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Biobased Epicatechin Conjugates Protect Erythrocytes and Nontumoral Cell Lines from H_2O_2-Induced Oxidative Stress

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This paper reports the study of the protective action of epicatechin and epicatechin derivatives, obtained by depolymerizing polymeric flavanols in the presence of cysteine or cysteamine, on red blood cells (RBC) and nontumoral cell lines challenged by exogenous H_2O_2. The epicatechin derivatives showed more effective antioxidant properties than epicatechin. Among them, 4β-(2-aminoethylthio)epicatechin 3-*O*-gallate showed the highest antioxidant activity against three markers of oxidative stress: hemolysis, lipid peroxidation, and cytotoxicity. Furthermore, as this compound lacks the pyrogallol group on the condensed flavanic structure, it might be safer than other potent gallocatechin-type polyphenols. These findings indicate that these epicatechin derivatives, which are byproducts of the agro-food industry show potential for application in the food and drug industries.

KEYWORDS: Catechins; antioxidants; polyphenols; lipid peroxidation

INTRODUCTION

Natural resources are limited, and there is currently increasing interest in the use of renewable sources and the integral exploitation of the raw materials. The recovery of high added value chemicals from residues and byproducts is a promising approach to sustainable development (*1*). Crops, such as grapes, olives, and citrus fruits, generate large amounts of byproducts that are rich sources of health-promoting phytochemicals, including carotenoids and polyphenolic compounds. A great deal of research effort is now being devoted to testing the putative benefits on health of new products obtained from agricultural byproducts (*2*).

Flavonoids, the most widely occurring group of phenolic phytochemicals, are present in fruits, vegetables, and food products and beverages derived from plants (*3*). They are diphenylpropane derivatives with a common structure consisting of two aromatic rings linked through three carbons ($C_6C_3C_6$); several families have been described on the basis of structural variations within the rings: flavonols, flavones, flavanols, isoflavones, anthocyanidins, and others. All of these families have ideal structural chemistry for free radical scavenging activities (*4*).

Grape pomace (skin, seeds, and stems) obtained after pressing in the wine industry is a good source of flavonoids, specifically of the flavanol group formed by catechins and proanthocyanidins (monomeric and oligomeric flavan-3-ols, respectively). Catechins have powerful antioxidant properties and can be protective against cancer and inflammatory and cardiovascular diseases (*5*, *6*). These compounds may exert their beneficial action by a combination of prophylactic and therapeutic effects related to both their radical scavenging capacity and their influence on the cell machinery by modulating the activity of a wide range of enzymes and cell receptors (*7*, *8*). Therefore, these grape pomace-derived catechins are suitable raw materials for the production of novel antioxidative compounds of possible relevance in biological, pharmacological, and nutritional fields.

Current interest in the recovery of high added value compounds derived from agricultural wastes previously led our laboratory to obtain biobased antioxidant compounds by depolymerizing polymeric flavanols in the presence of cysteine and cysteamine (*1*, *9*). Using diverse in vitro assay systems, we demonstrated their radical scavenging and antioxidant activity (*1*, *8*−*10*), cytotoxicity on nontumoral 3T3 fibroblasts and human HaCaT keratinocytes (*10*), and neuroprotective (*11*, *12*) and antiinflammatory properties (*13*, *14*); we also evaluated their role in cell cycle regulation by characterizing their antiproliferative and pro-apoptotic activity on diverse skin and colon tumoral cell lines (*8*, *15*). However, it must be noted that the significance and relevance of antioxidant evaluation depend strongly on the test method applied. Therefore, for the judicious choice of antioxidant compounds, here we evaluated the properties of epicatechin derivatives using a battery of assays to analyze the structure−activity relationships, particularly the effect of gallate and nonphenolic moieties on their antioxidant behavior. For this purpose, we (1) induced oxidative stress by H_2O_2 in intact human erythrocytes and in nontumoral cell cultures; (2) analyzed the markers of oxidative stress, namely, hemolysis, lipid peroxidation, and cytotoxicity; and (3) tested the protective capacity of the epicatechin conjugates against oxidative stress.

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MATERIALS AND METHODS

Chemicals. The thio-conjugates were synthesized from grapes and witch hazel following a previously published methodology (*1*, *8*). These compounds were obtained by depolymerizing polymeric flavanols (proanthocyanidins) in the presence of cysteamine or cysteine. The resulting conjugates were purified by ion-exchange and/or reverse-phase high-resolution chromatography. The compounds studied were (−)-epicatechin (**1**), 4β-(*S*-cysteinyl)epicatechin (**2**), 4β-(2-aminoethylthiol)epicatechin (**3**), 4β-(*S*-cysteinyl)epicatechin 3-*O*-gallate (**4**), 4β-(2-aminoethylthiol)epicatechin 3-*O*-gallate (**5**), 4β-(*S*-cysteinyl)epigallocatechin 3-*O*-gallate (**6**), 4β-(2-aminoethylthiol)epigallocatechin 3-*O*-gallate (**7**), and 4β-[*S*-(*O*-ethylcysteinyl)]epicatechin (**8**). The structures of the molecules studied are shown in **Figure 1**.

Hydrogen peroxide 30% (w/w) solution, sodium azide, and 2-thiobarbituric acid were purchased from Sigma (St. Louis, MO). Extra pure trichloroacetic acid (TCA) solution 20% w/v was from Scharlau (Sentmenat, Spain).

Red Blood Cell Assays. *Preparation of Erythrocyte Suspension*. Human blood was obtained from the Blood Bank of the "Hospital Vall d'Hebrón" (Barcelona, Spain) following the ethical guidelines of the hospital. The erythrocytes were washed three times in a phosphate-buffered saline (PBS) containing 123.3 mM NaCl, 22.2 mM Na_2HPO_4, and 5.6 mM KH_2PO_4, in distilled water (pH 7.4; 300 mOsmol/L) to remove plasma, platelets, and leucocytes. Cells were then resuspended in isotonic saline solution to a final cell density of 8×10^9 cells/mL. Sodium azide at 2 mM in PBS was added to the cell suspension (12.5% of hematocrit at the assay conditions), which was preincubated for 15 min in continuous rotation to allow inactivation of erythrocyte catalase by the sodium azide.

Erythrocyte Hemolysis. Hemolysis induced by H_2O_2 was evaluated following the technique desbribed by Grinberg et al., with slight modifications (*16*). A 12.5% erythrocyte suspension (250 μL) was incubated for 90 min at 37 °C in a shaker in the presence of H_2O_2 at a final concentration of 20 mM, to achieve maximal hemolysis; this concentration was chosen on the basis of previous studies (*17*) and confirmed in our laboratory (data not shown). The same test was performed to detect the antihemolytic activity of epicatechin and related compounds. Concentrations ranging between 12.5 and 150 μM of the compounds dissolved in PBS were added to the erythrocyte suspension in the presence of 20 mM H_2O_2 at 37 °C for 90 min. RBC controls were included in all of the assays to detect spontaneous hemolysis in the absence of oxidant agent or products. After the incubation time, cells were centrifuged and hemolysis was determined spectrophotometrically at 540 nm (release of hemoglobin). The percentage of hemolysis was calculated by comparing the absorbance (540 nm) of the supernatant of the samples with that of a control sample totally hemolyzed with distilled water. The IC_{50} (50% inhibitory concentration) of the hemolysis induced by H_2O_2 was determined for the compunds.

Erythrocyte Lipid Peroxidation. The concentration of malondialdehyde (MDA), a secondary product of lipid peroxidation, was determined indirectly by spectrophotometric measurement of the formation of thiobarbituric acid reactive substances (TBARS), following the method described by Stocks and Dormandy in 1972 with slight modifications (*18*). This method is based on the extraction of MDA from erythrocyte suspension by TCA solution and the subsequent reaction of this MDA with thiobarbituric acid (TBA), to yield a pink complex with maximum absorption at 532 nm (TBARS). We induced lipid peroxidation by incubating erythrocytes under the same conditions as those of the hemolysis assay (RBC suspensions plus 20 mM H_2O_2 alone or with a range of concentrations of a given compound for 90 min at 37 °C). Following incubation, the RBC suspension was mixed with 1 mL of TCA solution 20% w/v to remove potentially interfering substances (*19*). Samples were then centrifuged, and 1 mL of supernatant was mixed with 1 mL of 1% of TBA. Finally, samples were heated to 90 °C for 50 min, cooled rapidly, and centrifuged. The absorbance of the supernatant was measured at 532 and 600 nm to exclude possible impurities. The appropriate blanks and controls were run along with the test samples. The degree of lipid peroxidation was expressed in arbitrary absorbance units. In addition, IC_{50} values were calculated as the antioxidant concentration required to inhibit 50% of TBARS formation.

Cell Cultures and Cytoprotection against H_2O_2-Induced Damage. *Culture of 3T3 and HaCaT Cell Lines*. The mouse embryonic fibroblast cell line 3T3 and the spontaneously immortalized human keratinocyte cell line HaCaT were obtained from the "Banco de Células Eucariotas", Barcelona (Spain). Cells were cultured in DMEM (4.5 g/L glucose) supplemented with a 10% fetal bovine serum, 2 mM L-glutamine, 10 mM Hepes buffer, and 1% penicillin (10.000 units/mL)−streptomycin (10.000 μg/mL) mixture and maintained in a humidified atmosphere at 37 °C and 5% CO_2. When cells were approximately 80% confluent, they were harvested with trypsin/EDTA and seeded at a density of 8.5×10^4 cells/mL and 10×10^4 cells/mL for 3T3 and HaCaT cells, respectively, in 96-well plates and then incubated for 24 h at 37 °C and 5% CO_2.

Experimental Treatments. To assess the protective effects of the epicatechin and its thiol derivatives against H_2O_2-mediated oxidative damage in nontumoral cell lines (3T3 and HaCaT cells), the cells were preincubated overnight (18−20 h) with increasing concentrations (from 25 to 300 μM) of the test compounds in DMEM 5% FBS, previously sterilized by filtration. After preincubation time, the compounds were removed and the medium was exchanged before H_2O_2 was added. This procedure prevented a direct reaction between the epicatechin derivatives and the oxidant source in the medium (*20*). Next, H_2O_2 was dissolved in DMEM 0% FBS and added at a final concentration of 2 mM to 3T3 cells and 2.5 mM to HaCaT; these concentrations were chosen on the basis of the results of previous dose and time course assays to obtain a decrease of cell viability of at least 60% in order to detect the potential protective capacity of the compounds (data not shown). Cells were incubated with the oxidant agent for 2 h. After this, medium was removed and cells were washed with PBS. Finally, cell cultures were analyzed for cell viability. Control wells containing cells only with basal medium, medium with H_2O_2, and medium with compounds were included in each plate.

Cell Viability Assay. Cell viability was determined by the Neutral Red Uptake (NRU) assay following the method of Borenfreund and Puerner (*21*) and modified to remove the use of formaldehyde (*22*). Following treatments, the medium was removed and Neutral Red solution (50 μg/mL in RPMI medium without phenol red and serum) was added (100 μL per well) followed by incubation for 3 h at 37 °C and 5% CO_2. Finally, medium was aspirated, cells were washed twice with PBS, and a solution containing 50% ethanol absolute−1% acetic acid in distilled water was added to extract the dye absorbed into the viable cells. Plates were shaken for 10 min on a microtiter plate shaker, and absorbance of Neutral Red was determined at 550 nm in a Bio-Rad microplate reader. Results were given as the percentage of viability compared with control cells (the mean optical density of untreated cells was set to 100% viability).

Statistical Analysis. Results were expressed as mean ± SE of at least three independent experiments. Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test for multicomparison between epicatechin derivatives with respect to the H_2O_2 controls or Scheffé post hoc test to compare results between derivatives, using SPSS software (SPSS Inc., Chicago, IL). A value of $p < 0.05$ was judged to be statistically significant.

RESULTS AND DISCUSSION

Previous studies by our group evaluated the antiradical, antitumoral, neuroprotective, and cytotoxic activities of several epicatechin thiol derivatives (*8*−*15*). Given that flavonoid activity varies depending on the biological system used and the oxidative agent or the damage against which they may afford protection, here we examined the antioxidant potential of these derivatives using other oxidative models, for example, H_2O_2-induced lipid peroxidation (LPO) and hemolysis on human erythrocytes and H_2O_2-induced cell cytotoxicity.

Antioxidant Activity of Epicatechin Derivatives against RBC Oxidative Damage. Erythrocytes are widely used for studying mechanisms of oxidative damage in biomembranes. Due to their abundance in polyunsaturated fatty acids and membrane proteins and their high cellular concentration of oxygen, iron, and hemoglobin, erythrocytes are highly susceptible to oxidative

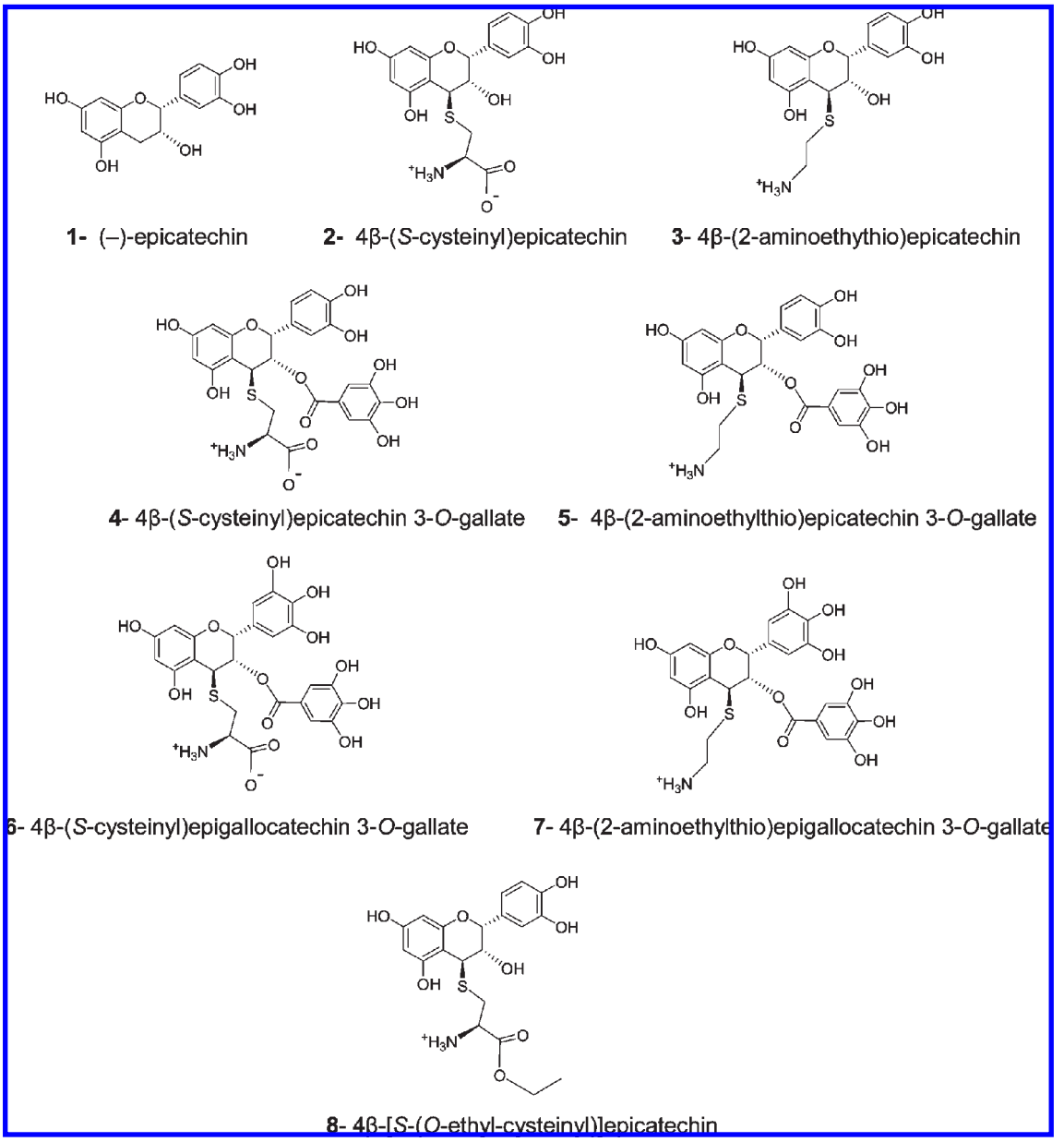

Figure 1. Chemical structures of epicatechin and epicatechin conjugates.

stress. Oxygen radical generating systems have been shown to cause lipid peroxidation, protein degradation, hemoglobin−spectrin cross-linking, glycosylation of proteins, inactivation of enzymes, membrane lipid bilayer perturbation, and hemolysis in RBCs (*19*). Hydrogen peroxide is considered to be a key reactive oxygen species (ROS) and an attractive oxidant model because of its relatively high stability, diffusion, and involvement in cell signaling cascades.

Here we examined the degree of lipoperoxidation in erythrocytes treated with 20 mM H_2O_2 by the formation of TBARS after reaction between MDA and TBA. Incubation of RBC with H_2O_2 caused a marked MDA production, thereby indicating oxidative injury of cell membranes (**Figure 2**). When epicatechin and derivatives were added to the solution, a different degree of protective capacity against H_2O_2-induced lipid damage was observed.

All of the compounds tested in the range of 50−150 μM significantly ($p < 0.05$) protect against H_2O_2TBARs formation, except compounds **1** and **2**, which did not demonstrate effect at any tested concentrations. Compound **5** also showed significant protection at the lowest concentrations (12.5 and 25 μM). Galloylated compounds (**4**−**7**) showed the strongest protective effects (around 75% LPO inhibition at the highest concentrations tested), whereas non-gallate compounds were less efficient. Several authors have reported a direct relationship between the protection against lipid peroxidation and the degree of galloylation of compounds (*5*, *23*, *24*), which is consistent with our findings.

The non-phenolic part of the molecule also affected the protective activity of the conjugates. Cysteamine conjugates showed slightly better performance than cysteine ones, although differences were not significant. Compound **8** showed higher antioxidant inhibition of LPO than **2**, which is attributed to the ethyl ester group, as postulated elsewhere (*25*). Product **5** was the most effective inhibitor of the compounds tested at the lowest concentration (45% inhibition at 12.5 μM).

The antioxidant activity of the epicatechin conjugates was also tested by measuring their antihemolytic action in H_2O_2-stressed RBC. When H_2O_2 was added to an RBC suspension in our assay conditions, a great degree of hemolysis was observed (75%) (**Figure 3**). Epicatechin and the related compounds varied

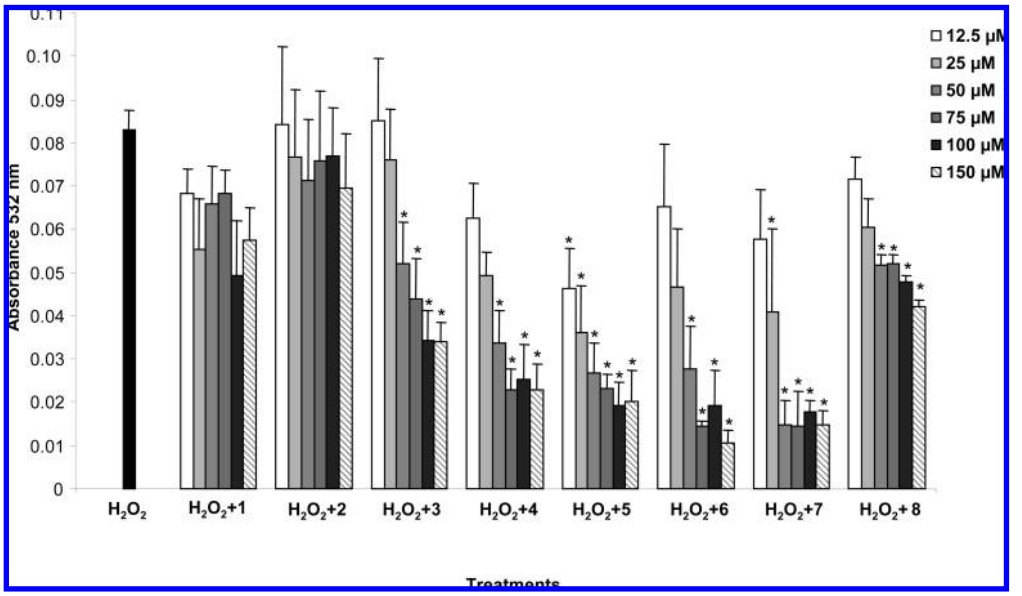

Figure 2. TBAR concentrations in erythrocytes treated with 20 mM H_2O_2 with or without epicatechin derivatives. The concentrations of the MDA−TBA complex are indicated as absorbance at 532 nm. Marked compounds are statistically different from the control with 20 mM H_2O_2 alone (∗, $p < 0.05$, denotes statistically significant differences).

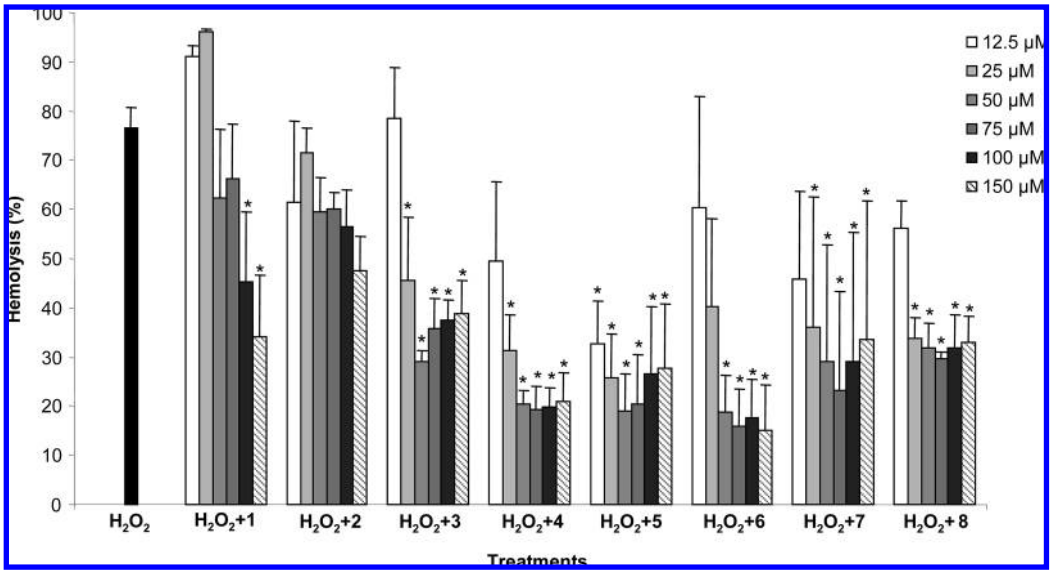

Figure 3. Effect of epicatechin derivatives on H_2O_2-induced hemolysis in erythrocytes treated with 20 mM H_2O_2 or H_2O_2 plus compounds at a range of concentrations. ∗ indicates significant difference from H_2O_2 group ($p < 0.05$).

in their capacity to limit the extent of hemolysis. The presence of cysteine and cysteamine increased the antihemolytic activity of epicatechin. Compounds with gallate groups showed the greatest protective action against RBC lysis; these data agree with those reported for LPO inhibition. It should be noted that compounds **2** and **8** showed better activity in antihemolitic assays than in lipoperoxidation inhibition and, again, compound **5** was the most efficient derivative, especially in the low concentration range tested.

We analyzed dose−response curves of lipid peroxidation and hemolysis assays and calculated the IC_{50} values (concentration inducing 50% inhibition of MDA production and 50% inhibition of hemolysis induced by H_2O_2). The four compounds containing 3-*O*-gallate (**4**−**7**) showed the lowest IC_{50} values, which corroborate the higher efficiency caused by the presence of this moiety. We obtained a strong correlation ($r = 0.972$) between the IC_{50} values for both assays (**Figure 4**). This finding implies that the compounds have a similar effectiveness in the two antioxidant assays. However, IC_{50} values for hemolysis were slightly lower than those for LPO.

The flavonol derivaties may have the capacity to prevent the passage of H_2O_2 through the erythrocyte membrane and its further reaction with hemoglobin, thus preventing damage and generation of ROS associated with hemolysis. However, these products are ineffective at the membrane level, as observed by the higher IC_{50} for LPO. The protective effects of these compounds have been attributed mostly to their antioxidative activities by scavenging free radicals and metal chelating. The tendency of bulky hydrophobic conjugates such as **8** to penetrate deeper into the membrane bilayer than small compounds such as **2** or **3** has been observed in previous studies (*26*). In addition, the introduction of a protected cysteine moiety facilitates the incorporation of the polyphenol into the lipid, and this may carry an improved capacity to protect membrane lipid from peroxidation.

The distribution of flavonoids in biological systems depends, among other factors, on their relative hydrophilicity/hydrophobicity

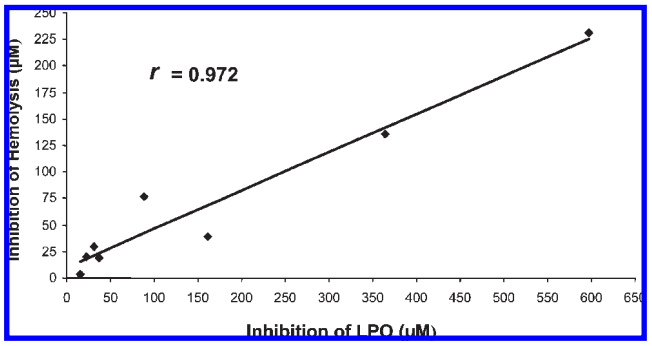

Figure 4. Correlation between the IC_{50} values for lipid peroxidation and the IC_{50} values for hemolysis (IC_{50} is the concentration of product that causes 50% inhibition of LPO or hemolysis). Each point represents one of the eight products tested.

and on their interactions with particular macromolecules (*27*). These factors will determine the local concentration of flavonoids, which will affect their capacity to regulate cellular events. Previous studies showed that (−)-epicatechin, (+)-catechin, and their related procyanidins adsorb to membranes through associations with the polar headgroups of phospholipids, thereby creating a flavonoid-rich environment. This enrichment 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. It has been proposed that the location of flavonoids in the membrane dictates their antioxidant capacity (*30*).

We observed that the presence of a gallate group increased the antioxidant activity, which was more significant in the case of compound **5**. Our results are consistent with other studies showing the role of the galloyl group in oxidative activity (*24*, *29*). The hydroxyl group on galloyl moieties contributes to the antioxidative and antiradical activity, making the compounds capable of not only donating more hydrogen atoms but also providing more chelating sites to scavenging catalytic cations (*5*, *8*, *33*). All of the compounds except **2** tended to be more active than epicatechin in protecting RBC against H_2O_2-induced hemolysis and, moreover, prevented peroxidative damage.

Antioxidant Protection against H_2O_2-Induced Cytotoxicity. Epicatechin conjugates show antiproliferative and apoptotic activities in several tumoral cells (*8*, *9*, *15*) and a low cytotoxic effect on nonmalignant cell lines (*10*). Here - we attempted to characterize the protective capacity of the epicatechin derivatives in nontumoral cells exposed to H_2O_2-induced damage. Several studies support the use of the mouse embryo fibroblast NIH 3T3 cell line as a sensitive cellular model for the evaluation of oxidative stress induced by H_2O_2 (*31*).

When 3T3 were exposed to 2 mM H_2O_2 for 2 h, cell viability decreased by around 80% (**Figure 5**). Overnight preincubation of cells with epicatechin (**1**) and its conjugates at concentrations ranging between 25 and 300 μM, before exposure to H_2O_2, resulted in an antioxidant-specific modulation of cell viability. An increase of cell viability indicates a protection against H_2O_2 cell cytotoxicity. Products **1**, **2**, **7**, and **8** did not show a statistically significant effect on cell viability, whereas product **3** showed the best performance at the highest concentration and a clear dose−response behavior. Compounds **5** and **6** showed more efficient protection at intermediate concentrations. The galloylated compounds lost effectiveness at the highest concentrations. This observation may be related to the previously reported pro-oxidant effect of phenolic compounds and, especially, to the gallate group content of molecules (*24*, *32*). Some of the highest concentrations assayed were found to be cytotoxic in our previous studies (*10*). This finding implied that it was not possible to totally inhibit H_2O_2-induced cell damage by increasing the concentration of galloylated compounds, because they may act as pro-apoptotic and pro-oxidants at high concentrations.

The cysteamine derivatives (**3** and **5**) were more effective than the corresponding cysteine derivatives (**2** and **4**), but this pattern was not observed in the compounds with a pyrogallol structure (**6** and **7**). Under certain conditions, flavonoids containing the pyrogallol structure in the B ring such as (−)-epigallocatechin and (−)-epigallocatechin-gallate, may participate in redox cycling via the production of the active superoxide radical anion ($O_2^{\bullet -}$) and subsequently hydrogen peroxide (*5*, *8*). The pro-oxidant action often attributed to flavanols is due to great extent to the pyrogallol group on ring B present in gallocatechins. In contrast, the gallate moiety, which has the trihydroxy structure coupled with the carbonyl group, may not produce superoxide because of the stability of its radical.

The activity of flavanols depends on the concentration tested, the oxidant agent used to cause oxidative damage, the incubation conditions, and the cell line assayed. We therefore considered it appropriate to test the protective activity of compounds in another cell model, the keratinocyte HaCaT cell line. Keratinocytes are the structural backbone of the epidermis. H_2O_2 produces oxygen radicals that cause oxidative damage directly to normal human keratinocytes, and there is increasing evidence that oxidative stress-induced cytotoxicity of keratinocytes participates in the pathogenesis of skin diseases (*33*). In 3T3 protective assays, we concluded that the most efficient derivatives were **5** and **6** because at intermediate concentration they showed significant biological antioxidant activity against ROS-induced damage. We therefore tested the protective capacity of these two epicatechin derivatives against H_2O_2-induced injury in HaCaT cells.

Exposure of HaCaT cells to H_2O_2 at 2.5 mM for 2 h led to a loss of approximately 55% cell viability (**Figure 6**). A dose-dependent increase in cell viability by the previous treatment with compounds **5** and **6** was observed, with significant diferences only at the highest concentrations. Cell viability increased around 1.6-fold in the presence of product **5** at 200 μM, and this compound was more effective than **6** at low concentrations. There was a slight loss of viability at the highest concentration tested. This result could be attributed to the presence of the gallate group, and these data are consistent with the findings of previous cytotoxic assays (*10*).

Distinct cell types differ in their sensitivity to oxidative damage (*34*), and it is tempting to speculate that this difference is linked to variation in endogenous levels of ROS protection. The 3T3 cell line is considered to be more sensitive to irritants and oxidizing agents than the HaCaT keratinocytes (*35*); however, the use of keratinocytes is recommended for certain purposes because in vivo they are the first cells to be exposed to exogenous agents (*36*, *37*).

We preincubated the cells with conjugates before H_2O_2 addition because preliminary assays with the flavanol epicatechin showed no protective effect when it was simultaneously incubated with the oxidant (data not shown). Other papers have emphasized this preincubation with polyphenolic compounds to achieve suitable protective effects in other cell models (*20*, *33*, *38*). The more marked protective effect reported when flavanol preincubation is carried out before H_2O_2 addition suggests that flavonoids exert a protective effect against cell

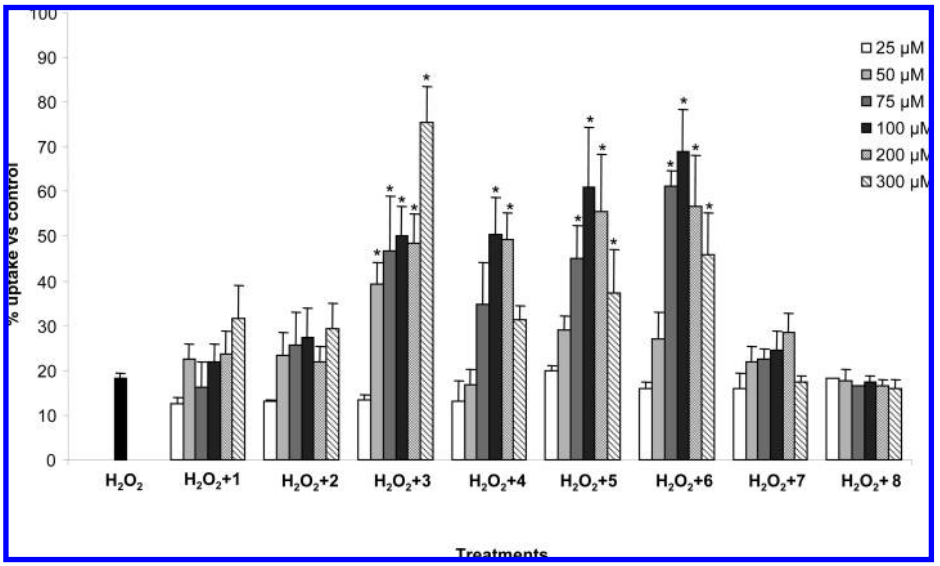

Figure 5. Comparative protective effect of epicatechin derivatives toward H_2O_2-induced cytotoxicity on 3T3 cells. Cells were preincubated overnight with the compounds, washed, and incubated with 2 mM H_2O_2 for 2 h. Cell viability is expressed as percentages of the untreated cells (100%). ∗, $p < 0.05$, denotes statistically significant differences with respect to 2 mM H_2O_2 control.

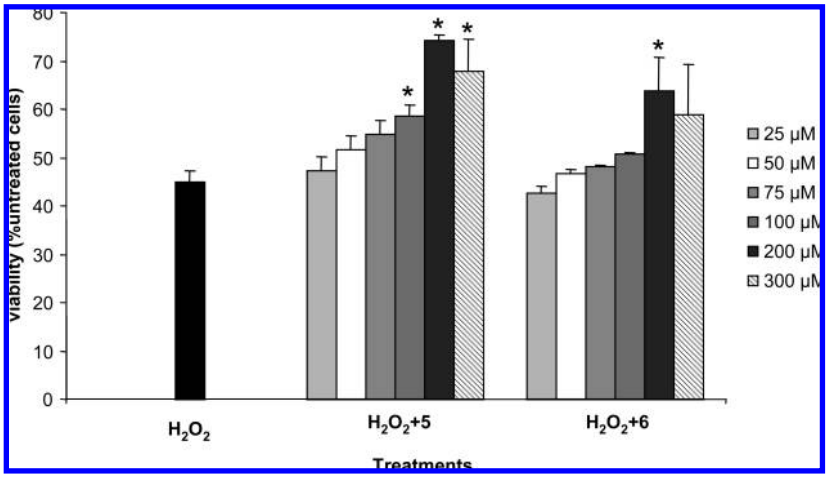

Figure 6. Comparative protective effect of compounds **5** and **6** against H_2O_2-induced cytotoxicity on HaCaT cells. Cells were preincubated overnight with the compounds, washed, and incubated with 2.5 mM H_2O_2 for 2 h. Cell viability is expressed as percentage of the untreated cells (100%). ∗, $p < 0.05$, denotes significant differences with respect to 2.5 mM H_2O_2 control.

damage by a mechanism other than the mere scavenging of ROS. Therefore, the preincubation with these compounds could enhance their antioxidant potential (*39*, *40*).

Compound **5** showed the highest effectiveness as antioxidant agent against the three markers of oxidative stress studied here, namely, hemolysis, lipid peroxidation, and cytotoxicity. Its effect may result from the combination of different antioxidant activities including direct free radical scavenging, enhancement of endogenous defense systems, metal chelation, and membrane fluidity. Furthermore, as this compound lacks the pyrogallol group on the condensed flavanic structure, it may be safer than other potent gallocatechin-type polyphenols such as compound **6**.

These findings indicate that these epicatechin derivatives, which are byproducts of the agro-food industry, show potential for application in the food and drug industries based on in vitro studies. However, special attention should be paid to the fact of the limitation of the use of these methodologies and the need of more well-designed human studies to provide clear evidence of health protective effects as has been recently reviewed (*41*, *42*).

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