which the anisotropy value increased but did not reach statistical
196 significance for DPH. In agreement with our previous study with liposomes and
197 thermal analysis (18), the current data suggests that epicatechin and its
198 derivatives distribute in the core of the bilayer, but also that they may interact
199 with its external part. This effect on membrane fluidity is important because, as
200 previously reported, changes in membrane fluidity can markedly affect the rate
201 of lipid oxidation (15,19). Previous studies suggest that the increase in
202 membrane rigidity hinders the diffusion of free radicals, reduces the kinetics of
203 oxidative reactions and thus inhibits lipid peroxidation (20).

204 The capacity of flavanols and procyanidins to protein-binding and interact with
205 the polar head groups of membrane phospholipids, suggests that these
206 compounds maintain membranes' integrity by preventing the access of
207 deleterious molecules to the hydrophobic region of the bilayer by accumulation
208 at the membranes' surface, both outside and inside the cells (15). In this way,
209 Arora et al., (20) described that genistein and other flavonoids and isoflavonoids
210 partitioned preferentially into the hydrophobic core of the model membrane,

where they modified the lipid packing order. Consequently, the increased membrane rigidity would result in inhibition of lipid peroxidation due to a slowdown of free radical reactions. In addition, a flavonoid-rich environment is created that could limit the access of oxidants to the bilayer and control the rate of propagation of free radical chain reactions occurring in the hydrophobic core of membrane (6).Therefore, among the antioxidant mechanisms that could explain the protective effects of epicatechin and its thio derivatives their ability to alter membrane fluidity besides to their membrane location should be taken into account.

Interestingly, the presence of AAPH also increased anisotropy values, indicating a decrease in membrane fluidity and an effect of the oxidant agent on the lipid components of the bilayer. However, the effect of epicatechin and its derivatives on anisotropy values were not affected by the presence of AAPH. In AAPH-induced peroxidation, free radicals are formed in the solution and attack the membranes from the external medium. The lack of effect of AAPH in presence of the antioxidants, combined with the decrease in membrane fluidity that they produced, may suggest that the antioxidant activity of these flavonoids could also be explained by their capacity to prevent the access of free radicals to the bilayer, although contribution of radical trapping can be also considered.

The incorporation of antioxidants in ordered membrane lipid bilayers determines great disordering of acyl chains, increasing membrane fluidity. In contrast, an increase in membrane lipid packing is detected by the incorporation of flavonoids between the acyl chains of the phospholiplids in disordered lipid

bilayers, resulting in rigidifying of the membrane. The effects exerted by several antioxidants on membrane fluidity resemble those of cholesterol, suggesting a positive correlation between rigidifying effects of the antioxidant in membrane lipid bilayers and its antioxidant capacity (21).

Oxidative damage in cell membranes also leads to alterations in shape. The effect of AAPH and epicatechin and its derivatives on cell morphology was studied by scanning electron microscopy. Untreated erythrocytes appeared as typical biconcave shapes (Fig. 3a), while exposure to AAPH resulted in a significant change to echinocytic or acanthocytic shapes (Fig. 3b). Epicatechin and its conjugates did not restore normal erythrocyte morphology after AAPH treatment (Fig. 3d). On the contrary, erythrocytes incubated with epicatechin and its conjugates also showed abnormal shapes, mainly echinocytic ones, as observed in the case of Cys-EcG, confirming their interaction with the cell membrane (Fig. 3c). According to the bilayer-couple hypothesis, the changes induced by foreign molecules are due to differential expansions of the two leaflets of the plasma membrane (22). Echinocytic and acanthocytic shapes would appear when molecules are inserted in the outer leaflet, causing surface expansion. Therefore, our observation that epicatechin and its conjugates induced the formation of echinocytic and acanthocytic shapes probably indicates that the antioxidants studied are mainly located at the outer leaflet of the membrane.

Given the membrane fluidity changes and the induction of altered erythrocyte shapes due to AAPH and antioxidant products, the next step was to analyze membrane proteins from erythrocytes. Erythrocytes exposed to oxidative stress

show altered transport capacity through the anion exchange band 3 protein. Peroxyl radicals derived from AAPH decrease erythrocyte anion transport capacity (23). Results of SDS-PAGE electrophoresis of erythrocyte ghosts are shown in Figure 3. The well-established normal distribution of the major membrane cytoskeletal proteins is shown in lane 1, which contains untreated erythrocyte ghosts. AAPH treatment produced changes in the protein pattern, leading to a remarkable protein loss of band-3 proteins, as seen in lane 2, confirming previous results from other authors (24,25). However, the antioxidants did not alter the protein pattern (lanes 4, 6 and 8), as has been recently observed for epicatechin (26). Moreover, epicatechin and epicatechin conjugates protected proteins from AAPH oxidative insult, as band 3 is fully recovered (lanes 5, 7 and 9). These results are confirmed by densitometry analysis, as shown in Table 2. After AAPH treatment, the amount of band-3 proteins dropped to 33% of that of untreated ghosts, but the co-treatment of erythrocytes with AAPH and the flavanols avoid the effects of the oxidant agent on that protein. These data suggest that antioxidants prevent the access of peroxyl radicals to band-3 proteins located in the lipid bilayer.

It has been reported that epicatechin conjugates with sulfur-containing moieties are strong free radical scavengers with cell-protecting activities, which may be in part modulated by their capacity to bind to biological membranes (18). Moreover, interaction of the conjugates with model membranes pointed out that the nonpolyphenolic moiety significantly influenced the membrane behavior of the whole molecules. We have previously demonstrated that Ec derivatives are better antioxidants against AAPH-induced hemolysis (7) than the former

283 compound, but only a direct relationship between the protection against lipid
284 peroxidation and the degree of galloylation of compounds was proven (7). In
285 this sense, distinctive membrane interaction was expected due to the presence
286 of both the cysteinyl group and galloil acid. However, no impact was registered
287 here not for membrane fluidity, morphology and/or protein profile that could be
288 related with differences emerging from their antioxidant protective effect both in
289 front of AAPH and H2O2 (6,7).

290

291 In summary, our results demonstrate that epicatechin and its conjugates spread
292 out in the core of the bilayer, but might also interact with its external part. They
293 stabilize the membrane through a decrease in lipid fluidity blocking the access
294 of the peroxyl radical to erythrocyte membranes, which may contribute to their
295 ability to inhibit oxidative haemolysis. These observations may enhance our
296 understanding of how these substances develop their antioxidant protective
297 activity over biological membranes. Taken together with our previously reported
298 data (6,7), which showed that these epicatechin derivatives are safe for normal
299 cells, this study reinforces the notion that the use of agricultural wastes as a
300 source of high value-added products confers potential health benefits.

301

302 **Acknowledgement**

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References

1. Rice-Evans, C.A.; Miller, N.J.; Paganga, G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic. Biol. Med*. **1996**, 20, 933-956.

2. Proteggente, A.R,; Pannala, A.S.; Paganga, G.; Van Buren, L.; Wagner, E.; Wiseman, S.; Van De Put, F.; Dacombe, C.; Rice-Evans, C.A. The antioxidant activity of regularly consumed fruit and vegetables reflects their phenolic and vitamin C composition. *Free Radic Res*. **2002**, 36, 217-33.

3. Rice-Evans, C.A. Implications of the mechanisms of action of tea polyphenols as antioxidants in vitro for chemoprevention in humans. *Proc. Soc. Exp. Biol. Med*. **1999**, 220, 262-266.

4. Salah, N.; Miller, N.J.; Paganga, G.; Tijburg, L.; Bolwell, G.P.; Rice-Evans, C. Polyphenolic flavanols as scavengers of aqueous phase radicals and as chain-breaking antioxidants. *Arch. Biochem. Biophys*. **1995,** 322, 339-346.

5. Torres, J.L.; Bobet, R. New flavanol-derivatives from grape (*Vitis vinifera*) byproducts. Antioxidant aminothylthiol-flavan-3-ol conjugates from a polymeric waste fraction used as a source of flavanols. *J Agric Food Chem*. **2001**, 49, 4627-4634.

6. Ugartondo, V.; Mitjans, M.; Torres, J.L.; Vinardell, M.P. Biobased epicatechin conjugates protect erythrocytes and nontumoral cell lines

from H2O2-induced oxidative stress. *J Agric Food Chem.* **2009**, 57, 4459-4465.

7. Ugartondo, V.; Mitjans, M.; Lozano, C.; Torres, J.L.; Vinardell, M.P. Comparative study of the cytotoxicity induced by antioxidant epicatechin conjugates obtained from grape. *J. Agric. Food Chem*. **2006**, 54, 6945-6950.

8. Beckman, K.B.; Ames, B.N. The free radical theory of aging matures. *Physiol. Rev*. **1998**, 78,547-581.

9. Sato, Y.; Sato, K.; Suzuki, Y. Mechanism of free radical-induced hemolysis of human erythrocytes: comparison of calculated rate constants for hemolysis with experimental rate constants. A*rch. Biochem. Biophys*. **1999**, 366, 61-69.

10. Lozano C.; Julià, Ll.; Jimenez, A.; Touriño, S.; Centelles, J.J.; Cascante, M.; Torres, J.L. Electron-transfer capacity of catechin derivatives and influence on the cell cycle and apoptosis in HT29 cells. *FEBS J*. **2006**, 273, 2475-2486.

11. Shinitzky, M.; Barenholz, Y. Fluidity parameters of lipid regions determined by fluorescence polarization. *Biochim. Biophys. Acta*. **1978**, 515, 367-394.

12. Fairbanks, G.; Theodore, L.; Steck, S.; Wallach, D.F.H. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry*. **1971**, 10, 2606-2617.

13. Torres, J. L.; Lozano, C.; Juliá, L.; Sánchez, F. J.; Anglada, J.; Centelles, J. J.; Cascante, M. Cysteinyl-flavan-3-ol conjugates from grape procyanidins. Antioxidant and antiproliferative properties. *Bioorg. Med. Chem.* **2002**, 10, 2497-2509.

14. Srour, M.A.; Bilto, Y.Y.; Juma, M.; Irhimeh, M.R. Exposure of human erythrocytes to oxygen radicals causes loss of deformability, increased osmotic fragility, lipid peroxidation and protein degradation. *Clin. Hemorheol. Microcirc*. **2000**, 23, 13-21.

15. Verstraeten, S.V.; Keen, C.L.; Golub, M.S.; Oteiza, P.I. Membrane composition can influence the rate of Al3+-mediated lipid oxidation: effect of galactolipids. *Biochem. J.* **1998**, 333, 833-838.

16. Kaiser, R.D.; London, E. Location of diphenylhexatriene (DPH) and its derivatives within membranes: comparison of different fluorescence quenching analyses of membrane depth. *Biochemistry*. **1998**, 37, 8180–8190.

17. Mély-Goubert, B.; Freedman, M.H. Lipid fluidity and membrane protein monitoring using 1,6-diphenyl-1,3,5-hexatriene. *Biochim. Biophys. Acta.***1980**, 601, 315–317.

18. Lázaro, E.; Castillo, J. A.; Ràfols, C.; Rosés, M.; Clapés, P.; Torres, J. L. Interaction of Antioxidant Biobased Epicatechin Conjugates with Biomembrane Models. *J. Agric. Food Chem.* **2007**, 55, 2901-2905.

19. Schnitzer, E.; Pinchuk, I.; Lichtenberg, D. Peroxidation of liposomal lipids. *Eur. Biophys. J.* **2007**, 36, 499-515.

20. Arora, A.; Byrem, T.M.; Nair, M.G.; Strasburg, G.M. Modulation of liposomal membrane fluidity by flavonoids and isoflavonoids. *Arch. Biochem. Biophys*. **2000**, 373, 102-109.

21. Tsuchiya, H. Structure-dependent membrane interaction of flavonoids associated with their bioactivity. *Food. Chem.* **2010**, 120, 1089-1096.

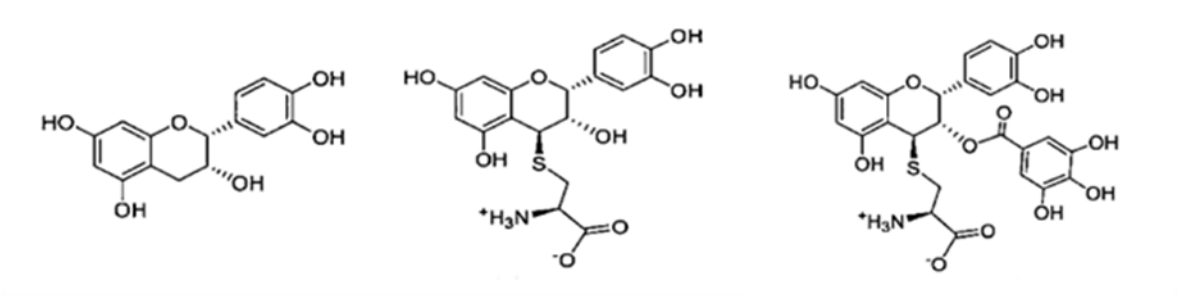
22. Lim, H.W.G; Wortis, M.; Mukhopadhyay, R. Stomatocyte-discocyte-echinocyte sequence of the human red blood cell: evidence for the bilayer- couple hypothesis from membrane mechanics. *Proc. Natl. Acad. Sci. USA.* **2002**, 99, 16766-16769.

23. Celedón, G.; González, G.; Ferrer, V.; Lissi, E.A. Peroxyl-oxidized erythrocyte membrane band 3 protein with anion transport capacity is degraded by membrane-bound proteinase. *Free Rad. Res.* **2004**, 38, 1055-1059.

24. Celedón, G.; González, G.; Lissi, E.A.; Hidalgo, G. Free radical-induced protein degradation of erythrocyte membrane is influenced by the localization of radical generation. *IUBMB Life.* **2001**, 51, 377-380.

25. Celedon, G.; Gonzalez, G.;Pino, J.; Lissi, E.A. Peroxynitrite oxidizes erythrocyte membrane band 3 protein and disminishes its anion transport capacity. *Free Rad. Res.* **2007,** 41, 316-323.

26. Chen, R.; Wang, J.B.; Zhang, X.Q.; Ren, J.; Zeng, C.M. Green tea polyphenol epigallocatechin-3-gallate (EGCG) induced intermolecular cross-linking of membrane proteins. *Arch. Biochem. Biophys.* **2011**, 507, 343-349.



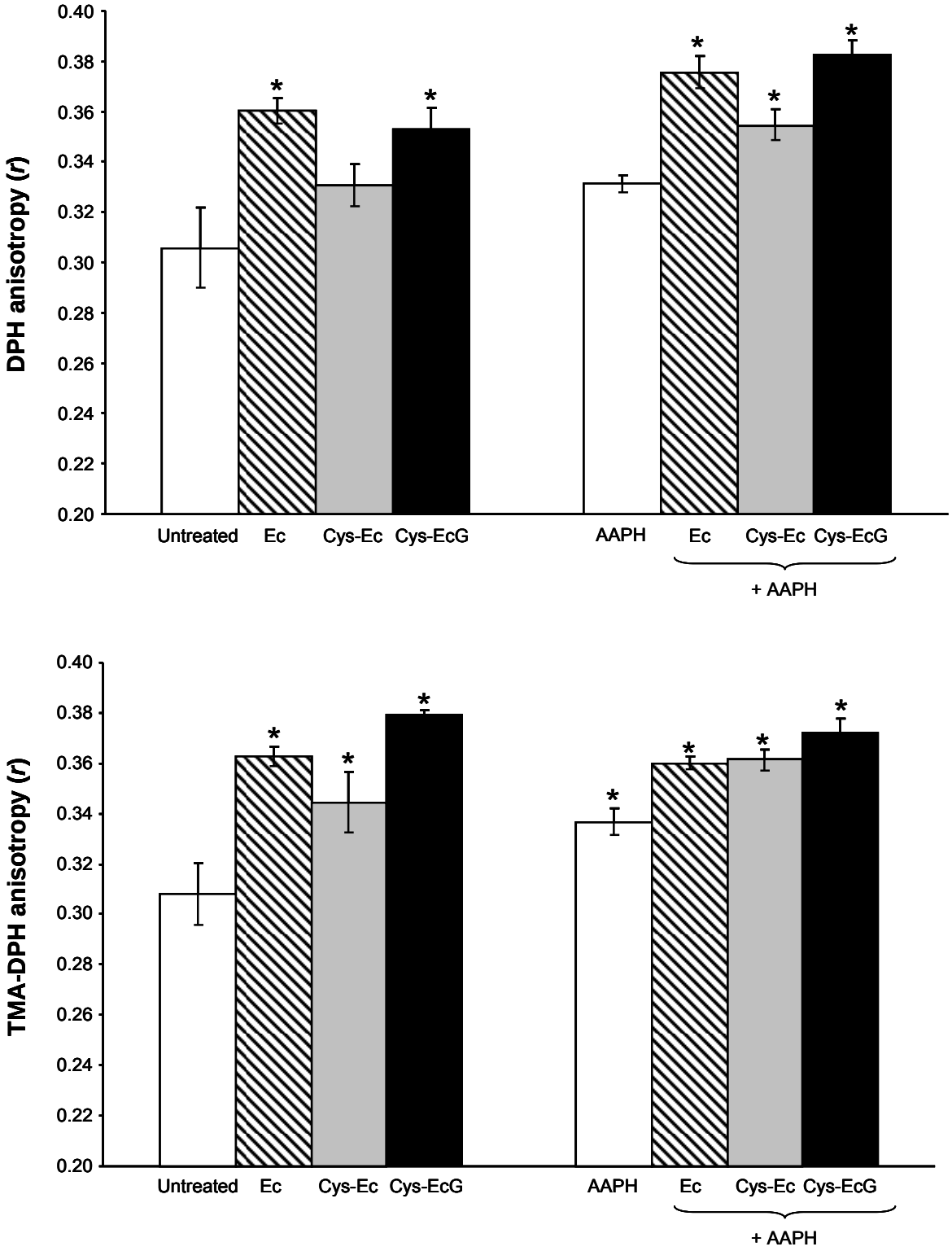
394

395

396 **Figure 1**. Chemical structure of epicatechin and cysteinyl conjugates. (1)

397 epicatechin; (2) 4β-(*S*-cysteinyl)epicatechin; (3) 4β-(*S*-cysteinyl)epicatechin 3-

398 *O*-gallate.



399

400 **Figure 2**. Steady-state anisotropy of the fluorescence probes, DPH and TMA-
401 DPH, incorporated into erythrocyte membranes. Results are expressed as

mean ± SEM of at least three independent experiments. Anisotropy measurements are represented by *r* values. *Significantly different when compared to values obtained for untreated cells (Dunnett's *post hoc* test, $p<0.05$).

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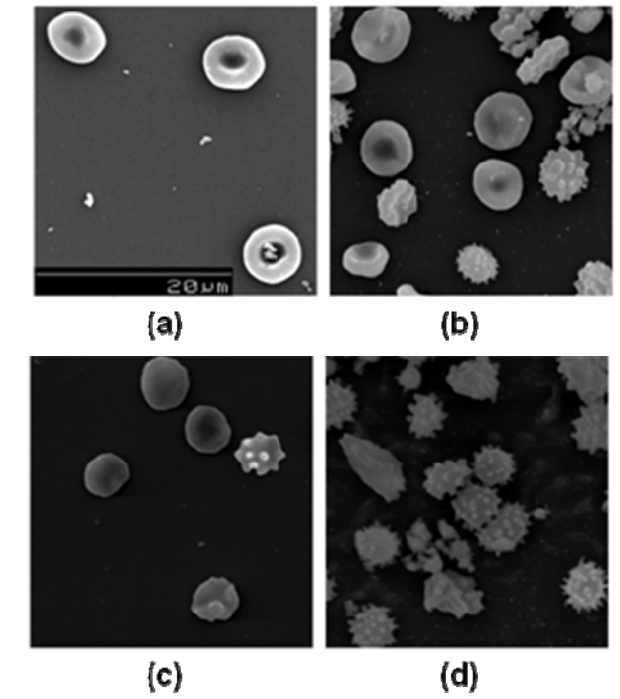

407

408 **Figure 3.** Effects of AAPH and Cys-epicatechin on the morphology of human
409 erythrocytes. Human erythrocytes observed by SEM: (a) untreated erythrocytes,
410 (b) erythrocytes treated with 100 mM of AAPH, (c) erythrocytes treated with 75
411 µM of Cys-EcG, (d) erythrocytes treated with 100 mM of AAPH and 75 µM of
412 Cys-EcG.

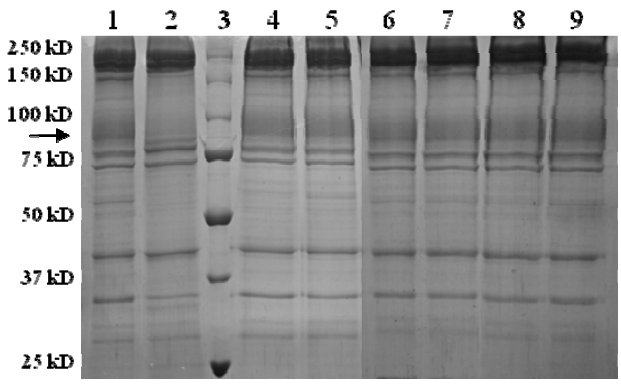

Figure 4. Effect of epicatechin and its conjugates and AAPPH in human erythrocyte membrane proteins analyzed using SDS-PAGE. Lane 1: untreated erythrocytes; lane 2: erythrocytes treated with 100 mM of AAPH; lane 3: molecular weight marker; lane 4: erythrocytes treated with 75 μM of Cys-EcG; lane 5: treated with 100 mM of AAPH and 75 μM of Cys-EcG; lane 6: erythrocytes treated with 75 μM of Ec; lane 7: erythrocytes treated with 100 mM of AAPH and 75 μM of Ec; lane 8: erythrocytes treated with 75 μM of Cys-Ec; lane 9: erythrocytes treated with 100 mM of AAPH and 75 μM of Cys-Ec. The arrow shows the location of band-3 proteins.

Table 1. Effect of AAPH and epicatechin and its derivatives on band-3 protein of erythrocyte membranes. The amount of band-3 protein in the SDS-PAGE gel was determined by densitometry and the percentage was calculated from the amount of control cells (untreated erythrocytes), using actin as the "internal standard".

Condition	Band 3*
Ec	114.8
Cys-Ec	144.1
Cys-EcG	123.9
AAPH	33.4
AAPH + Ec	95.9
AAPH + Cys-Ec	104.5
AAPH + Cys-EcG	137.9

***** Expressed as percentage of untreated

430 TOC

431