

## **Grape epicatechin conjugates prevent erythrocyte membrane protein oxidation**

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1 **Abstract**

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

19

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

## 22 Introduction

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

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

45 peroxidation and protein oxidation, including the redistribution of oxidized band-  
46 3 proteins to form haemolytic holes.

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

## 58 **Material and Methods**

59 **Chemicals.** Conjugates were prepared by acidic depolymerisation of  
60 proanthocyanidins obtained from grape (*Vitis Vinifera*) pomace and from hazel  
61 (*Hamamelis virginiana*) bark, essentially as described in Torres and Bobet  
62 (2001) (5) and Lozano et al. (2006) (10). We aimed to generate bio-based  
63 antioxidants with modified physicochemical and biological properties. The  
64 following compounds were studied: (-)-epicatechin (**1**), 4 $\beta$ -(S-  
65 cysteinyl)epicatechin (**2**) and 4 $\beta$ -(S-cysteinyl)epicatechin 3-O-gallate (**3**) (Figure  
66 1). 2,2'-Azobis(amidinopropane) dihydrochloride (AAPH) was purchased from  
67 Sigma (St. Louis, MO). Fluorescent probes DPH (1,6-diphenyl-1,3,5-hexatriene)  
68 and TMA-DPH (1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,4-hexatriene p-

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

75 **Blood Samples and Preparation of Red Blood Cells.** Blood samples were  
76 obtained from healthy donors by venipuncture (Tissue and Blood Bank of  
77 Hospital Vall d'Hebron, Barcelona, Spain), following the ethical guidelines of the  
78 hospital, and were collected in citrated tubes. Red blood cells (RBCs) were  
79 separated from the plasma and buffy coat by centrifugation at 1,000 g for 10  
80 min. The erythrocyte layer was washed three times in phosphate-buffered  
81 isotonic saline (PBS) containing 22.2 mM  $\text{Na}_2\text{HPO}_4$ , 5.6 mM  $\text{KH}_2\text{PO}_4$ , 123.3  
82 mM NaCl, and 10.0 mM glucose in distilled water (pH 7.4). The cells were then  
83 suspended in an isotonic saline solution at a density of  $8 \times 10^9$  cells/ml.

84 **Erythrocyte treatments.** Aliquots of 250  $\mu\text{l}$  of the red cell suspension were  
85 treated with AAPH (100 mM, 90 minutes) in constant agitation at 37°C. Samples  
86 were also studied in presence of 75  $\mu\text{M}$  of Ec and cysteinyl conjugates in  
87 presence and absence of the oxidant product. Untreated controls were included  
88 in all experiments to monitor spontaneous haemolysis. To avoid potential  
89 interferences attributed to tonicity fluctuations, this variable was previously  
90 monitored when treatments were added and subsequently corrected with the  
91 solvent, if necessary.

92 **Fluorescence emission anisotropy measurements.** To determine cell  
93 membrane fluidity, DPH and TMA-DPH fluorescent probes were selected. To  
94 carry out the steady-state fluorescence anisotropy measurements of the probes  
95 in treated and untreated red blood cells, the erythrocyte suspensions  
96 (hematocrit of 0.01%) in PBS were labelled with the fluorescent dyes (final  
97 concentration in samples  $10^{-6}$  M) at room temperature for 1 hour in dark  
98 conditions. Steady-state anisotropy measurements were carried out with an AB-  
99 2 spectrofluorometer SLM-Aminco using polarizers in the L configuration in a  
100 quartz cuvette under constant stirring at room temperature. Samples were lit  
101 with linearly (vertically or horizontally) polarized monochromatic light ( $\lambda_{ex} = 365$   
102 nm); and the fluorescence intensities ( $\lambda_{em} = 425$  nm), emitted parallel or  
103 perpendicular to the direction of the excitation beam (slit-widths: 8 nm), were  
104 recorded. Fluorescence anisotropy ( $r$ ) was calculated automatically by software  
105 provided with the instrument, according to:

$$106 \quad r = ( I_{vv} - I_{vh}G ) / ( I_{vv} + 2I_{vh}G ),$$

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

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

116 in 0.1 M PBS, 4°C 1 h). Then cells were dehydrated in a graded series of  
117 ethanol. Finally, the samples were transferred into isoamyl acetate for critical  
118 point drying with liquid CO<sub>2</sub> and coated with gold. Specimens were examined in  
119 a Hitachi 2300 electron microscope, operating at 15 kV. Samples were  
120 processed and examined at the Serveis Científicotècnics of the Universitat de  
121 Barcelona.

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

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

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

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

### 143 **Results and discussion**

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

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



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

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

173 One of the important parameters relating to the structure and functional state of  
174 the cell membrane is membrane fluidity (11). Membrane-active flavonoids are  
175 believed to show antioxidant activity by rigidifying membranes (15). To  
176 determine whether membrane fluidity was modified by epicatechin and its  
177 conjugates in presence or absence of the oxidant agent, the fluorescent probes  
178 DPH and TMA-DPH were incorporated into the membranes of erythrocytes.  
179 DPH is a hydrophobic molecule that is incorporated into the region near the  
180 centre of the bilayer. Differences in the fluorescence polarization of this probe  
181 may reflect a direct effect on the motion of the lipid molecules in the core region  
182 of the bilayer (16). The TMA-DPH molecules are believed to accumulate and  
183 remain almost exclusively in the outer leaflet of the cell membrane, since their  
184 polar heads (trimethylammonium groups) are anchored at the lipid-water  
185 interface, while hydrocarbon moieties enter the lipid part of the membrane.  
186 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  $0.308 \pm$   
192  $0.012$  and  $0.306 \pm 0.016$ , 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,

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

220

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

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

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

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

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

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

276 It has been reported that epicatechin conjugates with sulfur-containing moieties  
277 are strong free radical scavengers with cell-protecting activities, which may be  
278 in part modulated by their capacity to bind to biological membranes (18).  
279 Moreover, interaction of the conjugates with model membranes pointed out that  
280 the nonpolyphenolic moiety significantly influenced the membrane behavior of  
281 the whole molecules. We have previously demonstrated that Ec derivatives are  
282 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 H<sub>2</sub>O<sub>2</sub> (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 peroxy 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**

303 Fund AGL 2006-12210-C03-02/ALI, MCT (Spain).

304 **References**

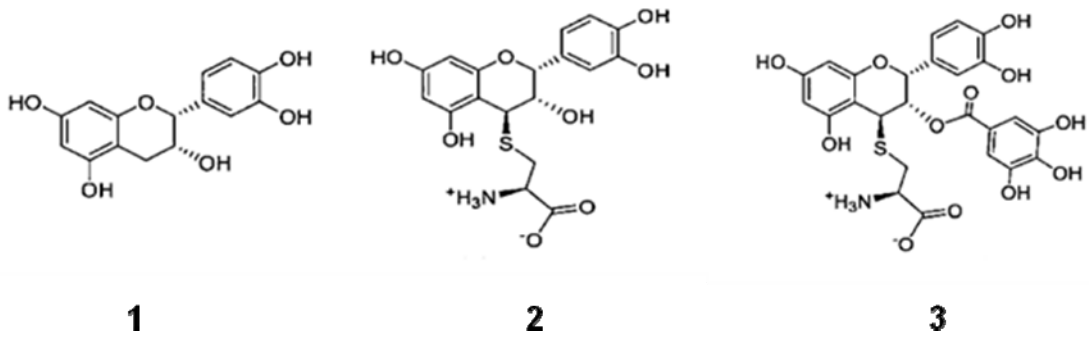
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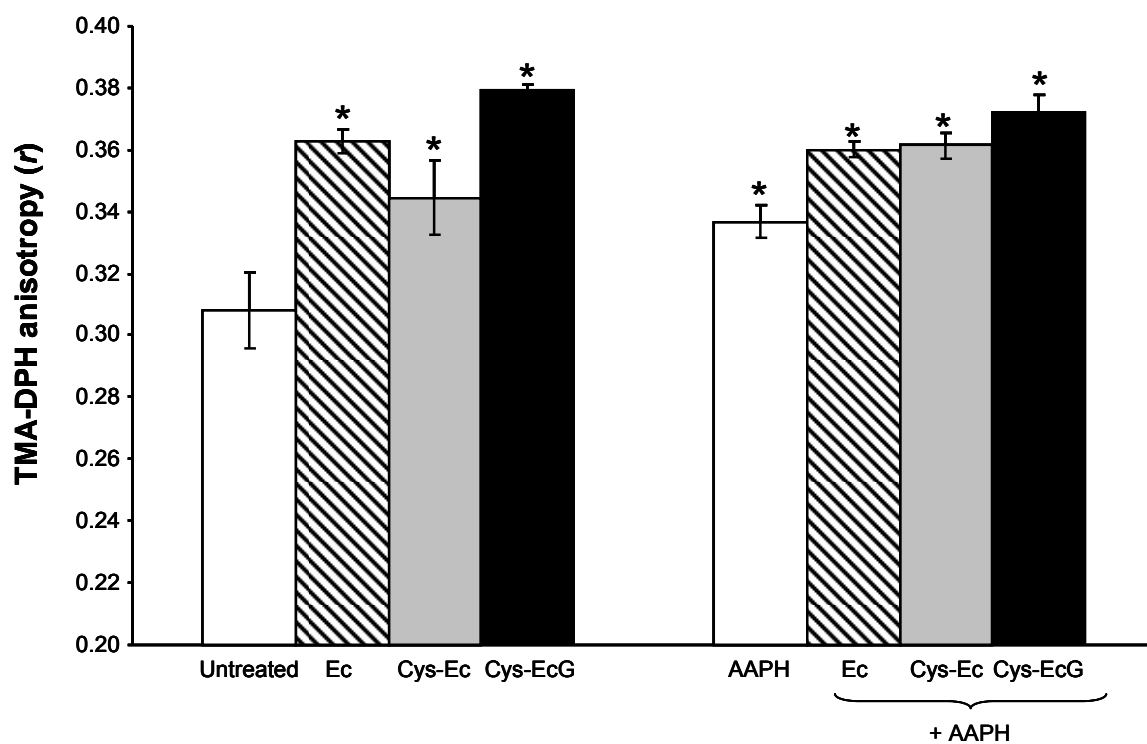
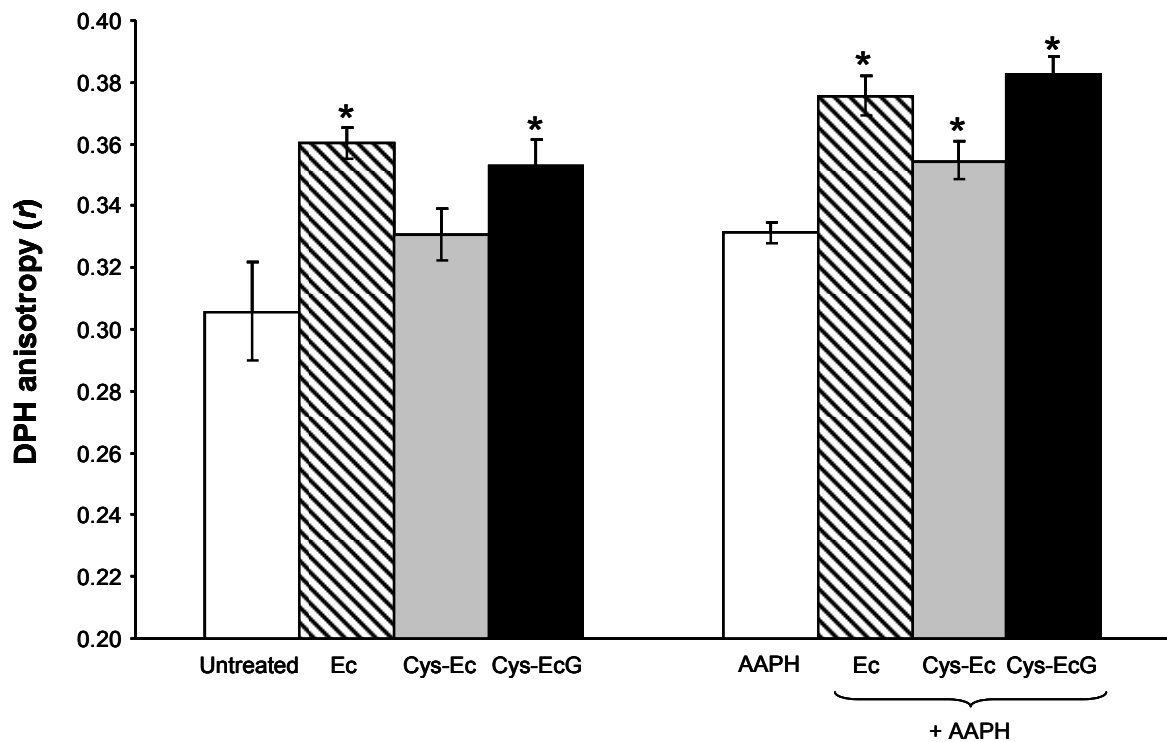
394

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-

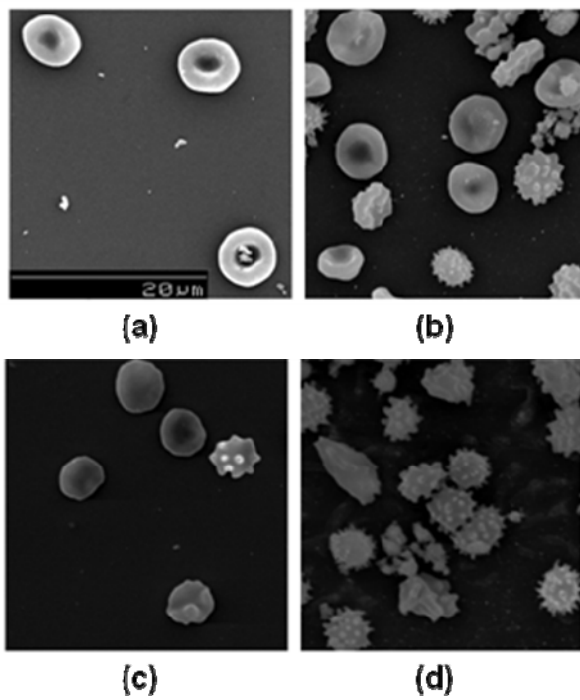
398 O-gallate.



399

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

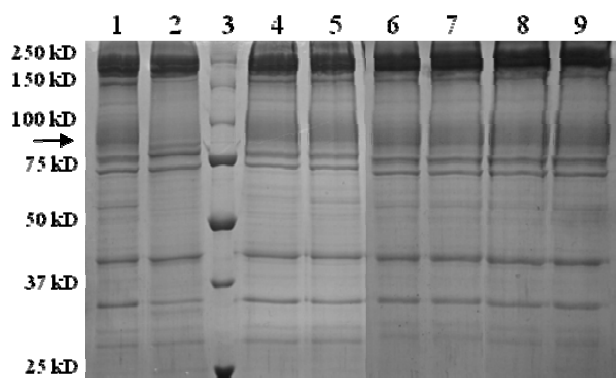
402 mean  $\pm$  SEM of at least three independent experiments. Anisotropy  
403 measurements are represented by  $r$  values. \*Significantly different when  
404 compared to values obtained for untreated cells (Dunnett's *post hoc* test,  
405  $p < 0.05$ ).



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.

413



414

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

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

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

429

\* Expressed as percentage of untreated



430 TOC

431

