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Caracterización de derivados polifenólicos obtenidos de fuentes naturales. Citotoxicidad y capacidad antioxidante frente a estrés oxidativo en modelos celulares

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3. RESULTADOS

3.1. Estudio de la actividad biológica de derivados de epicatequina

Bloque I. Actividad antioxidante y citotoxicidad

ARTÍCULO 1

Estudio comparativo de la citotoxicidad inducida por conjugados de Epicatequina antioxidantes obtenidos de uva

Comparative study of the cytotoxicity induced by antioxidant epicatechin conjugates obtained from grape

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Resumen

Los flavonoides son sustancias de naturaleza polifenólica que tienen numerosas actividades biológicas, siendo la mejor descrita, su capacidad de actuar como antioxidantes. El creciente interés en el estudio de las propiedades de los flavonoides, en especial de la familia de flavanoles, formada por catequinas y proantocianidinas, ha llevado a nuestro grupo a estudiar una serie de conjugados de Epicatequina obtenidos a partir de la despolimerización de flavanoles poliméricos de uva en presencia de cisteamina o cisteína. Estos compuestos han mostrado una prometedora actividad antiradicalaria y antiproliferativa en células tumorales, pero es necesario garantizar la seguridad de estos compuestos para las células normales del organismo y seleccionar los menos tóxicos entre los que presentan mejor actividad. Con este fin se ha evaluado la relación entre su actividad citotóxica y antioxidante y cómo la estructura de estos derivados de epicatequina tiene influencia en sus características. La citotoxicidad se ha evaluado determinando la captación de rojo neutro (NRU) después de 24, 48 y 72 horas de exposición a diferentes concentraciones de los productos y determinándose la CI_{50} para cada uno de ellos en dos líneas celulares, una de fibroblastos y una de queratinocitos. La actividad antioxidante se ha determinado mediante la capacidad protectora de los compuestos frente a la hemólisis inducida por radicales. Los efectos citotóxicos de los compuestos se observaron a concentraciones de 3 a 7 veces superiores que las concentraciones antioxidantes después de los períodos de exposición. Los compuestos con un grupo galato en su estructura presentaron

una mayor toxicidad que los productos equivalentes sin ese grupo. En general los compuestos más activos como antioxidantes resultaron ser los más citotóxicos, sin embargo es interesante destacar que el derivado éster etilo exhibió baja citotoxicidad, pero mostró la actividad antioxidante más potente. Los resultados indicaron que se puede obtener una actividad antioxidante efectiva con estos productos en un rango de concentración que es seguro para las células normales del organismo. Además, los datos obtenidos sugieren nuevas aplicaciones farmacéuticas para estos compuestos y pueden ayudarnos a delimitar las potenciales dosis terapéuticas para su correcto uso.

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Comparative Study of the Cytotoxicity Induced by Antioxidant Epicatechin Conjugates Obtained from Grape

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We studied the cytotoxicity of epicatechin conjugates obtained by depolymerization of grape polymeric flavanols in the presence of cysteamine or cysteine and the resulting conjugates purified by ion exchange and/or reversed-phase high-resolution chromatography and compared it to their antioxidant capacity. The studies were carried out on fibroblast and keratinocyte cell lines. The cytotoxic effects of these products were observed at concentrations 3−7-fold higher than the antioxidant concentration after exposure for 24, 48, and 72 h. The compounds with a gallate group were more toxic than the corresponding products without one. It is interesting to note that the esther ethyl derivative exhibited low cytotoxicity but had the most potent antioxidant activity. The results indicated that effective antioxidant activity can be obtained from these products in a concentration range that is safe for the normal cell. This finding suggests new pharmaceutical applications and may also help us to identify the potential therapeutic dose.

KEYWORDS: Cytotoxicity; catechins; antioxidants; polyphenols; grapes

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. They are micronutrients that are abundant in our diet, and evidence for their role in the prevention of degenerative diseases such as cancer and cardiovascular diseases is emerging. Their structure varies from simple molecules, such as phenolic acids, to highly polymerized compounds, such as condensed tannins.

The most common group of plant phenolics is the flavonoid family present in fruit and vegetables and also in food products and beverages made from plants such as olive oil, tea, and red wine (*1*). Flavonoids are subdivided into several families according to molecular structure: flavonols, flavanols, isoflavones, anthocyanidins, flavones, and flavanones.

The data that have been collected, particularly in recent years, have shown that flavonoids perform a wide variety of biological actions. The best-described property of almost every flavonoid group is their capacity to act as antioxidants. They scavenge reactive oxygen species, inhibit free radical-induced membrane lipid oxidation, and inhibit the oxidation of low-density lipoproteins (*2*). They have also been reported to have antiinflammatory, antihemorrhagic, antineoplastic, antiviral, antibacterial, antiallergic, and hepatoprotective properties and to inhibit platelet aggregation and capillary permeability (*3*). There is growing interest in the study of flavonoids, particularly the flavanol group formed by catechins (monomer form) and proanthocyanidins (polymer form). Catechin and epicatechin are the main flavanols in fruit, whereas epicatechin gallate, epigallocatechin, and epigallocatechin gallate are found in certain seeds of leguminous plants, in grapes, and in tea (*4*).

Some catechins, in addition to their free radical scavenging capacity, are inhibitors of key enzymes involved in the cell cycle. They also induce apoptosis in different cell lines and inhibit the expression of certain tumor-related genes. All these activities make catechins excellent candidates for acting as preventive agents against cancer, cardiovascular diseases, and premature aging (*5*).

The current interest in these compounds has led our group to use polyphenols present in wastes generated in wine making as raw materials for the preparation of a family of polyphenolic compounds with antioxidant properties. Grape pomace, consisting of skin, seeds, and stems obtained after pressing in the wine industry, is a rich source of polyphenols, including catechins (monomeric and oligomeric flavan-3-ols) and glycosylated flavonols (*5*). A new family of polymeric polyphenols from the grape (*Vitis vinifera*) has been obtained by acid depolymerization in the presence of natural amino acids such as cysteamine and cysteine, thus providing new thiol conjugate derivatives. These are promising products since they are more potent than their underivatized counterparts and include ionic groups, which may be used to modulate their action within different physicochem-

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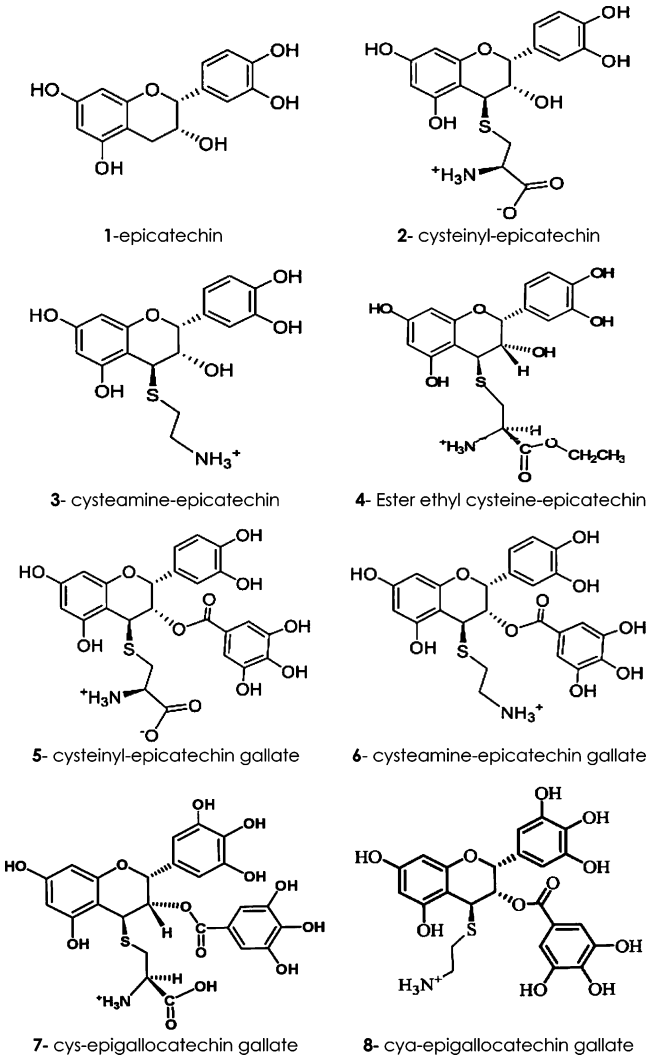

Figure 1. Chemical structures of epicatechin and epicatechin derivatives.

ical and biological environments (*6*). Preliminary studies performed by our group have demonstrated the potent free radical scavenger activity of these novel catechin derivatives in the 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH) assay (*7*) and their antioxidant potential by a biological system, the inhibition of red blood cell lysis after addition of AAPH, a well-known peroxyl radical initiator (*8*). Furthermore, we have investigated the influence of these compounds on the proliferation of different tumoral cell lines and their capacity to induce apoptosis (*9*).

The possibility of obtaining a chemopreventive agent that both minimizes ROS formation and induces apoptosis in tumor cells seems attractive. Our products appear to have a beneficial effect on several key mechanisms involved in the pathogenesis of cancer (*10*); however, these compounds must be safe, so they should not be toxic to normal body cells. A chemical that can inhibit cell proliferation at low or moderate concentrations may be considered to have basic toxic effects (*11*), and it is necessary to delimit these effects. Anticancer drugs are designed to kill cells, but this activity should be selective for tumor cells. Therefore, it seems reasonable to utilize, in the primary screening stage, in vitro toxicity assays to select the least toxic compounds among the most active ones (*12*). The determination of toxicity can be used to define concentrations at which chemopreventive effects can be further characterized (*13*).

In this work, we have determined cell viability through a neutral red uptake assay in human keratinocyte, HaCaT, and murine fibroblast 3T3 cell lines to specify the in vitro cytotoxic effects of our new catechin derivatives. We have evaluated the relationship between potential cytotoxic properties and the antioxidant activity of these compounds and how the structure of these new epicatechin derivatives influences these characteristics. The combination of these properties permits us to study the potential health benefits of these derivatives in depth and to identify their possible applications in different pharmaceutical formulations as chemopreventive agents.

MATERIALS AND METHODS

Chemicals. The conjugates used in this study were prepared from grape (*Vitis vinifera*) pomace, essentially as described above (*7*, *14*). Briefly, these bio-based antioxidant compounds were obtained by depolymerization of grape polymeric flavanols (proanthocyanidins) in the presence of cysteamine or cysteine, and the resulting conjugates were purified by ion exchange and/or reversed-phase high-resolution chromatography. 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**), 4β-(2-aminoethylthiol)epicatechin (**3**), 4β-[*S*-(*O*-ethylcysteinyl)]epicatechin (**4**), 4β-(*S*-cysteinyl)epicatechin 3-*O*-gallate (**5**), 4β-(2-aminoethylthiol)epicatechin 3-*O*-gallate (**6**), 4β-(*S*-cysteinyl)epigallocatechin 3-*O*-gallate (**7**), and 4β-(2-aminoethylthiol)epigallocatechin 3-*O*-gallate (**8**) (**Figure 1**). 2,2′-Azobis(amidinopropane) dihydrochloride (AAPH) were purchased from Sigma (St. Louis, MO).

Blood Samples and Preparation of Red Blood Cells. Blood samples were obtained from healthy donors by venipuncture (Blood Bank of Hospital Clinic, 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 1000*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 8×10^9 cells/mL.

Antioxidant Activity. We assessed the hemolysis of RBCs mediated by AAPH using a modification of the method described previously (*15*). The addition of AAPH (a peroxyl radical initiator) to the suspension of RBCs induces the oxidation of cell membrane lipids and proteins, thereby resulting in hemolysis. We incubated 250 μL of the erythrocyte suspension in the presence of AAPH at a final concentration of 100 mM for 150 min at 37 °C to achieve 100% hemolysis. Hemolysis was assessed by measuring the absorbance of the supernatant fraction, i.e., the hemoglobin release, at 540 nm in a Shimadzu spectrophotometer. The antihemolytic activity of (−)-epicatechin and related compounds was studied by adding several concentrations of the compounds, ranging from 12.5 to 200 μM, to the RBC suspension in the presence of 100 mM AAPH at 37 °C for 2.5 h. The IC_{50} (50% inhibitory concentration) of the hemolysis induced by AAPH was determined for each compound.

Cell Culture and Cytotoxicity Evaluation. *Culture of Cell Lines.* The spontaneously immortalized human keratinocyte cell line (HaCaT) and the mouse fibroblast cell line (3T3) were grown in DMEM (4.5 g/L glucose) supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 10 mM Hepes buffer, and a 1% penicillin (10 000 units/mL)/streptomycin (10 000 μg/mL) mixture at 37 °C and 5% CO_2. The cell lines were routinely cultured into 75 cm^2 culture flasks. When the cells were approximately 80% confluent, they were split by mild trypsinization and seeded into the central 60 wells of 96-well plates at a density of 10×10^4 cells/mL for HaCaT and 8.5×10^4 cells/mL for 3T3 for a 24 h exposure, 6.5×10^4 and 2.5×10^4 cells/mL, respectively for a 48 h exposure, and 5.5×10^4 and 1.5×10^4 cells/mL, respectively, for a 72 h exposure (*16*). The 96-well plates were incubated at 37 °C and 5% CO_2 for 24 h. The triplicate runs were done with different passage cells.

Experimental Treatments. After being incubated for 1 day, cells were exposed to increasing concentrations (from 23 μM to 4 mM) of the new bio-based antioxidants sterilized by filtration and dissolved in

DMEM supplemented with 5% FBS, 2 mM L-glutamine, 10 mM Hepes buffer, and a 1% antibiotic mixture. Controls, containing culture medium only, were included in each plate and were independent for each of the different samples that were tested. Plates were incubated at 37 °C and 5% CO_2 for 24, 48, or 72 h.

NRU Assay. The NRU assay was performed as described by Borenfreund and Puerner (*17*), modified to eliminate the use of formaldehyde (*18*). After the samples had been exposed to the test agents for 24, 48, or 72 h, medium was aspirated and replaced with 100 μL of NR solution (50 μg/mL in RPMI without phenol red and serum) per well. After incubation for 3 h at 37 °C and 5% CO_2, medium was aspirated, cells were washed twice in PBS, and a solution containing 50% absolute ethanol and 1% acetic acid in distilled water was added (100 μL/well) to extract the dye. After the sample had been shaken for 10 min on a microtiter plate shaker, the absorbance of the neutral red was read at a wavelength of 550 nm in a Bio-Rad 55 microplate reader.

Statistical Analysis. All NRU experiments were performed at least three times using three wells for each concentration of the product. The cytotoxicity of each product was expressed as a percentage of viability compared with control wells (the mean optical density of untreated cells was set to 100% viability) in terms of its IC_{50} (concentration of the product that causes 50% inhibition of growth or death of the cell population), calculated from the dose−response curves by linear regression analysis. NRU assay results are expressed as the percentage of uptake of neutral red dye by the lysosomes.

All data were compared by a one-way analysis of variance (ANOVA) and Student's *t*-test using SigmaPlot (SPSS Inc., Chicago, IL). $P < 0.05$ was considered to denote significance (*19*).

RESULTS AND DISCUSSION

Catechins are thought to exert protective effects against cancer and inflammatory and cardiovascular diseases. These protective effects have been mainly attributed to their antioxidative action, scavenging free radicals (*20*).

The antioxidant activity of the compounds that were studied (**Figure 1**) is presented in **Table 1** and expressed as the IC_{50} or the concentration inducing 50% inhibition of the hemolysis induced by AAPH. In general, all the compounds exhibit a tendency to be more active than epicatechin (lower IC_{50}), and the order of antioxidant power is as follows: **4** > **6** > **7** > **5** > **8** > **2** > **3** > **1**. We combined our compounds with cysteine and cysteamine amino acids to improve their extraction and isolation by cation exchange chromatography, to improve their performance and to promote their activity, because there are reports about the antioxidant activity of these amino acids (*21*). The amino and thiol groups introduced in position 4 of the new epicatechin derivatives modulate the reactivity of molecules and exert a strong influence above the A-ring of epicatechin. This can increase the antioxidant activity because it improves the epicatechin capacity to transfer electrons and eliminate protons itself (*7*).

The most potent antioxidant was compound **4** with an activity 10-fold higher than that of epicatechin. In all cases, the presence of a gallate group produced an increase in the antioxidant activity, as demonstrated in other studies (*7*, *22*). The number of hydroxyl groups connected with the aromatic ring, in the ortho or para position relative to each other, enhances the antioxidative and antiradical activity of phenolic acids. The hydroxyl groups on the galloyl moieties contribute to antioxidative activity, making the compounds capable not only of donating more hydrogen atoms but also of providing more chelating sites for scavenging catalytic cations (*23*−*25*). The epicatechin derivatives obtained by depolymerization of grape polymeric flavanols in the presence of cysteamine or cysteine have shown an effective protective action on RBCs challenged by exogenous oxidants and are more effective antioxidant protectors than epicatechin.

Table 1. Antioxidant Activity of the Products Determined by Their Antihemolytic Action Expressed as the IC_{50} or Concentration Inducing 50% Inhibition of the Hemolysis Induced by AAPH (mean ± the standard error of the mean)

product	IC_{50} (μM)	product	IC_{50} (μM)
1	119.8 ± 10.16	**5**	47.5 ± 12.09
2	74.9 ± 29.43	**6**	36.3 ± 8.19
3	89.4 ± 20.95	**7**	35.4 ± 3.73
4	12.9 ± 6.53	**8**	52.82 ± 4.26

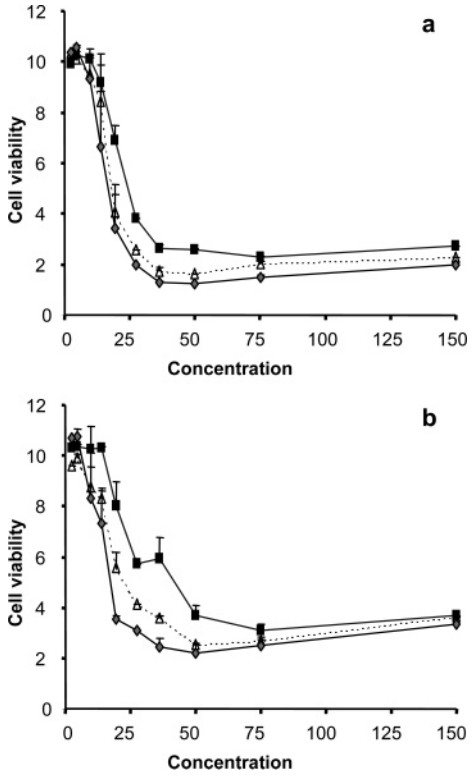

Figure 2. Comparative cytotoxicity of compounds toward proliferation of 3T3 (**a**) and HaCaT (**b**) cells after exposure for 24 (■), 48 (△), and 72 h (◆). The data are presented as the mean percentages of the control ± the standard error of the mean.

Previous studies by our group have demonstrated the antiproliferative and apoptotic activity of these compounds in melanoma cells, so these compounds are promising molecules to be considered in new strategies seeking to target cancer cells (*7*, *9*). In the search for new anticancer drugs, the most common screening methods employ cytotoxicity tests against a panel of cancer cell lines (*26*). Anticancer drugs are designed to kill cells, but this activity should be selective for tumor cells. Because of that, it is necessary to demonstrate that they are less toxic to normal cells than to tumoral cells. The cytotoxic effects of epicatechin and the new derivatives were evaluated via the cell membrane integrity of HaCaT and 3T3 cells using a colorimetric assay that measures the ability of live cells to take up neutral red dye.

In this study, we demonstrate that all the tested compounds had cytotoxic effects, as shown by the decrease in the rate of neutral red uptake; nevertheless, they are less cytotoxic to nonmalignant cell lines than to cancer cell lines, as previously described (*9*). **Figure 2** shows the cytotoxicity induced by product **6** in HaCaT and 3T3 cells after exposure for 24, 48, and 72 h. There is an increase in the cytotoxicity after the

Table 2. Cytotoxicity of Epicatechin and Its Derivatives in HaCaT Human Keratinocytes and 3T3 Mouse Fibroblasts Evaluated as IC_{50} (the dose inhibiting viability to 50%) (mean ± the standard error of the mean)

	IC_{50} for HaCaT keratinocytes (mM)			IC_{50} for 3T3 fibroblasts (mM)		
product	24 h	48 h	72 h	24 h	48 h	72 h
1	9.90 ± 0.99	2.22 ± 0.74	0.85 ± 0.22	3.80 ± 0.35	1.00 ± 0.20	0.78 ± 0.23
2	4.80 ± 1.28	1.05 ± 0.46	0.57 ± 0.14	2.10 ± 0.70	0.32 ± 0.05	0.32 ± 010
3	4.90 ± 1.30	0.82 ± 0.23	0.49 ± 0.11	2.70 ± 1.13	0.34 ± 0.06	0.32 ± 0.08
4	1.40 ± 0.39	0.56 ± 0.01	0.47 ± 0.02	0.40 ± 0.04	0.27 ± 0.05	0.22 ± 0.03
5	0.66 ± 0.07	0.34 ± 0.05	0.29 ± 0.06	0.48 ± 0.01	0.20 ± 0.04	0.12 ± 0.01
6	0.42 ± 0.03	0.27 ± 0.02	0.21 ± 0.00	0.28 ± 0.01	0.201 ± 0.01	0.18 ± 0.03
7	0.43 ± 0.09	0.18 ± 0.01	0.13 ± 0.01	0.26 ± 0.02	0.14 ± 0.01	0.12 ± 0.02
8	0.29 ± 0.01	0.18 ± 0.01	0.16 ± 0.01	0.39 ± 0.04	0.19 ± 0.02	0.16 ± 0.00

increase in the exposure period with a concentration-dependent decrease in the rate of neutral red uptake. Similar curve profiles were observed with the other compounds. Typical concentration−response curves were recorded for all the compounds, and their IC_{50} values were calculated and are listed in **Table 2**. All the new catechin derivatives have more cytotoxic effects than epicatechin, although not in all cases were significant differences found.

The cytotoxicity of the epicatechin derivatives was similar in the two cell lines, although different sensitivities of the HaCaT keratinocytes and 3T3 fibroblasts to the compounds studied were observed, the 3T3 line being the most sensitive, with the lowest IC_{50} of all the compounds that were tested (no statistical differences were noted for all the IC_{50} values). Such a difference between 3T3 fibroblasts and human keratinocytes has been observed previously (*27*) and is related to morphologic and physiologic differences between them, particularly variation in the ability to deal with oxidative stress. We used the 3T3 neutral red uptake assay because this test is recommended by the U.S. National Institute of Environmental Health Sciences (NIEHS) Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). The use of the HaCaT, nontumorogenic, spontaneously immortalized cell line has the advantage of providing an almost unlimited supply of identical cells, ensuring high intralaboratory and interlaboratory reproducibility (*28*).

Moreover, in all cases, the presence of gallate strengthens the cytotoxic effect of the product. Other authors have demonstrated that the more toxic polyphenolic tea catechins contained a gallic moiety while the least cytotoxic, catechin and epicatechin, did not (*29*, *30*).

Though the compounds that have been studied are more cytotoxic than epicatechin, they exhibit higher antioxidant activity, as shown previously. The cytotoxicity index of each compound obtained from the NRU assay (CI_{50}) correlates with the antioxidant activity: the compound that is most active as an antioxidant is also the most cytotoxic. There is a correlation ($r = 0.8034$ and $r = 0.8892$) between the antioxidant activity of the compounds at 24 h cytotoxicity for HaCaT and 3T3, respectively (**Figure 3**). A similar correlation was found for the 48 and 72 h exposure periods. If we do not consider compound **4**, then there is an increase in the correlation ($r = 0.9429$ and $r = 0.9690$, respectively). According to this observation, we could postulate that this compound is less cytotoxic than what might be supposed due to its antioxidant activity. It should theoretically be 10-fold more cytotoxic to HaCaT and 5-fold more cytotoxic to 3T3 on the basis of its antihemolytic capacity ($IC_{50} = 12.89\ \mu M$).

The presence of the ethyl ester group not only significantly increases the cytotoxicity of the compound but also promotes its antioxidant activity with regard to its ethyl ester counterpart

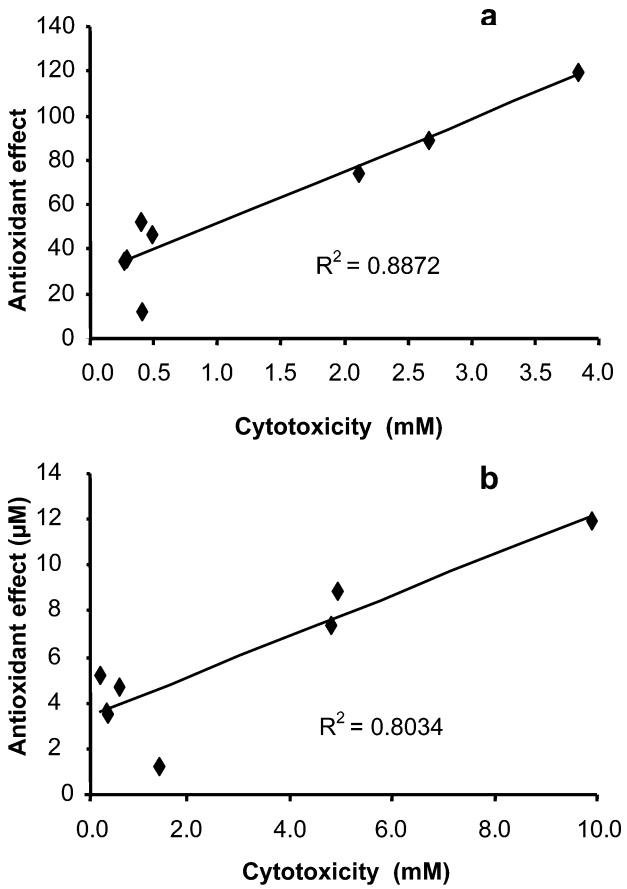

Figure 3. Relationship between the antioxidant activity expressed as the concentration of the product that causes 50% inhibition of hemolysis induced by AAPH (IC_{50}) in micromolar and the cytotoxicity expressed as the concentration of the product that causes 50% inhibition of growth or death of the cell population (IC_{50}) in millimolar on 3T3 (**a**) and HaCaT (**b**) cells. Each point represents each of the eight products that were studied.

(product **2**). Similar results have been observed by other authors in the case of ferulic acid ethyl ester, which exhibits high radical scavenging activity (*31*, *32*). Other authors have demonstrated that ethyl esterification of phenolic acids enhanced their lipophilicity and their protective effect against two types of oxidative stress: copper-catalyzed peroxidation of LDL and radical attack of erythrocyte membranes by AAPH (*33*). Their results indicate that esterification of phenolic acids increases the lipophilicity of their ethyl esters and may facilitate incorporation in the lipid layer of the LDL particle and the exertion of their antioxidant effect at the site of lipoperoxidation (*34*). The chemical synthesis of ethyl esters derived from natural phenolic acids may produce interesting new drugs for the prevention of oxidative diseases.

Our group is interested in searching for new applications for the family of polyphenolic compounds that were synthesized (*35*). In previous studies by our group, the apoptotic activity of some of these compounds has been characterized. These studies concluded that the most active derivative and therefore the best candidate for a potential chemopreventive agent would be **5** (*9*). In this work, we have continued characterizing the epicatechin derivatives and have presented antioxidant activity in a biological system (RBC) and the cytotoxic effect on two nontumoral cell lines. The new compounds have exhibited better antioxidant activity than epicatechin, although their cytotoxic effect was stronger. Nevertheless, the effective antioxidant concentrations are smaller than the cytotoxic concentration (the compounds are antioxidants at noncytotoxic concentrations). As expected, the strongest antioxidant products were also the most cytotoxic, especially due to the antioxidant power of the gallate group of the molecules, which is also responsible for the increase in their cytotoxicity. It is interesting to note that esther ethyl derivative **4** exhibited a very low cytotoxicity, although it revealed the most potent antioxidant activity.

On the basis of the results of this study and previous studies, we have observed that cancerous cells were more sensitive than normal cells to the growth inhibitory effect of the new family of epicatechin conjugates (*9*). These findings are in accord with other studies based on the comparative cytotoxicity of the epigallocatechin gallate between normal and tumoral cells (*36*, *37*). It has been postulated that normal cells in potentially frequent contact with plant-derived polyphenols, such as cells found in the oral mucosa, have developed a tolerance to mitigate cytotoxicity, whereas normal cells from internal organs and tumor cells are, in general, sensitive to it (*38*).

The modifications introduced into epicatechin during the recovery of agricultural byproducts not only maintain its properties but also enhance the potential uses. The results open up the possibility of using raw materials as sources of high-value added products with potential health benefits (*8*).

Thus, the results indicate that effective antioxidant activity can be obtained from these products in a concentration range that is safe for the normal cell. This finding suggests new pharmaceutical applications and may also help us to identify the potential therapeutic dose.

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LITERATURE CITED

(1) Kanupriya; Prasad, D.; Sai Ram, M.; Kumar, R.; Sawhney, R. C.; Sharma, S. K.; Ilavazhagan, G.; Kumar, D.; Banerjee, P. K. Cytoprotective and antioxidant activity of *Rhodiola imbricata* against *tert*-butyl hydroperoxide induced oxidative injury in U-937 human macrophages. *Mol. Cell. Biochem.* **2005**, *275* (1−2), 1−6.

(2) Kitagawa, S.; Sakamoto, H.; Tano, H. Inhibitory Effects of Flavonoids on Free Radical-Induced Hemolysis and Their Oxidative Effects on Hemoglobin. *Chem. Pharm. Bull.* **2004**, *52* (8), 999−1001.

(3) Puiggròs, F.; Llópiz, N.; Ardévol, A.; Bladé, C.; Arola, L. l.; Salvadó, M. J. Grape Seed Procyanidins Prevent Oxidative Injuty by Modulating the Expression of Antioxidant Enzyme Systems. *J. Agric. Food Chem.* **2005**, *53*, 6080−6.

(4) Manach, C.; Scalbert, A.; Morand, C. Polyphenols: Food sources and bioavailability. *Am. J. Clin. Nutr.* **2004**, *79*, 727−47.

(5) Torres, J.; Varela, B.; García, M. T.; Carilla, J.; Matito, C.; Centelles, J. J.; Cascante, M.; Sort, X.; Bobet, R. Valorization of Grape (*Vitis vinifera*) Byproducts. Antioxidant and Biological Properties of Polyphenolic Fractions Differing in Procyanidin Composition and Flavonol Content. *J. Agric. Food Chem.* **2002**, *50*, 7548−55.

(6) Selga, A.; Sort, X.; Bobet, R.; Torres, J. L. Efficient One Pot Extraction and Depolymerization of Grape (*Vitis vinifera*) Pomace Procyanidins for the Preparation of Antioxidant Thio-Conjugates. *J. Agric. Food Chem.* **2004**, *52*, 467−73.

(7) Torres, J. L.; Lozano, C.; Julià, 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*, 2497−509.

(8) Mitjans, M.; Martínez, V.; Del Campo, J.; Abajo, C.; Lozano, C.; Torres, J. L.; Vinardell, M. P. Novel epicatechin derivatives with antioxidant activity modulate interleukin-1β release in lipopolysaccharide-stimulated human blood. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5031−4.

(9) Lozano, C.; Torres, J.; Julià, L., Jiménez, A.; Centelles, J. J.; Cascante, M. Effect of new antioxidant cysteinyl-flavanol conjugates on skin cancer cells. *FEBS Lett.* **2005**, *579*, 4219−25.

(10) Yang, C. S.; Landau, J. M.; Huang, M. T.; Newmark, H. L. Inhibition of carcinogenesis by dietary polyphenolic compounds. *Annu. Rev. Nutr.* **2001**, *21*, 381−406.

(11) Soares, V. C.; Varanda, E. A.; Raddi, M. S. In vitro basal and metabolism-mediated cytotoxicity of flavonoids. *Food Chem. Toxicol.* **2006**, *44*, 835−8.

(12) Popiolkiewicz, J.; Polkowski, K.; Skierski, J. S.; Mazurek, A. P. In vitro toxicity evaluation in the development of new anticancer drugs: Genistein glycosides. *Cancer Lett.* **2005**, *229*, 67−75.

(13) Glei, M.; Matuschek, M.; Steiner, C.; Böhm, V.; Persin, C.; Pool-Zobel, B. L. Initial in vitro toxicity testing of functional foods rich in catechins and anthocyanins in human cells. *Toxicol. in Vitro* **2003**, *17*, 723−9.

(14) Torres, J. L.; Bobet, R. New flavanol-derivatives from grape (*Vitis vinifera*) byproducts. Antioxidant aminoethylthiol-flavan-3-ol conjugates from a polymeric waste fraction used as a source of flavanols. *J. Agric. Food Chem.* **2001**, *49*, 4627−34.

(15) Miki, M.; Tamai, H.; Mino, M.; Yamamoto, Y.; Niké, E. Free radical chain oxidation of rat red blood cells by molecular oxygen and its inhibition by α-toxopherol. *Arch. Biochem. Biophys.* **1987**, *258*, 373−80.

(16) Babich, H.; Krupka, M. E.; Nissim, H. A.; Zuckerbraun, H. L. Differential in vitro cytotoxicity of (−)-epicathechin gallate (ECG) to cancer and normal cells from the human oral cavity. *Toxicol. in Vitro* **2005**, *19*, 231−42.

(17) Borenfreund, E. P. J. A. Rapad colorimetric assay to cellular growth and survival: Application to proliferation and cytotoxicity assay. *Toxicol. Lett.* **1983**, *24*, 119−24.

(18) Riddell, R. J.; Clothier, R. H.; Ball, M. An evaluation of three in vitro cytotoxicity assays. *Food Chem. Toxicol.* **1986**, *24* (6/7), 469−71.

(19) Sánchez, L.; Mitjans, M.; Infante, M. R.; Vinardell, M. P. Assessment of the Potential Skin Irritation of Lysine-Derivative Anionic Surfactants Using Mouse Fibroblast and Human Keratinocytes as an Alternative to Animal Testing. *Pharmacol. Res.* **2004**, *21*, 1637−41.

(20) Kondo, K.; Kurihara, M.; Miyata, N.; Suzuki, T.; Toyoda, M. Mechanistic studies of catechins as antioxidants against radical oxidation. *Arch. Biochem. Biophys.* **1999**, *362*, 79−86.

(21) Sagristá, M. L. L.; García, A.; Madariaga, M. A.; Mora, M. Antioxidant and pro-oxidant effect of thiolic compounds *N*-acetyl-L-cysteine and glutathione against free radical-induced lipid peroxidation. *Free Radical Res.* **2002**, *36* (3), 329−40.

(22) Sroka, Z. Antioxidative and antiradical properties of plant phenolics. *Z. Naturforsch., C* **2005**, *60* (11−12), 833−43.

(23) Kondo, K.; Kurihara, M.; Miyata, N.; Suzuki, T.; Toyoda, M. Scavenging mechanisms of (−)-epigallocatechin gallate and (−)-epicatechin gallate on peroxyl radicals and formation of superoxide during inhibitory action. *Free Radical Biol. Med.* **1999**, *27*, 855−63.

(24) Bors, W.; Michel, C.; Stettmaier, K. Electron paramagnetic resonance studies of radical species of proanthocyanidins and gallate esters. *Arch. Biochem. Biophys.* **2000**, *374*, 347−55.

(25) Lozano, C.; Julia, L.; 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 Lett.* **2006**, *273* (11), 2475−86.

(26) Carmichael, J. Current Issues in Cancer: Cancer chemotherapy: Identifying novel anticancer drugs. *BMJ [Br. Med. J.]* **1994**, *308*, 1288−90.

(27) Clothier, R.; Willshaw, A.; Cox, H. The use of human keratinocytes in the EU/COLIPA international in vitro phototoxicity test validation study and the ECVAM/COLIPA study on UV filter chemicals. *ATLA, Altern. Lab. Anim.* **1999**, *27*, 247−59.

(28) Spielmann, H.; Balls, M.; Dupuis, J.; Pape, W. J.; Pechovitch, G.; de Silva, O.; Holzhütter, H. G.; Clothier, R.; Desolle, P.; Gerberick, F.; Liebsch, M.; Lovell, W. W.; Maurer, T.; Pfannenbecker, U.; Potthast, J. M.; Csato, M.; Sladowski, D.; Steiling, W.; Brantom, P. The international EU/COLIPA in vitro phototoxicity validation study results of phase II (blind trial). Part I: The 3T3 NRU phototoxicity test. *Toxicol. in Vitro* **1998**, *12*, 305−27.

(29) Galati, G.; Lin, A.; Sultan, A. M.; O'Brien, P. J. Cellular and in vivo hepatotoxicity caused by green tea phenolic acids and catechins. *Free Radical Biol. Med.* **2006**, *40*, 570−80.

(30) Schmidt, M.; Schmitz, H. J.; Baumgart, A.; Guédon, D.; Netsch, M. I.; Kreuter, M. H.; Schmidlin, C. B.; Schrenk, D. Toxicity of green tea extracts and their constituents in rat hepatocytes in primary culture. *Food Chem. Toxicol.* **2005**, *43*, 307−14.

(31) Mohmmad-Abdul, H.; Butterfield, D. A. Protection against amyloid β-peptide (1-42)-induced loss of phospholipids asymmetry in synaptosomal membranes by tricyclodecan-9-xanthogenate (D609) and ferulic acid ethyl ester: Implications for Alzheimer's disease. *Biochim. Biophys. Acta* **2005**, *1741*, 140−8.

(32) Kikukaki, H.; Hisamoto, M.; Hirose, K.; Akiyama, K.; Taniguchi, H. Antioxidant properties of ferulic acic and its related compounds. *J. Agric. Food Chem.* **2002**, *50*, 2161−8.

(33) Sultana, R.; Ravagna, A.; Mohmmad-Abdul, H.; Calíbrese, V.; Butterfield, D. A. Ferulic acid ethyl ester protects neurons against amyloid β-peptide(1-42)-induced oxidative stress and neurotoxicity: Relationship to antioxidant activity. *J. Neurochem.* **2005**, *92*, 749−58.

(34) Chalas, J.; Claise, C.; Edeas, M.; Messaoudi, C.; Vergnes, L.; Abella, A.; Lindenbaum, A. Effect of ethyl esterification of phenolic acids on low-density lipoprotein oxidation. *Biomed. Pharmacother.* **2001**, *55*, 54−60.

(35) Mitjans, M.; Del Campo, J.; Abajo, C.; Martinez, V.; Selga, A.; Lozano, C.; Torres, J. L.; Vinardell, M. P. Immunomodulatory Activity of a New Family of Antioxidants Obtained from Grape Polyphenols. *J. Agric. Food Chem.* **2004**, *52*, 7297−9.

(36) Chen, Z. P.; Schell, J. B.; Ho, C. T.; Chen, Y. Green tea epigallocatechin gallate shows a pronounced growth inhibitory effect on cancerous cells but not on their normal counterparts. *Cancer Lett.* **1997**, *129*, 173−9.

(37) Chen, C.; Shen, G.; Hebbar, V.; Hu, R.; Owuor, E. D.; Kong, A. N. T. Epigallocatechin-3-gallate-induced stress signals in HT-29 human colon adenocarcinoma cells. *Carcinogenesis* **2003**, *24*, 1369−78.

(38) Yamamoto, T. J.; Lewis, J.; Wahata, D.; Dickinson, B.; Singh, W. B.; Bollarg, E.; Ueta, T.; Osaki, T.; Athar, M.; Shuster, G.; Hsu, S. Roles of catalase and hydrogen peroxide in green tea polyphenols-induced chemopreventive effects. *J. Pharmacol. Exp. Ther.* **2004**, *308*, 317−23.

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Bloque II. Actividad protectora frente al estrés oxidativo inducido por el H_2O_2

ARTÍCULO 2 (MANUSCRITO)

Protección ejercida por conjugados de Epicatequina de origen biológico contra el estrés oxidativo inducido por el H_2O_2 en eritrocitos y líneas celulares no tumorales

Biobased epicatechin conjugates protect erythrocytes and non tumoral cell lines from H_2O_2-induced oxidative stress

Vanessa Ugartondo, Montserrat Mitjans, Josep Lluís Torres y

María Pilar Vinardell

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Resumen

Se ha estudiado el potencial efecto antioxidante y la acción protectora de la Epicatequina y de una serie de derivados de la misma, obtenidos por despolimerización de flavanoles poliméricos en presencia de cisteína y cisteamina. Dichos estudios se han realizado utilizando eritrocitos humanos y líneas celulares no tumorales sometidos al daño oxidativo inducido por H_2O_2 exógeno. Los derivados de Epicatequina mostraron unas propiedades antioxidantes más efectivas que la Epicatequina en ambos modelos. Este mejor comportamiento podría explicarse por la actividad antiradicalaria incrementada de estos compuestos, su diferente modo de interacción con la membrana celular y su capacidad de elevar los niveles intracelulares de GSH y por tanto las defensas celulares. Los datos demostraron que estos compuestos eran capaces de proteger a las células de la toxicidad inducida por estrés oxidativo en un rango de concentraciones seguro para las células normales. Entre los derivados, el 4β-(2-aminoetiltiol)epicatequina 3-*O*-galato presentó la actividad antioxidante más elevada con los tres marcadores de estrés oxidativo evaluados: hemólisis, peroxidación lipídica y citotoxicidad. Además, debido a que a este compuesto le falta el grupo pirogalol en el anillo B de la estructura flavánica condensada, podría resultar ser más seguro que otros polifenoles del tipo galocatequinas que al tener este grupo pirogalol pueden actuar como prooxidantes en determinadas condiciones. El grado de hemólisis se determinó midiendo la liberación de hemoglobina (lectura espectrofotométrica a 540 nm), la peroxidación lipídica, por la formación de TBARs (absorbancia a 532 nm) y la citotoxicidad, mediante el ensayo de viabilidad de captación del Rojo Neutro (NRU assay). Los resultados indican que los derivados de epicatequina evaluados son buenos candidatos como suplementos nutricionales y presentan potencial para ser utilizados en la industria alimentaria y farmacéutica.

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Biobased epicatechin conjugates protect erythrocytes and non tumoral cell lines from H_2O_2- induced oxidative stress

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ABSTRACT

We studied 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 non-tumoral cell lines challenged by exogenous H_2O_2. The epicatechin derivatives showed more effective antioxidant properties than epicatechin. Among them, 4β-(2-aminoethylthiol)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, are good candidates for nutraceuticals and 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 citric 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 beneficial on health of new products obtained from agricultural byproducts (*2*).

Flavonoids, the most widely occurring group of phenolic phytochemicals, are present in fruit, 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, antocyanidins and others. All these families have ideal structural chemistry for free radical-scavenging activities (*4*).

Grape pomace (skin, seeds, and steams) 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 lab 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*, *9*, *10*), cytotoxicity on non-tumoral 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 characterising their anti-proliferative 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 several antioxidant compounds using a battery of assays that provide diverse and complementary information for the proper interpretation of the results. To increase our knowledge about the biological properties of our catechin derivatives, we studied their protective capacity using other oxidative models, namely H_2O_2-induced RBC oxidative damage and H_2O_2-induced cell cytotoxicity.

This study aims to take a step forward in the characterisation of the antioxidant properties of our epicatechin derivatives and analyse the structure-activity relationships, particularly the effect of gallate and non-phenolic moieties on their antioxidant behaviour. For this purpose, we: (1) induced oxidative stress by H_2O_2 in intact human erythrocytes and in non-tumoral 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.

A better understanding of antioxidant properties in biological systems may contribute to the development of compounds with enhanced properties and to the widening of their range of applications. Data on the behaviour of cell-protecting antioxidants in H_2O_2-oxidative models may contribute to elucidate their mechanism of action and of these compounds and open up new approaches to evaluate agro-food byproducts.

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 exchanged 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-ethyl-cysteinyl)]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 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, 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 x 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 pre-incubated 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 described by Grinberg et al., with slight modifications (*16*). A 12.5% erythrocyte suspension (250 µL) was incubated for 90 min at 37 ^oC 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 the results of previous studies 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 µM and 150 µM of the compounds solved in PBS were added to the erythrocyte suspension in the presence of 20 mM H_2O_2 at 37 ^oC for 90 min.

RBC controls were included in all 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 hemolysed with distilled water. The IC_{50} (50% inhibitory concentration) of the hemolysis induced by H_2O_2 was determined for the compounds.

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 (TBAR) substances, following the method described by Stocks and Dormandy in 1972 with slight modifications (*17*). This method is based on the extraction of MDA from erythrocyte suspension by trichloroacetic acid (TCA) solution and the subsequent reaction of this MDA with thiobarbituric acid (TBA), to yield a pink complex with maximum absorption at 532 nm (TBAR substances). We induced lipid peroxidation by incubating erythrocytes under the same conditions as those of the hemolysis assay (RBC suspensions plus H_2O_2 20 mM alone or with a range of concentrations of a given compound for 90 minutes at 37 ^{o}C). Following incubation, the RBC suspension was mixed with 1 mL of TCA solution 20 % w/v to remove potentially interfering substances (*18*). Samples were then centrifuged and 1 ml of supernatant was mixed with 1 mL of 1% of TBA. Finally, samples were heated to 90 ^{o}C for 50 minutes, cooled rapidly and centrifuged. The absorbance of the supernatant was measured at 532 nm 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 TBAR 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 medium (4.5 g/L glucose) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 mM Hepes buffer, and 1% penicillin (10.000 U/mL) – streptomycin (10.000 µg/mL) mixture and maintained in a humidified atmosphere at 37 ^{o}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 x 10^4 cells/mL and 10 x 10^4 cells/mL for 3T3 and HaCaT cells respectively, in 96-well plates and then incubated for 24 h at 37 ^oC 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 non-tumoral cell lines (3T3 and HaCaT cells), the cells were pre-incubated overnight (18-20 hours) with increasing concentrations (from 25 μM to 300 μM) of the test compounds in DMEM 5% FBS, previously sterilized by filtration. After pre-incubation time, the excess of compounds was removed and the medium was exchanged before adding H_2O_2. This procedure prevented a direct reaction between the epicatechin derivatives and the oxidant source in the medium (*19*). 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 (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 analysed 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 Borenfreund and Puerner (*20*), and modified to remove the use of formaldehyde (*21*). 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 ^oC, 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 microtitre-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 the SPSS software (SPSS Inc., Chicago, IL, USA). A value of $p<0.05$ was judged as statistically significant.

RESULTS AND DISCUSSION

Previous studies by our group evaluated the antiradical, antitumoral, neuroprotective and cytotoxic activities of several of epicatechin thiol derivatives (*8-15*). Given that flavonoid activity varies depending on to 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, e.g, 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 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 RBC (*18*). Hydrogen peroxide is considered a key reactive oxygen species (ROS) and an attractive oxidant model because of its relatively high stability, diffusion, and involvement in cell signalling cascades. We therefore considered H_2O_2-induced RBC damage a suitable oxidative stress model to evaluate the protective role of epicatechin and its thiol derivatives and to complete their characterization as antioxidants. Lipid peroxidation produces a number of unstable intermediary metabolites from cellular molecules. MDA, one of the intermediates released, has been widely used as a marker of the extent of lipid peroxidation; MDA is a highly reactive molecule that can impairs a variety of membrane-related functions, thereby eventually leading to diminish erythrocyte survival. The reaction of MDA with TBA is considered a sensitive assay for measuring lipid peroxidation (*22*).

Here we examined the degree of lipoperoxidation in erythrocytes treated with 20 mM H_2O_2 by the formation of TBAR 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**). Our results indicated that when epicatechin and its derivatives were added, MDA formation was inhibited. This observation indicates that these compounds vary in their degree of protective capacity against H_2O_2-induced lipid damage.

All the compounds tested in the range of 50 μM to 150 μM statisticatly ($p<0.05$) protect against H_2O_2-TBAR formation, except compounds **1** and **2**. Compound **5** also showed significant protection at the lowest concentrations (12.5 μM and 25 μM). Galloylated compounds (**4**, **5**, **6** and **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). It has recently been described that the non-phenolic part of the conjugates, particularly the cysteamine moiety, promotes the internalization of cysteine into cells and helps to maintain the intracellular glutathione level (*12*). This enhancement of the endogenous antioxidant defence system may significantly contribute to the cell antioxidant activity of the epicatechin derivatives.

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 a RBC suspension in our assay conditions, a great degree of hemolysis was observed (75%) (**Figure 3**). Epicatechin and the related compounds varied 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 (more than 55% inhibition at the lowest dose 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**, **5**, **6**, and **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. IC_{50} values for hemolysis were slightly lower than those for LPO. These data suggested that lipid peroxidation and hemolysis are induced by distinct mechanisms. A model of competition between lipid and protein oxidation has been proposed to explain the mechanism of free radical-induced hemolysis (*26*).

The flavonol derivatives 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 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. In addition, compounds could interact with membranes at two additional levels, by increasing membrane fluidity, and thus reducing the rate of lipid peroxidation, and by limiting the access of certain molecules to hydrophobic region of the membrane (*27*). The location of the derivatives in the membrane bilayer and the resulting restriction of its fluidity may hinder the diffusion of H_2O_2 and its consequent damaging effects (*28*). It has been observed in previous studies the tendency of bulky hydrophobic conjugates such as **8** to penetrate deeper into the membrane bilayer than small compounds such as **2** or **3** (*29*). 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 and on their interactions with particular macromolecules. These factors will determine the local concentration of flavonoids, which will affect their capacity to regulate cellular events. Previous reports 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*, *31*).

Regarding the flavanols tested in our study, two main chemical characteristics could influence their antioxidant capacity, namely the non-phenolic moiety and the

presence of galloyl and gallic groups. The non-phenolic part of the molecule appears to affect the activity of the conjugates, particularly their capacity to penetrate biological membranes (*32*) and may also modulate the endogenous defense systems. In contrast, the gallate groups of these conjugates provide greater scavenging power through their additional hydroxyl groups (*11*).

The combination of our compounds with cysteine and cysteamine improved their extraction and isolation by cation exchange chromatography, enhanced their performance and promoted their activity. Furthermore, the nonphenolic part of the molecules might determine whether the phenolics are available for action on surface receptors, inside the membrane, or even intracellularly (*29*).

We observed that the presence of a gallate group increased the antioxidant activity, which was more significant in the case of the compound **5**. Our results are consistent with other reports showing the role of the galloyl group in oxidative activity (*24*, *33*). 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*, *34*)**.** In general, all the compounds tended to be more active than epicatechin in protecting RBC against H_2O_2-induced damage. This behavior may be explained by the improved antiradical activity of these compounds, their different interaction with cellular membrane and their capacity to enhance GSH levels.

Antioxidant protection against H_2O_2-induced cytotoxicity. Epicatechin conjugates show antiproliferative and apoptotic activity in several tumoral cells (*8*, *9*, *15*) and a low cytotoxic effect on non-malignant cells lines (*10*). Here we attempted to characterise the protective capacity of the epicatechin derivatives in non tumoral cells exposed to H_2O_2-induced damage.

We selected the murine fibroblast 3T3 and the human keratinocyte HaCaT cell lines because we have previously performed the cytotoxicity assays of our compounds on these cell lines. In addition, 3T3 NRU assay is recommended by the U.S. National Institute of Environmental Health Science (NIEHS) Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and, several studies support the use of mouse embryo fibroblast NIH 3T3 cell line as a sensitive cellular model for the evaluation of oxidative stress induced by H_2O_2 (*35*).

When 3T3 were exposed to 2 mM H_2O_2 for 2 h, cell viability decreased by around 80% (**Figure 5**). The oxidant condition was previously determined by means of

dose-time exposure assays (data not shown). Overnight pre-incubation 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. Protection against H_2O_2 cytotoxicity was not statistically significant for any compound. Products **1**, **2**, **7** and **8** showed a very low protective capacity whereas product **3** showed the best performance at the highest concentration and was the only compound to show a dose-response behaviour. Again, the maintenance of intracellular glutathione levels triggered by the cysteamine moiety (*12*) may be partially responsible for the overall effect of **3**. 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 with the previously reported pro-oxidant effect of phenolic compounds, and especially with the gallate group content of molecules (*24*, *36*). 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, since 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 ($O_2^{\cdot -}$) and subsequently hydrogen peroxide (*5*, *8*). The pro-oxidant action often attributed to flavanols is due to a great extent to the pyrogallol group on ring B presented 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 (*37*). 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. The amount of H_2O_2 required to achieve a 50% decrease in viability in this cell line is 2.5 mM on the basis of results from previous assays (data not shown).

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 differences 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 (*38*) 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 (*39*); however, the use of keratinocytes is recommended for certain purposes since *in vivo* they are the first cells to be exposed to exogenous agents (*40*, *41*).

We pre-incubated 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 reports have emphasized this preincubation with polyphenolic compounds to achieve suitable protective effects in other cell models (*19*, *37*, *42*). 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 damage by a mechanism other than the mere scavenging of ROS. Several studies have proposed the interaction of flavonoids with cell membranes and intracellular proteins, and it has been reported that grape flavanols activate antioxidative enzymes and the glutathione cycle. Therefore, the pre-incubation with these compounds could enhance the antioxidant potential of them (*43*, *44*).

Hydrogen peroxide diffuses into the cell nucleus and cause damages in DNA by generating the highly reactive hydroxyl radical $^{\cdot}OH$ in close proximity to DNA (*45*). This observation and reports of other studies (*46*, *47*) of the involvement of

apoptotic processes in hydrogen peroxide-induced cell death in fibroblasts and other cells suggest that the capacity of flavanols to protect cells is not necessarily dependent only on their capacity to scavenge free radicals or ROS. Instead, they may also have the capacity to interact with cell-signalling cascades, to influence cell transcriptional mechanisms, down-regulate pathways leading to cell death and modulate the repair potential of damaged DNA. However, the mechanisms of this enhanced protection against DNA injury are complex and remain to be elucidated.

Our results demonstrate that compound **1** and its related derivatives from grape pomace and other natural sources are biological antioxidants in cell culture experimental systems. Moreover, in a range of concentrations that are safe to normal cells, these compounds protect cells from oxidative stress-induced toxicity.

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 radicals 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**.

The chemical characteristics (especially by their antioxidant capacity) and physical properties (structure able to react in biochemical processes) of these polyphenolic compounds reflect their high potential as chemopreventive agents. The advance of research into this field may allow: (1) a reduction of the environmental impact caused by the industrial wastes from wine manufacturing and other agricultural and forestry management activities; (2) improved extraction of high added value substances, (3) the production of new flavonoid conjugates with distinct physico-chemical and biological properties, that are promising chemopreventive agents, dietary supplements, and antioxidant substances.

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LITERATURE CITED

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

(2) Yilmaz, Y.; Toledo, R.T. Major flavonoids in grape seeds and skins: antioxidant capacity of catechin, epicatechin, and gallic acid. *J. Agric. Food Chem.* **2004**, *52,* 255-260.

(3) Rice-Evans, C. Flavonoid Antioxidants. *Curr. Med. Chem.* **2001**, *8*, 797-807.

(4) Dai, F.; Miao, Q.; Zhou, B.; Yang, L.; Liu, Z. Protective effects of flavonols and their glycosides against free radical-induced oxidative hemolysis of red blood cells. *Life Sci*. **2006**, *78*, 2488-2493.

(5) Kondo, K.; Kurihara, M.; Miyata, N.; Suzuki, T.; Toyoda, M. Scavenging mechanisms of (−)-epigallocatechin gallate and (−)-epicatechin gallate on peroxyl radicals and formation of superoxide during the inhibitory action. *Free Radic. Biol. Med.* **1999**, *27*(7-8), 855-863.

(6) Middleton, E.; Kandaswami, C.; Theoharides, T.C. The effect of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol. Rev*. **2000**, *52*, 673-751.

(7) Chung, F.L.; Schwartz, J.; Herzog, C.R.; Yang, Y.M. Tea and cancer prevention: studies in animals and humans. *J. Nutr.* **2003**, *133*, 3268S-3274S.

(8) Lozano, C.; Julià, L.; Jiménez, 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.

(9) Torres, J.L; Lozano, C.; Julià, 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.

(10) 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.

(11) Torres, J.L; Lozano, C.; Mather, P. Conjugation of catechins with cysteine generates antioxidant compounds with enhanced neuroprotective activity. *Phytochemistry* **2005**, *66*, 2032-2037.

(12) Maher, P.; Lewerenz, J.; Lozano, C.; Torres, J.L. A novel approach to enhancing cellular glutathione levels. *J. Neurochem*. **2008**, *107(3)*, 690-700.

(13) Mitjans, M.; Martínez, V.; Del Campo, J.; Abajo, C.; Lozano, C.; Torres, J.L.; Vinardell, M.P. Novel epicatechin derivatives with antioxidant activity modulate interleukin-1α release in lipopolysaccharide-stimulated human blood. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5031-5034.

(14) Mitjans, M.; Del Campo, J.; Abajo, C.; Martínez, V.; Selga, A.; Lozano, C., Torres, J.L.; Vinardell, M.P. Immunomodulatory Activity of a New Family of Antioxidants Obtained from Grape Polyphenols. *J. Agric. Food Chem*. **2004**, *52*, 7297-7299.

(15) Lozano, C.; Torres, J.L.; Julià, L.; Jiménez, A.; Centelles, J.J.; Cascante, M. Effect of new antioxidant cysteinyl-flavanol conjugates on skin cancer cells. *FEBS Lett.* **2005**, *579*, 4219-4225.

(16) Grinberg, L.N.; Newmark, H.; Kitrossky, N.; Rahamim, E.; Chevion, M.; Rachmilewitz, E.A. Protective effects of tea polyphenols against oxidative damage to red blood cells. *Biochem. Pharmacol.* **1997**, *54*(9), 973-978.

(17) Stocks, J.; Dormandy, T.L. The autooxidation of human red cell lipids induced by hydrogen peroxide. *British J. Haematol.* **1971**, *20*, 95-111.

(18) Srour, M.A.; Bilto, Y.Y.; Juma, M. Evaluation of different methods used to measure malonyldialdehyde in human erythrocytes. *Clin. Hemorheol. Microcirc.* **2000**, *23*(1), 23-30.

(19) Park, Y.H.; Han, D.W.; Suh, H.; Ryu, G.H.; Hyon, S.H.; Cho, B.K.; Park, J.C. Protective effects of green tea polyphenol against reactive oxigen species-induced oxidative stress in cultured rat calvarial osteoblast. *Cell Biol. Toxicol.* **2003**, *19*, 325-337.

(20) Borenfreund, E.; Puerner, J.A. Toxicity determined *in vitro* by morphological alterations and neutral red absorption. *Toxicol. Lett.* **1985**, *24*(2/3), 119-124.

(21) Riddell, R.J.; Clothier, R.H.; Ball, M. An evaluation of three *in vitro* cytotoxicity assays. *Food Chem. Toxicol.* **1986**, *24* (6/7), 469-471.

(22) Halliwell, B.; Chirico, S. Lipid peroxidation: its mechanism, measurement, and significance. *Am. J. Clin. Nutr.* **1993**; *57*, 715S-724S.

(23) Sroka, Z.; Cisowski, W. Hydrogen peroxide scavenging, antioxidant and anti-radical activity of some phenolic acids. *Food Chem. Toxicol*. **2003**, *41*, 753-758.

(24) Caturla, N.; Vera-Samper, E.; Villalaín, J.; Mateo, C.; Micol, V. The relationship between the antioxidant and the antibacterial preperties of galloylated catechins and the structure of phospholipid model membranes. *Free Rad. Biol. Med.* **2003**, *34* (6), 648-662.

(25) Sultana, R.; Ravagna, A.; Mohmmad-Abdul, H.; Calíbrese, V.; Butterfield, D.A. Ferulic acid ethyl ester protects neurons against amyloid ß-peptide(1-42)-induced oxidative stress and neurotoxicity: relationship to antioxidant activity. *J. Neurochem*. **2005**, *92*, 749-758.

(26) Zou, C.G.; Agar, N.S.; Jones, G.L. Oxidative insult to human red blood cells induced by free radical initiator AAPH and its inhibition by a commercial antioxidant mixture. *Life Sci*. **2001**, *69*(1), 75-86.

(27) Verstraeten, S.V.; Oteiza, P.I.; Fraga, C.G. Membrane effects of Cocoa Procyanidins in Liposomes and Jurkat T Cells. *Biol. Res*. **2004,** *37*(2), 293-300.

(28) Suwalsky, M.; Orellana, P.; Avello, M.; Villena, F.; Sotomayor, C.P. Human erythrocytes are affected *in vitro* by extracts of Ugni molinae leaves*. Food Chem. Toxicol*. **2006**, *44*(8), 1393-1398.

(29) Lázaro, E.; Castillo, J.A.; Ráfols, 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.

(30) Erlejman, A.G.; Verstraeten, S.V.; Fraga, C.G.; Oteiza, P.I. The interaction of flavonoids with membranes: Potential determination of flavonoid antioxidant effects. *Free Radic. Res.* **2004**, *38*(12), 1311-1320.

(31) Verstraeten, S.V.; Keen, C.L.; Schmitz, H.H.; Fraga, C.G.; Oteiza, P.I. Flavan-3-ols and procyanidins protect liposomes against lipid oxidation and disruption of the bilayer structure. *Free Radic. Biol. Med*. **2003**, *34*(1), 84-92.

(32) Alonso, C.; Ramón E.; Lozano, C.; Parra, J.L.; Torres, J.L.; Coderech, L. Percutaneous absorption of flavan-3-ol conjugates from plant procyanidins. *Drugs Exp. Clin. Res.* **2004**, *30*(1), 1-10.

(33) Sroka, Z. Antioxidative and antiradical properties of plant phenolics. *Z. Naturforsch. C*. **2005,** *60*(11-12), 833-843.

(34) Shi, J.; Yu, J.; Pohorly, J.E.; Kakuda, Y. Polyphenolics in grape seeds–Biochemistry and functionality. *J. Med. Food.* **2003**, *6*(4), 291-299.

(35) Pool-Zabel, BL.; Adlercreutz, H.; Glei, M.; Liegibel, U.M.; Sittlingon, J.; Rowland, I.; Wähälä, K.; Rechkemmer, G. Isoflavonoids and lignans have different potentials to modulate oxidative genetic damage in human colon cells. *Carcinogenesis.* **2000**, *21*(6), 1247-52.

(36) Fan, P.; Lou, H. Effects of polyphenols from grape seeds on oxidative damage to cellular DNA. *Mol. Cell Biochem.* **2004**, *267*, 67-74.

(37) Zhu, Q.X.; Shen, T.; Tu, D.Y.; Ding, R.; Liang, Z.Z.; Zhang, X.J. Protective effects of Ginkgo biloba leak extracts on trichloroethylene-induced human keratinocyte cytotoxicity and apoptosis*. Skin Pharmacol. Physiol*. **2005**, *18,* 160-169.

(38) Morley, N.; Clifford, T.; Salter, L.; Campbell, S.; Gould, D.; Curnow, A. Tea green polyphenol (–)-epigallocatechin gallate and green tea can protect human cellular DNA from ultraviolet and visible radiation-induced damage. *Photodermatol. Photoimmunol. Photomed*. **2005**, *21*, 15-22.

(39) Maier, K.; Schmitt-Landgraf, R.; Siegemund, B. Development of an *in vitro* test system with human skin cells for evaluation of phototoxicity. *Toxicol. In vitro*. **1991**, *5*, 457-461.

(40) Zanatta, C.F.; Ugartondo, V.; Mitjans, M.; Rocha-Filho, P.A.; Vinardell M.P. Low cytotoxicity of creams and lotions formulated with Buriti oil (*Mauritia flexuosa*) assessed by the neutral red release test. *Food Chem. Toxicol.* **2008**, *46*, 2776-2781.

(41) Dijoux, N.; Guingand, Y.; Bourgeois. C.; Durand, S.; Fromageot, C.; Combe, C.; Ferret, P.J. Assessment of the phototoxic hazard of some essential oils using modified 3T3 neutral red uptake assay. *Toxicol. In vitro.* **2006,** *20*, 480-489.

(42) Llópiz, N; Puiggròs, F.; Céspedes, E.; Arola, L.; Ardévol, A.; Bladé, C.; Salvadó, M.J. Antigenotoxic effect of grape seed procyanidin extract in Fao cells submitted to oxidative stress. *J. Agric. Food Chem*. **2004,** *52*, 1083-1087.

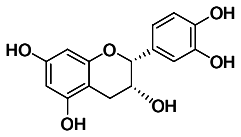
(43) Roig, R.; Cascón, E.; Arola, L.; Bladé, C.; Salvadó, M.J. Procyanidins protect Fao cells against hydrogen peroxide-induced oxidative stress. *Biochim. Biophys. Acta.* **2002**, *1572*(1), 25-30.

(44) Puiggròs, F.; Llópiz, N.; Ardévol A.; Bladé, C.; Arola, Ll.; Salvadó, M.J. Grape seed procyanidins prevent oxidative injury by modulating the expression of antioxidant enzyme system. *J. Agric. Food Chem*. **2005**, *53,* 6080-6086.

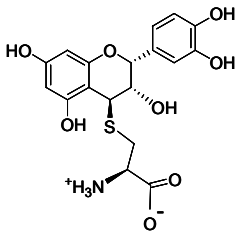
(45) Chen, X.; Nishida, H.; Konishi, T. Baicalin promoted the repair of DNA single strand breakage caused by H_2O_2 in cultured NIH3T3 fibroblast. *Biol. Pharm. Bull.* **2003**, *26*(2), 282-284.

(46) Teramoto, S.; Tomita, T.; Matsui, H.; Ohga, E.; Matsuse, T.; Ouchi, Y. (1999). Hydrogen peroxide-induced apoptosis and necrosis in human lung fibroblast: protective roles of glutathione. *Jpn. J. Pharmacol.* **1999**, *79*, 33-40.

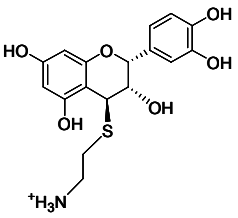
(47) Spencer, J.; Schroeter, H.; Kuhnle, G.; Srai, S.K.; Tyrrell, R.; Hakn, U.; Rice-Evans, C. Epicatechin and its *in vivo* metabolite, 3'-o-methyl epicatechin, protect human fibroblasts from oxidative-stress-induced cell death involving caspase-3 activation. *Biochem. J.* **2001**, *354*, 493-500.



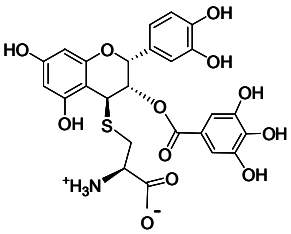
1- (–)-epicatechin



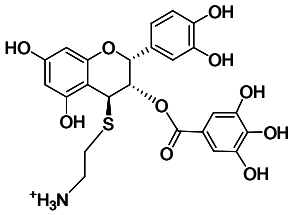
2- 4β-(*S*-cysteinyl)epicatechin



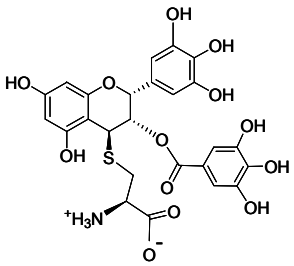
3- 4β-(2-aminoethythio)epicatechin



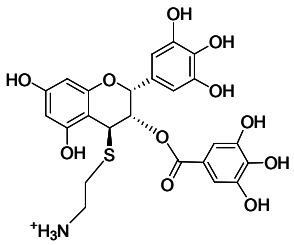
4- 4β-(*S*-cysteinyl)epicatechin 3-*O*-gallate



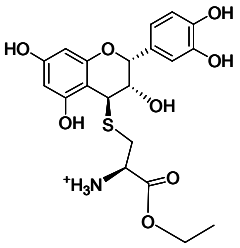
5- 4β-(2-aminoethylthio)epicatechin 3-*O*-gallate



6- 4β-(*S*-cysteinyl)epigallocatechin 3-*O*-gallate



7- 4β-(2-aminoethylthio)epigallocatechin 3-*O*-gallate



8- 4β-[*S*-(*O*-ethyl-cysteinyl)]epicatechin

Figure 1. Chemical structures of epicatechin and epicatechin conjugates.

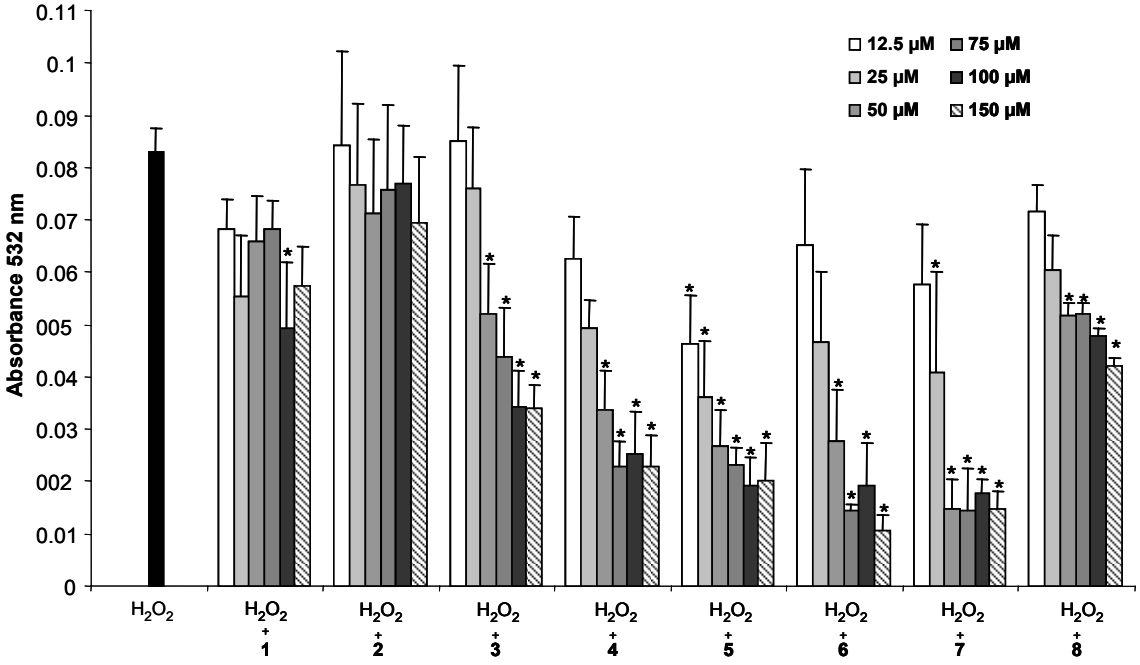


Figure 2. TBAR concentrations in erythrocytes treated with H_2O_2 20 mM with or without epicatechin derivatives. The concentrations of the MDA-TBA complex are indicated as absorbance at 532 nm. Marked compounds are statistically different to the control with H_2O_2 20 mM alone (*$p<0.05$ denotes statistically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

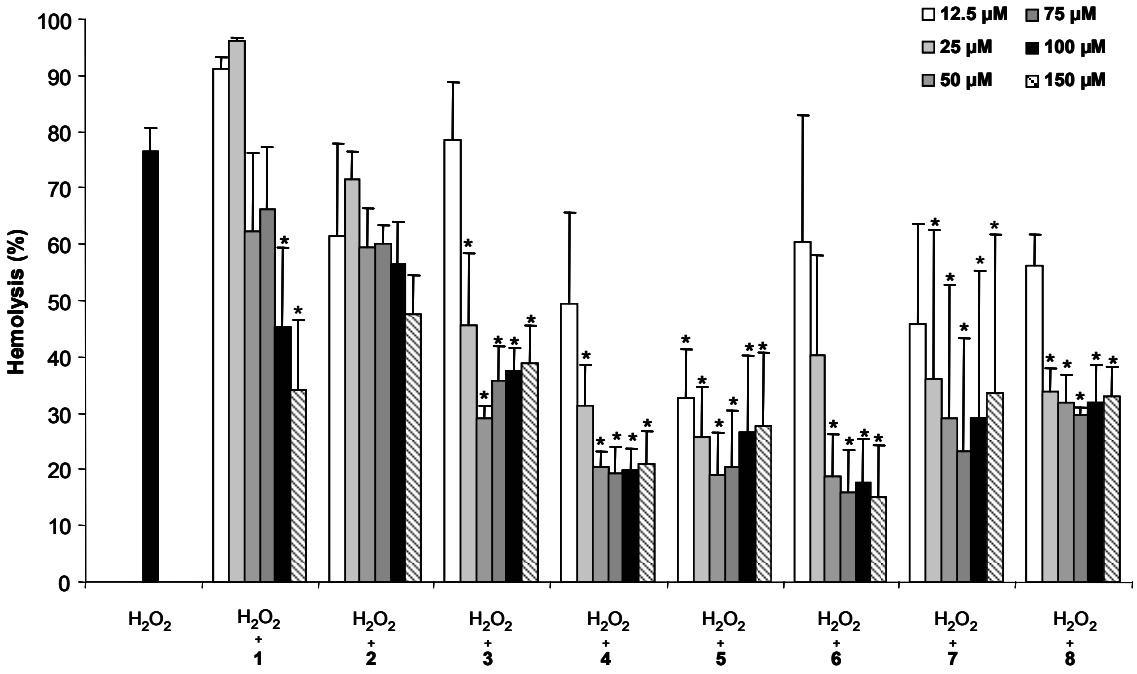


Figure 3. Effect of epicatechin derivatives on H_2O_2-induced hemolysis in erythrocytes treated with H_2O_2 20 mM or H_2O_2 plus compounds at a range of concentrations. *indicates a significant difference from H_2O_2 group ($p<0.05$).

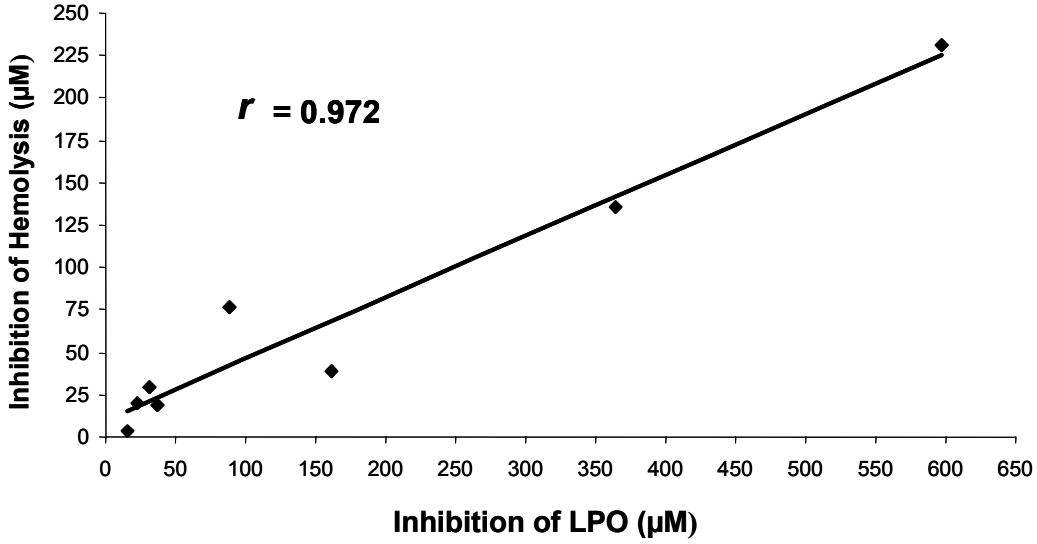


Figure 4. Correlation between the IC_{50} values for lipid peroxidation and IC_{50} values for hemolysis (IC_{50} is the concentration of product that causes 50% inhibition of LPO or hemolysis). Each point represents one of the eight products tested.

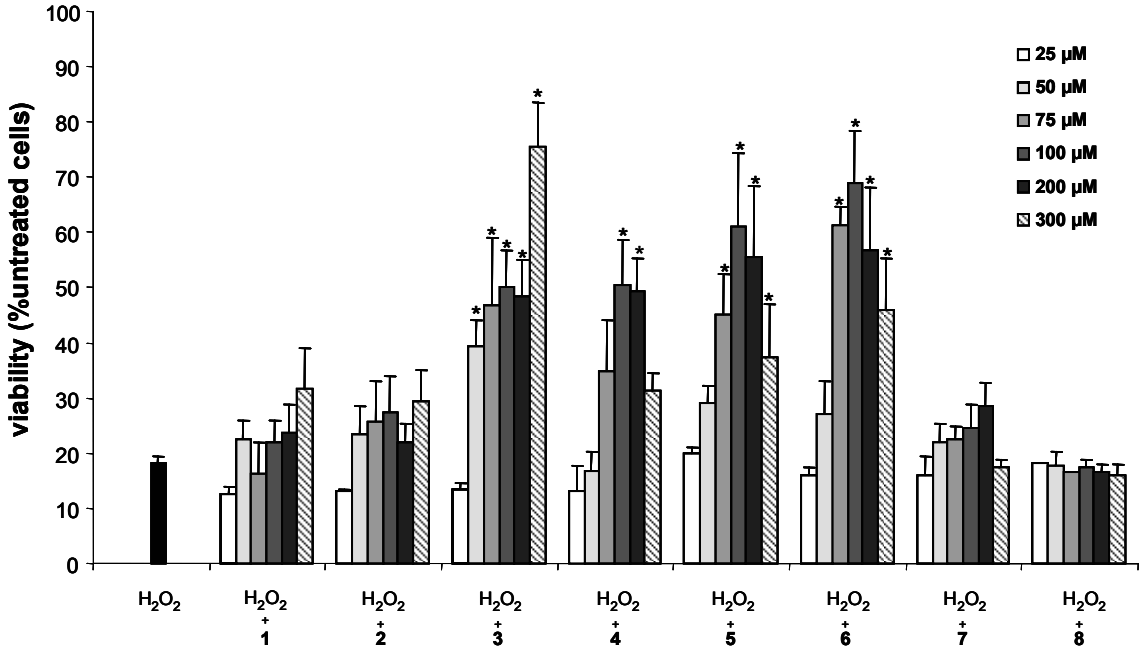


Figure 5. Comparative protective effect of epicatechin derivatives towards H_2O_2-induced cytotoxicity on 3T3 cells. Cells were preincubated overnight with the compounds, washed, and incubated with 2 mM H_2O_2 for 2 h. Cell viability are expressed as percentages of the untreated cells (100%). *$p<0.05$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

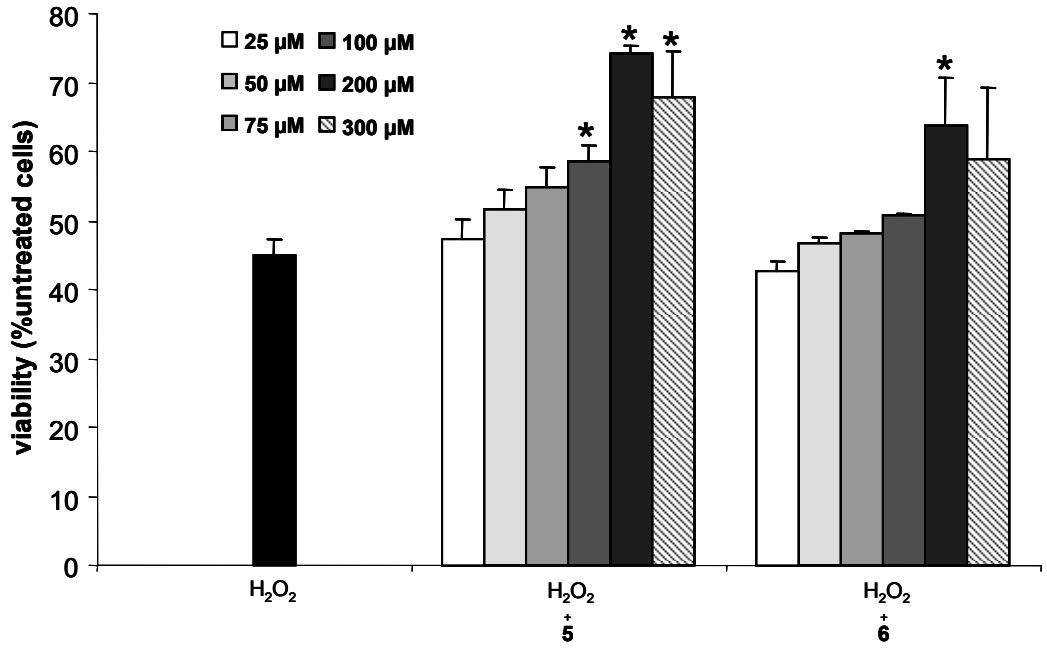


Figure 6. Comparative protective effect of compounds **5** and **6** against H_2O_2-induced cytotoxicity on HaCaT cells. Cells were pre-incubated overnight with the compounds, washed and incubated with 2.5 mM H_2O_2 for 2 h. Cell viability is expressed as percentages of the untreated cells (100%). *$p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

Bloque III. Interacción de la epicatequina y sus derivados con la membrana del eritrocito

INTRODUCCIÓN

La membrana celular es una barrera de difusión que protege el interior de la célula y debido a su localización, tanto su estructura como su función son muy propensas a sufrir alteraciones por la interacción con agentes extraños. Los eritrocitos (RBC) son un modelo celular ampliamente utilizado para investigar el daño oxidativo en biomembranas debido a su abundancia en ácidos grasos poliinsaturados y en proteínas de membrana, a su función de transportadores de O_2 y a la presencia de elevadas concentraciones de hemoglobina. Debido a estas características, los eritrocitos son muy susceptibles al estrés oxidativo y constituyen una de las primeras dianas del ataque de radicales libres. Además, aunque son menos especializadas que las otras células, comparten suficientes funciones en común (transporte activo y pasivo, producción de gradientes iónicos y eléctricos...) como para ser considerados unos buenos representantes de la membrana plasmática en general (Suwalsky y col., 2006).

La exposición de los eritrocitos a diferentes sistemas generadores de radicales del oxígeno, puede desencadenar peroxidación lipídica, cambios en la conformación de las proteínas y/o su degradación, cambios en la morfología celular, entrecruzamiento de proteínas y lípidos, inactivación de enzimas, perturbación de la bicapa lipídica de la membrana y hemólisis (Srour y col., 2000). Numerosos estudios han demostrado que una gran variedad de antioxidantes de origen natural, entre ellos los compuestos polifenólicos pueden proteger o incrementar la resistencia de los eritrocitos a este estrés oxidativo (Carini y col., 2000; Ajila y Prasada Rao, 2008).

Los flavonoides son compuestos fenólicos presentes en las plantas cuyos efectos beneficiosos para la salud son cada vez más aceptados, aunque los mecanismos a través de los cuales actúan aún son bastante desconocidos. Entre estos mecanismos se proponen la capacidad antioxidante, la quelación de metales, la inhibición enzimática y la regulación génica (Erlejman y col., 2004). Además, cada vez se acepta más la idea de que la localización de los flavonoides en la membrana podría dictar su capacidad antioxidante (Saija y col., 1995).

Con el objetivo de conocer el mecanismo de interacción de los flavanoles con la membrana celular del eritrocito, se han realizado una serie de ensayos preliminares del efecto de la epicatequina y una serie de tiol conjugados previamente obtenidos por nuestro grupo (Torres y Bobet, 2001; Lozano y col., 2006). En estos trabajos se han utilizado dos modelos para generar estrés oxidativo en eritrocitos, el AAPH (2,2´-Azobis (amidinopropano) dihidrochlorido) y el peróxido de hidrógeno (H_2O_2). La descomposición del compuesto azo AAPH a temperatura fisiológica genera radicales libres *in vitro* que atacan la membrana del eritrocito induciendo oxidación de los lípidos y proteínas y finalmente hemólisis. Por otro lado, el H_2O_2 se considera una especie reactiva del oxígeno clave debido a su alta estabilidad, difusión y participación en cascadas de señalización celular. Además, atraviesa fácilmente la membrana celular y provoca la aparición del radical hidroxilo ($OH^{\cdot}$), que reacciona con diferentes componentes celulares como lípidos y proteínas, produciendo finalmente daños en la célula (Lee y Jeong, 2007).

Se ha estudiado la interacción del flavanol epicatequina y sus derivados con la membrana de los eritrocitos y su capacidad para prevenir o revertir las alteraciones de membrana causadas por dos agentes oxidantes, el AAPH y el H_2O_2. Se han caracterizado (1) la capacidad de modulación de la fluidez de membrana, (2), los cambios morfológicos de los eritrocitos y (3), la alteración en el patrón de proteínas de membrana debido a los procesos oxidativos.

Estos estudios se han realizado fundamentalmente para poner a punto en nuestro laboratorio las técnicas correspondientes de fluidez, microscopia y electroforesis SDS-PAGE con eritrocitos. Por otro lado, permitirán plantear los posibles mecanismos de interacción de los flavonoides con las membranas celulares y los de protección frente al estrés oxidativo, para en un futuro poder profundizar en tales mecanismos con todos los compuestos de naturaleza flavonoide que integran nuestra línea de trabajo.

MATERIAL Y MÉTODOS

Productos químicos. Los tiol conjugados utilizados en este estudio se obtuvieron por despolimerización ácida de procianidinas procedentes de bagazo de uva (*Vitis vinífera*) y de corteza de hamamelis (*Hamamelis virginiana*) según el procedimiento descrito previamente (Torres y Bobet, 2001; Lozano y col., 2006). Los compuestos estudiados han sido: (−)-Epicatequina (**Ec**), 4β-(*S*-cisteinil)epicatequina (**Cys-Ec**), 4β-(*S*-cisteinil)epicatequina 3-*O*-galato (**Cys-EcG**), 4β-(*S*-cisteinil)epigalocatequina 3-*O*-galato (**Cys-EgcG**). (−)-Epicatequina, peróxido de hidrógeno, 2,2´-Azobis (amidinopropano) dihidrochlorido (AAPH), Ázida sódica, Albúmina sérica bovina (BSA), han sido suministradas por Sigma-Aldrich (St. Louis, MO); Tris y dodecil sulfato sódico (SDS), por Merck (Darmstandt, GE); Acrilamida 40%, Bisacrilamida 2%, TEMED, Persulfato de amonio, ß-Mercaptoetanol y Azul de Bromofenol, por GE Healthcare Bio-Sciences AB (Uppsala, SE); estándar de proteínas (Precision Plus Unstained Standards) por BioRad (España).

Preparación de la suspensión de eritrocitos. La sangre humana ha sido proporcionada por el Banco de Sangre del Hospital Vall d'Hebrón de Barcelona (España) siguiendo las guías éticas de la institución. Se han lavado los eritrocitos (RBC) tres veces en una solución de tampón fosfato (PBS) compuesta por 123,3 mM NaCl, 22,2 mM Na_2HPO_4 y 5,6 mM KH_2PO_4 en agua destilada (pH 7,4; 300 mOsmol/L) para eliminar el plasma, las plaquetas y los leucocitos. Después del último lavado, las células se resuspendieron en PBS a una densidad de 8 x 10^9 céls/mL. El hematocrito se ajustó al 12,5% en las condiciones de incubación de las muestras con los diferentes compuestos.

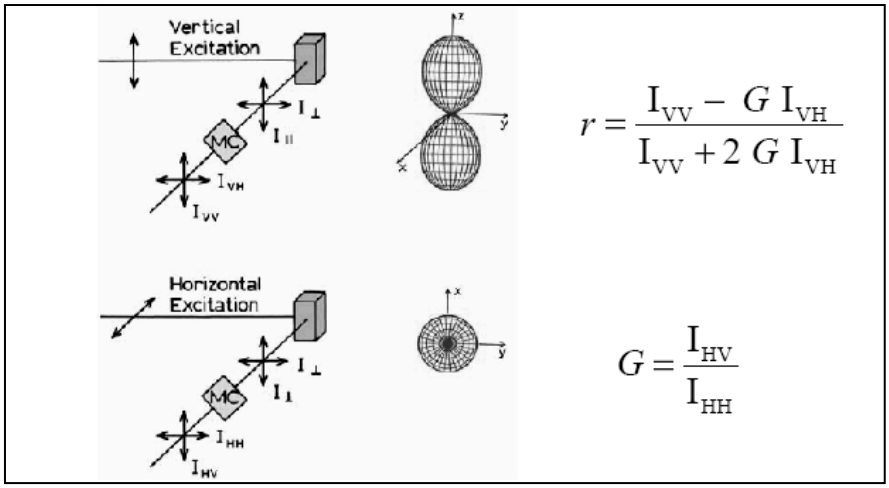
Las muestras de los ensayos realizados con H_2O_2 fueron preincubadas durante 15 minutos en rotación continua con ázida sódica a concentración 2 mM con el fin de inactivar la catalasa de los eritrocitos.

Tratamientos experimentales.

Efecto sobre la fluidez de membrana y la morfología de los eritrocitos. Para inducir las alteraciones en la membrana de los eritrocitos se utilizaron dos agentes oxidantes, el iniciador de radicales peroxilo AAPH y el peróxido de hidrógeno. Se incubaron 250 µL de la suspensión de eritrocitos con AAPH a una concentración final de 100 mM durante 90 minutos o con H_2O_2 a una concentración final de 20 mM durante 60 minutos. En ambos casos la incubación se realizó a 37 ºC y agitación constante. Estos períodos de incubación fueron previamente determinados mediante curvas de tiempo para establecer unas condiciones en las que los compuestos oxidantes no provocaran más del 30% de hemólisis. El mismo ensayo se realizó para estudiar la acción de la Ec y sus derivados sobre las alteraciones de membrana. Se añadieron los compuestos Ec, Cys-Ec y Cys-EcG (disueltos en PBS) a una concentración única de 75 µM en presencia o ausencia de los agentes oxidantes. Además, en cada ensayo se incluyeron controles de eritrocitos sin tratar.

Ensayos de oxidación de las proteínas de membrana. El diseño fue el mismo que para determinar la fluidez y los cambios morfológicos de los eritrocitos con la excepción que en los ensayos en los que el agente oxidante fue el H_2O_2 la incubación fue de 40 minutos. Además, se incluyó el Cys-EgcG, también a una concentración de 75 µM, tanto en los ensayos con AAPH como con H_2O_2.

Determinación de la anisotropía por emisión de fluorescencia. Para determinar la fluidez de membrana celular, se han elegido como marcadores extrínsecos el 1,6-difenil-1,3,5-hexatrieno (DPH) y la 1-(4-feniltrimetilamonio)-6-fenil-1,3,5-hexatrieno ρ-sulfonato de tolueno (TMA-DPH), ya que son sondas fluorescentes que se intercalan en distintas regiones de las membranas de los eritrocitos (Lúcio y col., 2007). El DPH es una molécula hidrofóbica que se incorpora en una zona central de la bicapa lipídica mientras que el TMA-DPH, en cambio, se acumula básicamente en la membrana externa debido a la presencia de grupos polares (grupos trimetilamonios). Para determinar la anisotropía de fluorescencia de las sondas en estado de equilibrio, se han añadido las sondas fluorescentes (concentración final de 10^{-6} M) a la suspensión de eritrocitos tratados y control con un hematocrito final de 0,01% en PBS. Posteriormente se han incubado a temperatura ambiente y en oscuridad, durante una 1 hora. Finalizado este período, se ha procedido a la determinación de anisotropía utilizando cubeta de cuarzo, en un espectrofluorímetro (AB-2 SLM-Aminco) y con polarizadores en configuración L, a temperatura ambiente. Las muestras se han iluminado con luz monocromática polarizada linealmente (vertical u horizontalmente) de longitud de onda 365 nm (λ_{ex}) y se han registrado las intensidades de fluorescencia emitida (λ_{em}= 425 nm) paralelas o perpendiculares a la dirección del rayo de excitación (ancho de banda 8 nm). La anisotropía de fluorescencia (*r*) se calculó automáticamente con el programa informático incorporado en el espectrofluorímetro mediante la fórmula que aparece en la Figura 32:



$$r = \frac{I_{VV} - G\, I_{VH}}{I_{VV} + 2\, G\, I_{VH}}$$

$$G = \frac{I_{HV}}{I_{HH}}$$

Figura 32. Esquema del procedimiento de excitación y emisión de la luz fluorescente. Se indica la fórmula para el cálculo de la anisotropía de fluorescencia (*r*).

Según la fórmula indicada anteriormente, Ivv representa la intensidad de emisión en paralelo e Ivh es la intensidad de emisión en perpendicular. Las intensidades de emisión se corrigen con el factor G = Ihv/Ihh (Shinitzky y Barenholz, 1978).

Estudios del efecto sobre la morfología de los eritrocitos. Después de los tratamientos correspondientes, se añadió a cada suspensión de eritrocitos 1 mL del fijador glutaraldehido al 5% en solución amortiguadora de fosfatos 0,1 M (pH 7,4) y se mantuvieron a 4 ºC durante 1 hora. A continuación se centrifugaron las muestras a 1000g durante 10 minutos, se descartaron los sobrenadantes y se añadió 1 mL del fijador glutaraldehido al 2,5%. Posteriormente se realizaron dos lavados de 15 minutos con solución amortiguadora de fosfato 0,1 M (pH 7,4) y se procedió a la postfijación con tetraóxido de osmio al 1 % en solución amortiguadora de fosfato. Después de permanecer en esta solución 1 hora a 4 ºC, se lavaron las muestras con agua destilada y a continuación se deshidrataron con solución de alcohol etílico de gradación creciente (50°, 70°, 90°, 96°, y absoluto). Finalmente, las muestras se secaron mediante la técnica del punto crítico, se montaron sobre soportes de aluminio y se recubrieron de oro. Una vez montadas las muestras, se observaron en un microscopio electrónico de barrido (SEM) con un potencial de aceleración de 15 kV (Hitachi S2300, Japón)(Figura 33) y se tomaron imágenes fotográficas a diversos aumentos en función de la calidad de las muestras. La preparación y observación de ha realizado en los Servicios Científico-técnicos de la Universidad de Barcelona (UB).

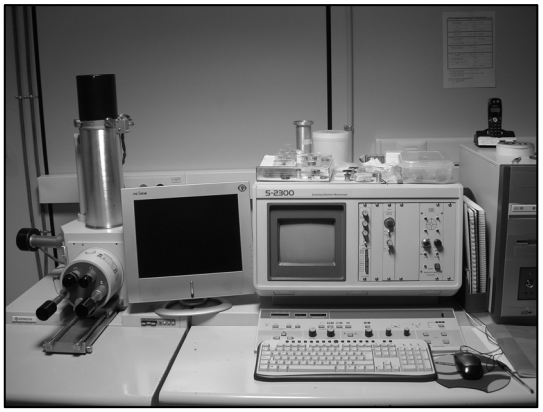


Figura 33. Microscopio electrónico de barrido modelo Hitachi S2300.

Evaluación del daño oxidativo en las proteínas de membrana de los eritrocitos en gel de poliacrilanida (SDS-PAGE). La oxidación de las proteínas de membrana de los eritrocitos se determinó por electroforesis en gel de poliacrilamida (SDS-PAGE) según el procedimiento descrito por Fairbanks y col., 1971, basado en el método clásico de Laemmli, 1970. Este sistema se caracteriza por emplear dos tipos de geles: el gel concentrador de alta porosidad (4,8% de acrilamida), que sirve para que la muestra se compacte, entrando a la separación como una banda estrecha, y el gel separador, de tamaño de poro más pequeño (7,5% de acrilamida) en el cual tiene lugar la separación de las proteínas según su tamaño. El montaje del sistema de electroforesis (Mini-Protean 3, BioRad) se hizo de acuerdo a las instrucciones del fabricante y una vez polimerizados los geles se depositaron en la cubeta con tampón de electroforesis (Figura 34). Después de someter los eritrocitos a los tratamientos experimentales antes descritos, se procedió a la determinación de la proteína presente en cada muestra mediante el método de Bradford (1976) utilizando el reactivo BioRad y la BSA como estándar, para cargar aproximadamente en el gel el equivalente a unos 15 µg de proteína. Una vez cargadas las muestras y el patrón de peso molecular (10-250 kD) se conectó la corriente, primero a 60 V durante 10 minutos y después a 200 V 35 minutos. Las bandas de proteínas se visualizaron mediante tinción con Azul Brillante de Coomassie durante 2 horas en agitación constante.

Análisis estadísticos. Los datos de la anisotropía por emisión de fluorescencia se expresan como la media ± ESM de tres experimentos independientes. Los datos se analizaron mediante un análisis de la varianza ANOVA seguido de los test post hoc Dunnett y Scheffé, utilizando el programa SPSS (SPSS Inc., Chicago, IL, USA). $p< 0,05$ se consideró estadísticamente significativo.

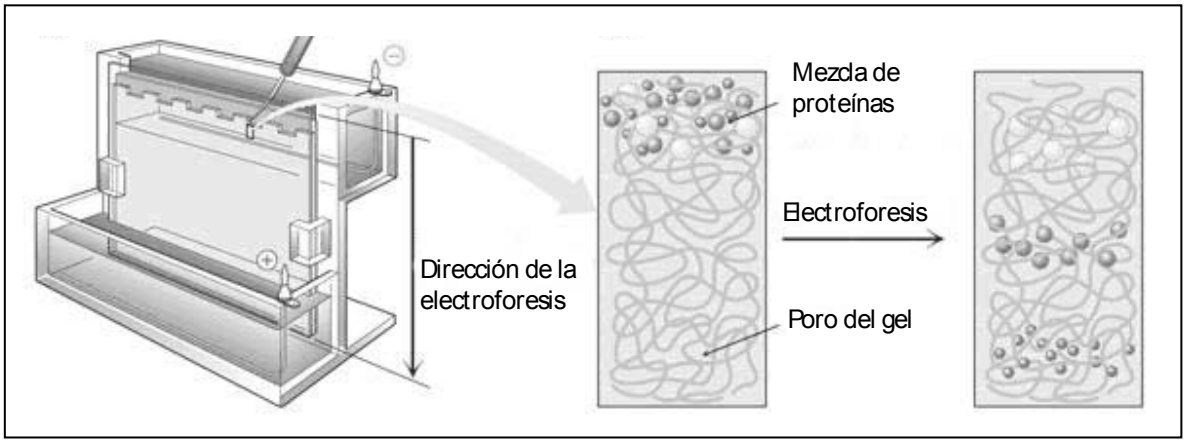


Figura 34. Cubeta de electroforesis y detalle del gel de poliacrilamida (imagen modificada de www.imb_jena.de/.../proteins_purification.html).

RESULTADOS Y DISCUSIÓN

Muchos estudios han mostrado la potencial capacidad de diversos compuestos antioxidantes procedentes de fuentes vegetales para alterar, contrarrestar o prevenir los efectos negativos del estrés oxidativo. Los polifenoles, y entre ellos los flavonoides, tienen un importante papel en la defensa de las células contra este insulto oxidativo (Tedesco y col., 2001).

Cuando los eritrocitos se someten a un importante estrés oxidativo, la membrana plasmática es a menudo la diana inicial del daño oxidativo, produciéndose diferente grado de oxidación de los lípidos y proteínas de membrana, entrecruzamiento entre estas moléculas y, a menudo, hemólisis. La peroxidación por radicales libres de los lípidos en las membranas celulares perturba sus funciones estructurales y protectoras implicando la aparición de diversas patologías como resultado de esta oxidación. Por otro lado, los mecanismos moleculares de la acción antioxidante de los polifenoles aún no están completamente caracterizados. Pero su lipofilicidad hace que las membranas ricas en lípidos sean importantes dianas para la interacción entre antioxidantes y radicales libres. Se ha sugerido que las propiedades antioxidantes podrían residir en la habilidad de las sustancias polifenólicas para insertarse en las membranas celulares y modificar el orden de los lípidos y la fluidez (Suwalsky y col., 2006) y que la diferente localización de los flavonoides vendría determinada principalmente por sus propiedades químicas.

En el presente trabajo nos hemos propuesto comprobar si las membranas pueden ser alteradas por una serie de compuestos antioxidantes de naturaleza flavanoide y si estos compuestos pueden reducir las modificaciones o daños en la membrana originados por la exposición a diferentes agentes oxidantes.

Fluidez de membrana

Uno de los parámetros más importantes relacionados con la estructura y funcionalidad de la membrana celular es su fluidez. La localización de los derivados de epicatequina y de los agentes oxidantes en las membranas y su efecto sobre la fluidez, se evaluaron a través de su capacidad de modificar la ordenación de los lípidos mediante anisotropía de fluorescencia utilizando las sondas DPH y TMA-DPH incorporadas en la membrana de los eritrocitos. La determinación de la anisotropía de sondas fluorescentes es una de las técnicas más ampliamente utilizadas en el estudio de la influencia de las diferentes moléculas sobre las propiedades biofísicas de la membrana (Tedesco y col., 2001). La localización de las sondas es esencial para una correcta interpretación de la polarización de la fluorescencia observada. Las moléculas de TMA-DPH se cree que se acumulan y permanecen casi exclusivamente en la capa más externa de la membrana celular, ya que sus grupos polares se anclan en la interfase acuosa-lipídica mientras que los grupos hidrocarbonados entran en la parte lipídica de la membrana. La DPH, por su parte, es una molécula hidrofóbica que se incorpora en la región cercana al centro de la bicapa (Figura 35).

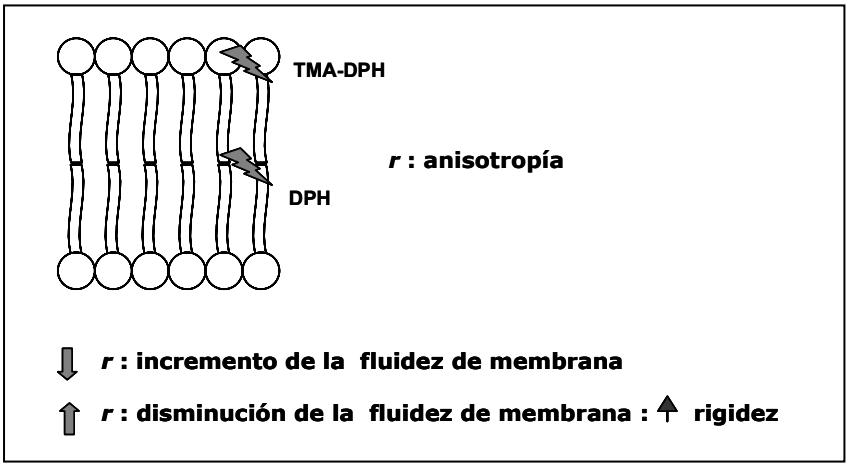


Figura 35. Localización de las sondas TMA-DPH y DPH en la membrana celular.

Los efectos ejercidos por los agentes oxidantes y la epicatequina y sus derivados sobre la fluidez de membrana, determinados mediante las sondas fluorescentes, se presentan en la Tabla 5. Los valores de anisotropía se representan por *r* y un incremento de dicho valor para una sonda es indicativo de un descenso de la fluidez de la membrana celular o, lo que es lo mismo, un aumento de su rigidez.

Tabla 5. Anisotropía de fluorescencia de las sondas en estado de equilibrio TMA-DPH y DPH incorporadas a las membranas de los eritrocitos

	(*r*) TMA-DPH (media ± ESM)	(*r*) DPH (media ± ESM)
Células no tratadas	0,3080 ± 0,0478	0,3059 ± 0,0704
Ec (75 µM)	0,3624 ± 0,0136*	0,3603 ± 0,0177*
Cys-Ec (75 µM)	0,3445 ± 0,0431*	0,3309 ± 0,0297
Cys-EcG (75 µM)	0,3792 ± 0,0063*	0,3529 ± 0,0314*
AAPH (100 mM)	0,3366 ± 0,0180*	0,3314 ± 0,0118
AAPH + Ec	0,3601 ± 0,0081*	0,3757 ± 0,0239*
AAPH + Cys-Ec	0,3613 ± 0,0155*	0,3518 ± 0,0189*
AAPH + Cys-EcG	0,3721 ± 0,0214*	0,3829 ± 0,0203*
Células no tratadas	0,3570 ± 0,0110	0,2497 ± 0,0576
Ec (75 µM)	0,3676 ± 0,0083	0,3762 ± 0,0127*
Cys-Ec (75 µM)	0,3648 ± 0,0130	0,3529 ± 0,0410*
Cys-EcG (75 µM)	0,3683 ± 0,0076	0,3495 ± 0,0294*
H_2O_2 (20 mM)	0,3579 ± 0,0104	0,3359 ± 0,0539*
H_2O_2 + Ec	0,3658 ± 0,0105	0,3548 ± 0,0506*
H_2O_2 + Cys-Ec	0,3625 ± 0,0135	0,3555 ± 0,0332*
H_2O_2 + Cys-EcG	0,3675 ± 0,0132	0,3406 ± 0,0286*

Media ± ESM de tres experimentos independientes.

* Diferencias significativas cuando se comparan con los valores obtenidos para las células no tratadas, $p<0,05$.

De los agentes oxidantes utilizados, se puede concluir que ambos causarían un incremento en la rigidez de la membrana (incremento del valor de *r*), sin embargo el efecto ejercido por el AAPH sobre la fluidez de la membrana estaría principalmente confinado a la porción exterior de la membrana (se observan diferencias significativas para la sonda TMA-DPH respecto a las células no tratadas), mientras que el ejercido por el H_2O_2 lo estaría en el interior de la bicapa (los valores de *r* para la sonda DPH son significativamente diferentes respecto a las células control). Este dato confirma las observaciones de Celedón y col., 2001 que indican que los radicales generados por el AAPH son efectivos promoviendo la oxidación de

las proteínas de membrana y la lisis celular y tienen poca habilidad para entrar en el eritrocito. Por el contrario, el H_2O_2 presenta una elevada capacidad de difusión y penetra en la membrana de los eritrocitos y reacciona con los componentes más internos de las mismas (Lee y Jeong, 2007). Respecto a los resultados obtenidos con la Ec y sus derivados se observa un incremento los valores de *r* para la sonda TMA-DPH y para la DPH respecto a los eritrocitos no tratados que indica un descenso en la fluidez de la membrana tanto en la región externa como en el núcleo interno de la bicapa. Estos datos sugieren que la posible localización de los flavonoides en la membrana no se restringe a una zona determinada, sino que abarca diferentes profundidades de la bicapa lipídica. El tratamiento conjunto de los eritrocitos con los agentes oxidantes y las moléculas de flavanoles, produjo nuevamente un incremento de la rigidez de la membrana, tanto en el caso del AAPH como en el del H_2O_2, respecto a las células no tratadas. Sin embargo no se observó ningún cambio significativo en los valores de *r* para ambas sondas al compararlos con los oxidantes solos.

Respecto al efecto de los compuestos polifenólicos sobre la fluidez de la membrana, existen diferentes trabajos que mantienen abierto el debate sobre los mecanismos de acción de estos compuestos. Arora y col., 2000, constataron en sus estudios que flavonoides como la naringenina y la rutina disminuían la fluidez en las regiones interiores de la membrana, mientras que la genisteina causaba un elevado incremento de la rigidez principalmente en el interior de la membrana, pero también contribuía, aunque en menor medida, a disminuir la fluidez en las porciones más externas de la membrana; Verstraeten y col., 2003, expusieron que la epicatequina a 25 μM no producía cambios significativos en los valores de polarización, indicando que la interacción de esta molécula con la membrana no causaba cambios en su fluidez; Tsuchiya, 2001, demostró que las catequinas reducían la fluidez de membrana de liposomas y que esta disminución era superior en las catequinas que contenían ésteres de galato. Otros autores defienden que cuando las membranas se hacen menos fluidas, pasan a ser más susceptibles a ser oxidadas debido a potenciales incrementos en la tasa de propagación. Al contrario, un incremento en la fluidez de membrana llevaría a una menor propagación de la oxidación lipídica (Verstraeten y col., 2004). El aumento de fluidez favorecería la capacidad antioxidante y las características secuestradoras de compuestos como los fenólicos, permitiendo una interacción más eficaz de las moléculas de antioxidantes con los radicales lipídicos (Lúcio y col., 2007). Pero también existe la hipótesis que defiende que los flavonoides, al imponer un mayor grado de orden estructural y rigidez en la membrana, reducirían la movilidad de los radicales libres en la bicapa lipídica. En consecuencia, el descenso en la fluidez de membrana conduciría a una inhibición de la peroxidación lipídica debido a un enlentecimiento de las reacciones radicalarias (Arora y col., 2000).

Los mecanismos moleculares de la acción antioxidante de los fenoles y flavonoides no han sido todavía elucidados y es aún un asunto de considerable debate. Los mecanismos normalmente aceptados para los efectos protectores de los flavonoides serían el secuestro de radicales y la quelación de metales. Pero además, estos compuestos podrían interaccionar con las membranas a otros dos niveles, modificando la fluidez de membrana y reduciendo, por tanto, la oxidación de lípidos o limitando el acceso de ciertas moléculas a la región hidrofóbica de la membrana para ejercer sus efectos deletéreos (Verstraeten y col., 2004). Se ha

sugerido que la habilidad de estos compuestos para situarse en las membranas celulares y la restricción resultante en su fluidez, podría dificultar la difusión de radicales libres y disminuir la cinética de las reacciones de los radicales libres (Arora y col., 2000). El efecto protector de los flavonoides estaría relacionado con el número de grupos hidroxilo en la molécula. La formación de puentes de hidrógeno entre los grupos hidroxilo de los flavonoides y los grupos de cabeza polar de los lípidos podría ser particularmente relevante para las interacciones de los flavonoides con la superficie de la membrana en la interfase agua-lípido (Erlejman y col., 2004) y ayudarían a mantener la integridad de las membranas, previniendo el acceso de moléculas hidrofóbicas, incluyendo las que afectan a la fluidez de la membrana o inducen daño a los componentes celulares.

Estas hipótesis pueden explicar el efecto de los productos estudiados en este trabajo ya que, tanto por si solos como en presencia de los agentes oxidantes inducen un incremento de la fluidez de la membrana.

Estudios del efecto sobre la morfología de los eritrocitos

El daño oxidativo que sufren los eritrocitos incluye cambios en las proteínas de membrana y en la estructura de los lípidos, que a su vez pueden inducir alteraciones en la superficie externa de las células.

El efecto de los sistemas generadores de radicales de oxígeno y de la Ec y sus derivados sobre la morfología de los eritrocitos se ha investigado estudiando los cambios de forma de estas células por microscopía electrónica de barrido (SEM). En la Figura 36 se muestran algunas de las micrografías obtenidas de los eritrocitos tratados con AAPH y H_2O_2, así como con la Ec y sus derivados Cys-Ec y Cys-EcG. Los eritrocitos no tratados aparecen con la típica forma de discocitos bicóncavos (Fig. 36a), mientras que después del tratamiento de las células con 20 mM de H_2O_2 durante 60 minutos (Fig. 36b) y con AAPH 100 mM 90 minutos (Fig. 36c) gran parte de la población celular presenta formas anormales, con predominio de formas crenadas, también conocidas como equinocitos o acantocitos. En esta condición alterada, los eritrocitos pierden su perfil normal y presentan una configuración espiculada y con protuberancias en su superficie. Estas alteraciones morfológicas provocadas por los sistemas generadores de radicales son consistentes con las de otros autores que también han constatado que el H_2O_2 induce la transformación de discocito a equinocito (Ajila y Prasada Rao, 2008; Singh y Rajini, 2008) y que el AAPH provoca la aparición de formas esféricas y sobre todo, crenadas (Sato y col., 1995). La formación de múltiples protuberancias en la membrana plasmática se considera una señal inicial de daño oxidativo de la célula y si estas elongaciones se extienden, pueden acabar rompiendo la membrana y liberando los componentes intracelulares. La peroxidación lipídica de la membrana y el daño en las proteínas del citoesqueleto se consideran factores importantes en la formación de estas protuberancias (Singh y Rajini, 2008).

La observación mediante SEM de los eritrocitos indica que tanto la Ec como sus derivados, inducen por sí mismos cambios en la forma normal de las células. La proporción de discocitos normales disminuye respecto a las células no tratadas y aparecen formas espiculadas (equinocitos) y formas aplanadas conocidas como leptocitos. La Cys-Ec y la Cys-EcG parecen alterar en mayor grado la forma normal de los eritrocitos que la Ec, pero no de una forma relevante. Estos resultados

preliminares indicarían que existe una interacción entre estos derivados flavonoides y la membrana de los eritrocitos que lleva a una modificación de las características morfológicas de estas membranas. No obstante, en nuestras condiciones experimentales, la Ec y sus derivados Cys-Ec y Cys-EcG, no consiguieron revertir de forma significativa las alteraciones morfológicas inducidas por el AAPH y el H_2O_2. En el caso del AAPH, la Ec resulta ser la más efectiva ya que la proporción de formas espiculadas respecto a las células tratadas sólo con AAPH parece disminuir, en cambio, la Cys-Ec y la Cys-EcG no consiguen modificar esta proporción. En los ensayos con H_2O_2, sin embargo, la Ec no presenta ningún efecto apreciable sobre las formas alteradas, pero sus tiol derivados disminuyen sutilmente la cantidad de formas alteradas. Los resultados de este estudio preliminar indican que, como en el caso de los generadores de radicales AAPH y H_2O_2, los flavanoles Ec y sus tiol derivados también inducen, aunque en una proporción inferior, alteraciones en la morfología de los eritrocitos provocando la transición de la forma normal de discocito a la forma de equinocito espiculado. Diversos trabajos han constatado que compuestos de naturaleza polifenólica, como extractos de piel de mango o polifenoles del té, son capaces de prevenir los cambios morfológicos inducidos por el H_2O_2 en eritrocitos (Ajila y Prasada Rao, 2008; Grinberg y col., 1997); en nuestro caso, no hemos observado esta prevención.

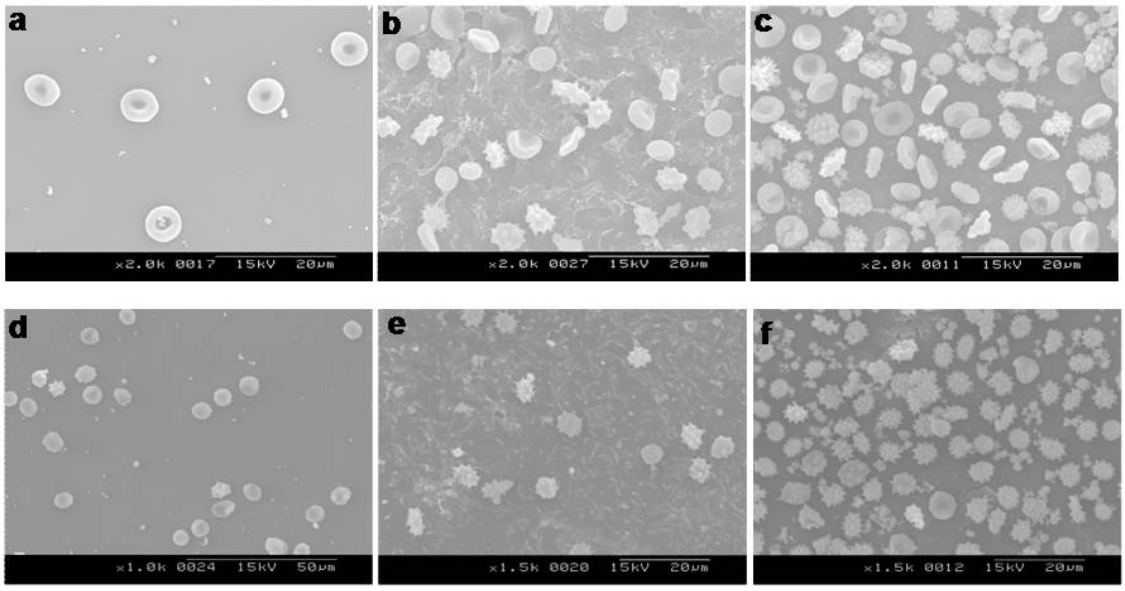


Figura 36. Microscopía electrónica de barrido de eritrocitos humanos. (a) eritrocitos no tratados; (b) eritrocitos incubados con H_2O_2 20 mM; (c) eritrocitos incubados con AAPH 100 mM; (d) eritrocitos incubados con Cys-Ec 75 µM; (e) eritrocitos incubados con H_2O_2 y Cys-Ec 75 µM; (f) eritrocitos incubados con AAPH y Cys-Ec 75 µM.

El daño oxidativo en las membranas celulares conduce a alteraciones en la rigidez y en la forma. El estrés oxidativo provoca la formación de equinocitos debido al daño en la membrana, lo que a su vez provocará cambios en la conformación estructural general del eritrocito y de su funcionalidad. De acuerdo con la hipótesis de la bicapa lipídica (Sheetz y Singer, 1974; Lim y col., 2002) los cambios de forma inducidos en los eritrocitos por moléculas extrañas se deben a expansiones

diferenciales de las dos monocapas de la membrana. Según esta hipótesis, los equinocitos espiculados se formarían cuando los compuestos se insertaran en la monocapa más externa de la bicapa (por lo tanto, el área de esta capa se incrementaría respecto a la capa interna), mientras que los estomatocitos, eritrocitos que presentan una morfología redondeada, con forma de copa, se formarían cuando los compuestos se insertaran en la monocapa interna (Singh y Rajini, 2008) (Figura 37).

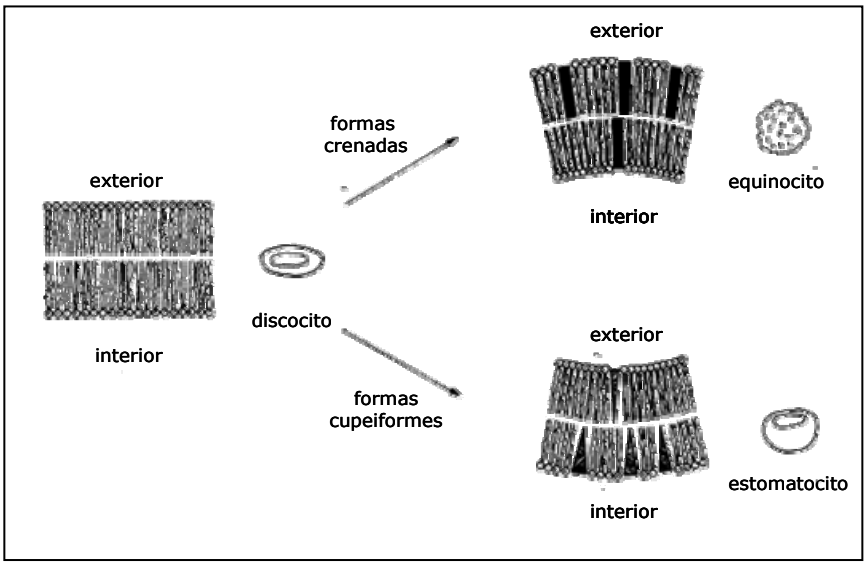


Figura 37. Representación esquemática de la hipótesis de la bicapa (Adaptado de Sheetz y Singer, 1974; Vermehren y Hansen, 1998).

Nuestros resultados preliminares muestran que la Ec y sus tiol conjugados no consiguen neutralizar del todo el efecto de los compuestos oxidantes sobre la membrana de los eritrocitos y que estos flavanoles por sí mismos, producen ciertas alteraciones en la morfología de los eritrocitos lo que confirma su interacción con las membranas celulares. De acuerdo con la hipótesis de la bicapa lipídica, el hecho de que se induzca la aparición de equinocitos indica que las moléculas de flavonoides se localizan principalmente en la monocapa más externa de la membrana de los eritrocitos. Además se ha demostrado que estos compuestos efectivamente causan cierta perturbación en el orden de los lípidos de la membrana disminuyendo su fluidez, como se ha visto en los ensayos de fluidez de membrana.

Oxidación de las proteínas de membrana de los eritrocitos

En la membrana de los eritrocitos, la bicapa lipídica está anclada a una red de proteínas esqueléticas a través de proteínas transmembrana. La Banda 3, la principal proteína integral de membrana, interacciona con este citoesqueleto que mayoritariamente incluye espectrina, actina, anquirina y proteína 4.1. La organización de esta red proteica particular en la superficie más interna de la membrana plasmática es la responsable de mantener la forma, estabilidad y deformidad de los eritrocitos (Vittori y col., 2002), por lo tanto se puede establecer una estrecha relación entre la organización de la membrana y la forma celular. Entre algunas de las alteraciones en las membranas de los eritrocitos que producen

los agentes oxidantes, encontramos una disminución del contenido de proteínas del citoesqueleto y una producción de proteínas de elevado peso molecular. Estas alteraciones pueden desencadenar anormalidades en la forma del eritrocito y, por tanto, perturbaciones en la microcirculación *in vivo*.

Las alteraciones en el patrón de proteínas de membrana producidas por los compuestos oxidantes y la Ec y sus derivados se observaron mediante electroforesis SDS-PAGE. Como agentes oxidantes nuevamente se utilizaron el AAPH y el H_2O_2, y los eritrocitos fueron tratados con las diferentes epicatequinas, con los oxidantes, o con ambos a la vez.

En la Figura 38 se presenta la separación por SDS-PAGE de las proteínas de eritrocitos tratados con AAPH y con dos de los compuestos fenólicos (Ec y Cys-Ec). Debido al tratamiento con AAPH (carril 7), parece que hay un cambio en el patrón de bandas, sobre todo en las correspondientes a la zona donde se localizaría la banda 3 (~ 95 kD). En las co-incubaciones de los oxidantes con los compuestos fenólicos (carriles 2 y 3) no se aprecia una recuperación de bandas respecto a la incubación con el agente oxidante solo, por lo que en nuestras condiciones de ensayo no podemos concluir que estos compuestos protejan la membrana del eritrocito del daño oxidativo infringido por el AAPH.

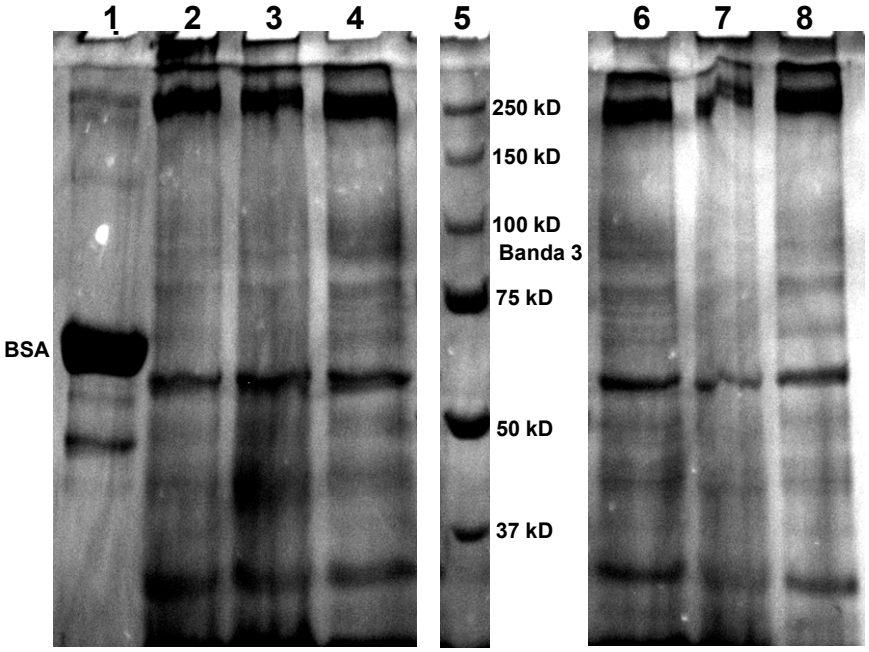


Figura 38. Electroforesis mediante SDS-PAGE (7,5%) de las proteínas de membrana de eritrocitos. Efecto del AAPH y diversos flavanoles. (1) control de BSA ;(2) eritrocitos tratados con AAPH 100 mM + Cys-Ec 75 µM durante 90 min; (3) eritrocitos tratados con AAPH 100 mM + Ec 75 µM durante 90 min; (4) eritrocitos tratados con Cys-Ec 75 µM durante 90 min; (5) Marcador de peso molecular; (6) eritrocitos tratados con Ec 75 µM durante 90 min; (7) eritrocitos oxidados con AAPH 100 mM durante 90 min; (8) eritrocitos control no tratados.

Se sabe que la oxidación de las proteínas de membrana de los eritrocitos inducida por el AAPH va acompañada por un incremento en la formación de proteínas de elevado peso molecular (HMW proteins), y por una disminución en el contenido de proteínas de bajo peso molecular (LMW proteins). Las HMW observadas podrían

formarse por entrecruzamiento directo y/o interacción de las proteínas LMW con los lípidos oxidados (Hseu y col., 2002). Una de las principales proteínas que se ve afectada por el tratamiento con AAPH es la Banda 3, cuya banda decrece. Se postula que esta proteína sufre entrecruzamientos y se polimeriza contribuyendo así a la aparición de las bandas correspondientes a proteínas HMW (Sato y col., 1995), indicativas de agregación proteica.

En la Figura 39 se muestra el patrón de bandas de eritrocitos tratados con H_2O_2 y con dos de los tiol derivados de la Ec (Cys-EcG y Cys-EgcG).

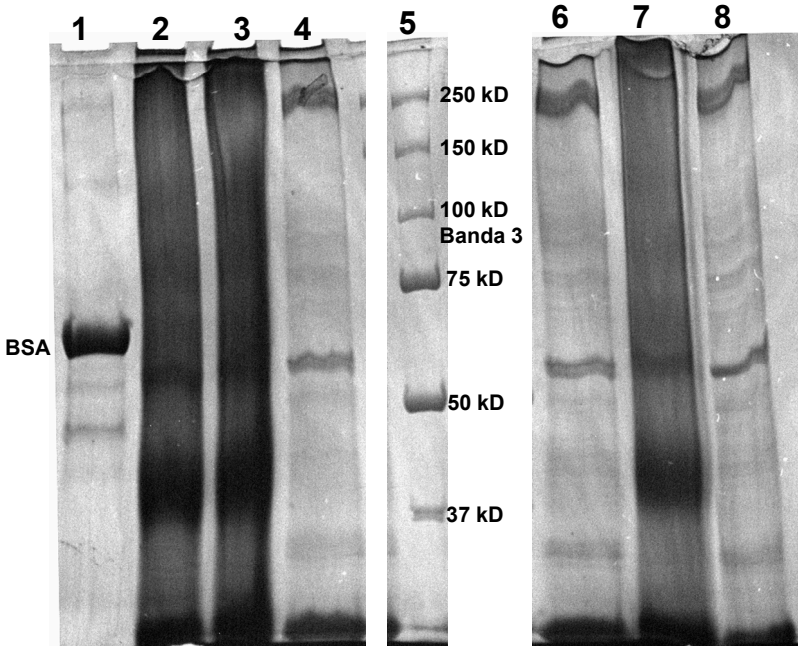


Figura 39. Electroforesis mediante SDS-PAGE (7,5%) de las proteínas de membrana de eritrocitos. Efecto del H_2O_2 y diversos flavanoles. (1) control de BSA; (2) eritrocitos tratados con H_2O_2 20 mM + Cys-EgcG 75 µM durante 40 min; (3) eritrocitos tratados con H_2O_2 20 mM + Cys-EcG 75 µM durante 40 min; (4) eritrocitos tratados con Cyc-EgcG 75 µM durante 40 min; (5) Marcador de peso molecular; (6) eritrocitos tratados con Cys-EcG 75 µM durante 40 min; (7) eritrocitos oxidados con H_2O_2 20 mM durante 40 min; (8) eritrocitos control no tratados.

En nuestros ensayos hemos visto que el H_2O_2 (carril 7) produce una desorganización total del patrón de proteínas respecto a las células no tratadas (carril 8) y se aprecia la formación de proteínas de bajo peso molecular. Sin embargo, la adición de los derivados de Ec (carriles 2 y 3) no evitó esta desestructuración, por lo que no se puede establecer una acción protectora de estos compuestos contra la degradación de las proteínas de membrana inducida por el H_2O_2. Por otro lado, el patrón de bandas normal de los eritrocitos no se vio afectado al tratar las células sólo con los flavonoides (carriles 4 y 6), observación que coincide con trabajos previos de otros autores (Singh y Rajini, 2008).

Varios estudios han mostrado que bajo el tratamiento con H_2O_2, la mayoría de las bandas por encima de la actina (~ 42kD) disminuyen, mientras que aparecen nuevas bandas en la región correspondiente a proteínas de bajo peso molecular

(indicativas de procesos de degradación de proteínas). Esto podría deberse a la rotura de las proteínas dañadas por las proteasas. La activación de la proteolisis se ha visto que se produce después de someter a los eritrocitos al ataque con diferentes generadores de radicales (Davis y Goldberg, 2001). Estos cambios pueden desencadenar alteraciones en la forma y función de estas células (Ajila y Prasada Rao, 2008). Estos estudios también demostraron que en el tratamiento conjunto de los eritrocitos con el peróxido y los antioxidantes, se recuperaba el patrón de bandas propio de las células no tratadas en diferentes grados, por lo que los antioxidantes protegerían la membrana de los eritrocitos de la degradación de proteínas inducida por el peróxido.

Así, la Ec y los derivados con cisteína ensayados en este estudio no modificarían el patrón de proteínas de los eritrocitos por sí mismos, pero tampoco protegerían contra la degradación de las proteínas de membrana inducida por el AAPH y el H_2O_2 en nuestras condiciones experimentales. En estos estudios preliminares tampoco hemos podido establecer un comportamiento diferente de los derivados de Ec relacionado con su diferente estructura, es decir, la presencia del grupo tiol (cisteína) y el diferente contenido en grupos galato.

CONCLUSIONES

Los radicales libres atacan a los componentes de las membranas de los eritrocitos, especialmente a los lípidos y proteínas, causando cambios en la estructura y función de las membranas. Numerosos compuestos de naturaleza polifenólica han sido propuestos como agentes antioxidantes capaces de prevenir, evitar o disminuir este daño oxidativo sobre las membranas (Tedesco y col., 2001). En el presente trabajo se han mostrado una serie de estudios preliminares que pretendían, por un lado, poner a punto las técnicas utilizadas en el mismo y, por el otro, intentar comprender mejor la interacción de un grupo de derivados flavanoides con la membrana del eritrocito y su posible efecto protector contra el daño inducido por diferentes sistemas generadores de radicales. Los resultados preliminares presentados en este trabajo muestran la importancia de considerar no sólo la estructura química de los flavonoides, sino también la naturaleza de las interacciones entre esas moléculas y las membranas al estimar su potencial capacidad antioxidante. Hemos visto que nuestros compuestos actuaban modificando la fluidez de membrana y la morfología de los eritrocitos, pero no hemos observado que alteraran el patrón proteico de las membranas. Numerosos trabajos defienden que, aparte de su reactividad hacia los radicales libres, las interacciones de los flavonoides con las bicapas lipídicas podrían contribuir a determinar su capacidad antioxidante y ser un mecanismo relevante en su protección contra la oxidación de la membrana (Verstraeten y col., 2004; Singh y Rajini, 2008) y contra la pérdida de integridad debida a agentes disruptores externos (Lúcio y col., 2007). Los flavonoides podrían estabilizar la membrana localizándose sobre todo en la interfase acuosa-lipídica de las membranas y su localización y las restricciones en la fluidez, podrían dificultar la difusión de los agentes oxidantes y evitar así sus efectos dañinos. Esta restricción en la difusión también se aplicaría a los propios radicales libres lo que podría disminuir la cinética de las reacciones oxidativas (Suwalsky y col., 2006). También se podría especular con el hecho que los cambios en la estructura de la membrana inducidos por

diversos flavonoides podrían modular la respuesta de las células a moléculas señalizadoras (Lúcio y col., 2007).

De cualquier forma, dado que los mecanismos moleculares de la acción antioxidante de los compuestos polifenólicos es aún un tema de debate abierto, se necesitan estudios y análisis más detallados para comprender mejor estos mecanismos protectores, y determinar si los resultados podrán extrapolarse a condiciones *in vivo* y si permitirán la aplicación de este tipo de compuestos en campos como la dermofarmacia o la quimioterapia.

Bloque IV. Actividad genotóxica de la epicatequina y sus tiol derivados en células 3T3

INTRODUCCIÓN

Los compuestos fitoquímicos presentes en la fruta y vegetales presentan efectos antioxidantes, antimutagénicos y anticarcinogénicos que los convierten en potenciales agentes para el tratamiento de la aterosclerosis, cáncer y otras enfermedades. Debido a sus potenciales beneficios para la salud, el consumo de productos que contienen estos compuestos ha aumentado entre la población. Pero existe poca información sobre el potencial riesgo para la salud humana que podrían suponer estos compuestos. Ciertas sustancias presentes en las plantas expresan actividades citotóxicas y genotóxicas y muestran correlación con la incidencia de ciertos tumores (Labieniec y Gabryelak, 2005). Por lo tanto, entender los beneficios para la salud y/o la toxicidad potencial de estos componentes vegetales es fundamental para garantizar su correcta y segura utilización.

Los compuestos polifenólicos derivados de las plantas, entre ellos los flavonoides, poseen una amplia variedad de propiedades farmacológicas, los mecanismos de las cuales han sido tema de considerable interés. Se les reconocen propiedades antioxidantes, y cada vez hay más evidencias de que podrían interferir en el proceso de desarrollo de tumores malignos (Urquiaga y Leighton, 2000); además, en años recientes se ha documentado que polifenoles como el resveratrol, curcumina y galocatequinas inducen apoptosis en varias líneas celulares. También se ha visto que algunos de estos flavonoides causan rotura oxidativa en las cadenas de ADN por sí mismos o en presencia de metales de transición como el cobre (Azmi y col., 2006). La mayoría de los polifenoles de las plantas poseen propiedades antioxidantes y prooxidantes y se ha propuesto que la acción prooxidante podría ser un mecanismo importante a través del cual ejercieran sus propiedades anticancerígenas e inductoras de apoptosis. Los flavonoides, a pesar de ser muy valorados por poseer un amplio espectro de actividades beneficiosas (Middleton y col., 2000), también pueden resultar genotóxicos. Así, por ejemplo, Sahu y Washington (1991) han descrito que la quercitina muestra propiedades genotóxicas *in vitro*. Sin embargo, hay que puntualizar que la dualidad de actuación antioxidante/prooxidante depende en parte del rango de concentraciones utilizado (Cemeli y col., 2008). En este sentido, hay estudios que describen que los flavonoides de la dieta a elevadas concentraciones inhiben el crecimiento, agotan el glutatión, disminuyen la viabilidad e inducen rotura del ADN en linfocitos humanos normales y en células transformadas (Duthie y col., 1997; Duthie y Dobson, 1999).

Actualmente hay mucho interés en identificar secuestradores de radicales libres o antioxidantes que inhiban el daño oxidativo del ADN y que eviten así fenómenos de mutagénesis y quizás cáncer (Fan y Lou, 2004). Uno de los pasos previos antes de investigar el potencial efecto protector de estos compuestos es comprobar la ausencia de daño sobre el ADN por ellos mismos. Es decir, es fundamental descartar posibles efectos genotóxicos y garantizar la seguridad de estos compuestos antioxidantes. Así, nos hemos propuesto determinar si el flavanol epicatequina y varios de sus derivados conjugados con cisteína obtenidos por nuestro grupo, eran capaces de inducir daño oxidativo en el ADN. Con este objetivo hemos realizado una serie de estudios preliminares utilizando como línea celular de

ensayo los fibroblastos 3T3 de ratón y el ensayo del cometa (Comet Assay) para evaluar la genotoxicidad de nuestros compuestos.

La electroforesis de células individuales en geles de agarosa (single-cell gel electrophoresis), más conocida como ensayo del cometa (Comet assay) por la morfología característica de las figuras observadas, es una técnica ampliamente utilizada para determinar y analizar la rotura del ADN en células individuales que puede ser aplicada en sistemas *in vitro*, *ex vivo* e *in vivo* (Azmi y col., 2006). Desde su desarrollo inicial (Östling y Johanson, 1984) y posteriores modificaciones para incrementar su sensibilidad (Singh y col., 1988), ha sido una técnica cada vez más utilizada como método para detectar y valorar sustancias genotóxicas (Tice y col., 2000) que además se ha ido modificando para ampliar los ámbitos de uso y sus aplicaciones. Además, ha resultado ser una herramienta muy útil para estudiar fenómenos relacionados con el estrés oxidativo en diferentes células y el uso de ciertos antioxidantes ha permitido entender mejor los mecanismos de daño ejercidos por una gran variedad de agentes, así como revelar los efectos antioxidantes/prooxidantes de varios compuestos (Azmi y col., 2006).

En este ensayo un pequeño número de células que han sido expuestas a agentes físicos o químicos, se engloban en un delgado gel de agarosa sobre un portaobjetos. Las células se lisan y el ADN puede desenrollarse y correr bajo diferentes condiciones de pH. El grado de migración del ADN puede correlacionarse con la extensión del daño en el ADN que se ha producido en cada célula individual. Las células con el ADN dañado presentan una migración incrementada desde el núcleo al ánodo y las células con el ADN roto parecen un "cometa" con una cabeza fluorescente brillante y una región de cola que se utiliza para evaluar el daño en el ADN (Duthie y Dobson, 1999; Singh y col., 1988).

Por lo tanto, con el objetivo de proporcionar una primera aproximación sobre la seguridad a nivel génico de los derivados semisintéticos de epicatequina e identificar potenciales riesgos para el organismo que podrían surgir de su uso, hemos centralizado este trabajo en el estudio de la inducción de rotura de ADN por estos compuestos en la línea celular de fibroblastos 3T3 mediante el ensayo del cometa.

MATERIAL Y MÉTODOS

Productos químicos. Los tiol derivados utilizados en este estudio fueron preparados a partir de bagazo de uva (*Vitis vinifera*) y de hamamelis (*Hamamelis virginiana*) según el procedimiento previamente publicado (Torres y Bobet, 2001; Lozano y col., 2006). Los compuestos estudiados han sido: (−)-Epicatequina (**Ec**), 4β-(*S*-cisteinil)epicatequina (**Cys-Ec**), 4β-(*S*-cisteinil)epicatequina 3-*O*-galato (**Cys-EcG**), 4β-(*S*-cisteinil)epigalocatequina 3-*O*-galato (**Cys-EgcG**). La (−)-Epicatequina, ciclofosfamida monohidrato y el peróxido de hidrógeno (H_2O_2) han sido suministradas por Sigma-Aldrich (St. Louis, MO); Fracción S9 (pooled male mouse liver S9, NADPH Regenerating system, solution A y B) por Gentest, BD Biosciences (Woburn, MA USA).

Cultivo celular. La línea celular de fibroblastos 3T3 se obtuvo del Banco de Células Eucariotas, Barcelona (España). Se cultivaron las células en frascos de 75 cm^2 en medio DMEM (4,5 g/L de glucosa) suplementado con 10% de suero fetal bovino (FBS), 2 mM de L-glutamina, 10 mM de tampón Hepes y una mezcla al 1% de penicilina (10000 U/mL)-estreptomicina (10000 µg/mL) y se mantuvieron en una incubadora a 37 ºC y 5% de CO_2. Cuando las células alcanzaron aproximadamente el 80% de confluencia, se tripsinizaron con tripsina/EDTA y se sembraron en placas de 96 pocillos a una densidad de 2,5 x 10^4 céls/mL y se incubaron durante 24 horas a 37 ºC y 5% de CO_2.

Tratamiento de las células. El diseño experimental consistió en determinar el efecto citotóxico y genotóxico de la epicatequina y tres de sus conjugados con cisteína en la línea celular de fibroblastos de ratón 3T3. Con este fin se preincubaron las células toda la noche (15 horas aproximadamente) en presencia de los diferentes flavanoles a las concentraciones adecuadas y disueltos en medio DMEM al 5% de suero fetal bovino (FBS) o en medio DMEM sin suero fetal, pero suplementado con el activador metabólico Fracción S9 (10 µg/mL). Un primer ensayo se realizó con la epicatequina a elevadas concentraciones (1, 3, 5, 7 mM) y un segundo ensayo con la epicatequina y los tres derivados, Cys-Ec, Cys-EcG y Cys-EgcG, a concentración de 300 µM. En cada ensayo se añadieron como controles células sólo con medio de cultivo, con y sin activador metabólico; SDS 0.02% en DMEM 5% de FBS, como control de citotoxicidad; H_2O_2 200 µM (en DMEM 5% FBS y DMEM sin FBS pero con Fracción S9), como control de compuesto genotóxico con y sin la presencia del activador metabólico S9; y la ciclofosfamida (CPh) 2 µg/mL (en DMEM 5% FBS y DMEM sin FBS pero con Fracción S9), como control de compuesto que sólo actúa como genotóxico al metabolizarse. Todas las soluciones fueron filtradas antes de ser añadidas a las células. Después del período de incubación, se recogieron los sobrenadantes de algunas de las condiciones de ensayo para determinar la presencia o no de metabolitos.

Ensayo de viabilidad celular. La viabilidad celular se determinó mediante el ensayo de captación del rojo neutro (NRU) realizado según Borenfreund y Puerner, 1985 y modificado para eliminar el uso del formaldehido (Riddell y col., 1986). Después de los tratamientos, se eliminó el medio de los pocillos destinados al ensayo de viabilidad, se efectuaron varios lavados con PBS y se añadió la solución de neutral red (50 µg/mL en medio RPMI sin rojo de fenol ni suero). Después de 3 horas de incubación a 37 ^oC, 5% CO_2, se aspiró el medio, se lavaron de nuevo las células dos veces con PBS y se añadió solución DESTAIN (50% de etanol absoluto/1% ácido acético en agua destilada) para extraer el colorante absorbido por las células viables. Se dejaron las placas 10 minutos agitándose en un agitador de microplacas y se determinó la absorbancia del rojo neutro en una longitud de onda de 550 nm en un lector de microplacas Bio-Rad 550. Los resultados se presentan como el porcentaje de viabilidad comparado con las células control (la densidad óptica media de las células no tratadas, sea en medio DMEM 5% de suero o DMEM sin suero pero con fracción S9, se consideran el 100% de viabilidad).

Ensayo del cometa. El grado del daño en el ADN se determinó mediante electroforesis de células individuales en geles de agarosa o ensayo del cometa. Después de los tratamientos y los lavados, se tripsinizaron los pocillos destinados al ensayo, se resuspendieron en medio DMEM 5% de FBS y se pasó la suspensión de células a Eppendorfs. Se procedió a la centrifugación, extracción del sobrenadante y

a la resuspensión del pellet de nuevo en medio DMEM 5%. A continuación se mezclaron las suspensiones celulares con 80 µL de agarosa de bajo punto de fusión al 0,9% (37 ºC) y se depositaron aproximadamente unos 75 µL de esta mezcla sobre portaobjetos previamente cubiertos con agarosa al punto de fusión normal al 1%. Inmediatamente se colocó un cubreobjetos encima de cada porta y se dejaron a Tª ambiente 10 minutos. A continuación se permite que la mezcla agarosa/células se congele colocando los portaobjetos a -20 ºC durante 6 minutos. Después de quitar los cubres se realizó la lisis alcalina (pH 10) sumergiendo los portas en tampón de lisis (2,5 M NaCl, 100 mM Na-EDTA, 10 mM Tris, 6g NaOH, 1% Tritón X-100, 1% lauril sarcosine) durante 1 hora a 4 ºC. Durante este tiempo se produce la lisis de las membranas celulares y de varias proteínas para dejar libre el ADN (en forma de nucleoide). Transcurrido este tiempo se colocaron los portaobjetos en las bandejas de electroforesis cubiertos por el tampón de electroforesis (1 mM Na_2EDTA, 300 mM NaOH en agua MiliQ pH>13) y se dejaron 40 minutos antes de conectar los electrodos para provocar el desenrollamiento, relajación y separación de las dos cadenas de ADN. La electroforesis de dejó progresar a 25 V y 300 mA, durante 30 minutos. Después de la electroforesis, se realizaron tres lavados de 5 minutos de los portas en solución Tris (0,4 M) a pH 7,5, 4ºC para eliminar los detergentes y la solución amortiguadora alcalina. Los portas se dejaron secar hasta el momento de su análisis al microscopio. Para realizar la visualización los portas se hidrataron en agua MiliQ durante 10 minutos y se tiñeron con 40 µL de DAPI (4,6 Diamino-2-Phenylindole Dihydrachloride Hydrate en una dilución de 5 µL/mL) durante otros 5 minutos. Las muestras han sido analizadas a 20x aumentos utilizando un microscopio NIKON E-600 con equipo de fluorescencia. Se analizaron 30 células (nucleoides) por porta. La extensión del daño en el ADN se expresó como el porcentaje de la fluorescencia total que migra en la cola para cada núcleo (% ADN en la cola) y se analizó mediante el programa de análisis COMET ASSAY IV (PERSPECTIVE INSTRUMENTS).

Análisis estadístico. Los datos se analizaron mediante un programa estándar SPSS® 1.4 (SPSS Inc., Chicago, IL, USA). El análisis estadístico se ha realizado mediante una prueba no paramétrica aplicando la U de Mann Whitney como estadístico de comparación. Se considera significancia si $p < 0,05$.

RESULTADOS Y DISCUSIÓN

La determinación de la citotoxicidad y la genotoxicidad son una parte fundamental de la evaluación de nuevos compuestos con potenciales usos en campos como la quimioterapia o la dermofarmacia, ya que se ha de garantizar que los productos ejerzan sus actividades de una forma segura con el mínimo efecto tóxico sobre el organismo (Savi y col., 2006). Con este objetivo se ha evaluado el potencial efecto genotóxico de una serie de derivados flavanólicos mediante la técnica del cometa. Esta prueba consiste en someter, en nuestro caso, una suspensión celular procedente de cultivos celulares expuestos a potenciales agentes tóxicos y englobada en un gel de agarosa aplicada sobre un portaobjetos, a una lisis alcalina, a un tratamiento para desenrollar el ADN y a una migración electroforética de corta duración. El ADN teñido con un fluorocromo apropiado se observa mediante un microscopio de fluorescencia. El material genético no dañado migrará agrupado en forma esférica, pero en caso de haberse producido

fragmentación del mismo, los fragmentos de menor tamaño migrarán una distancia superior, produciéndose una imagen parecida a la cola de un cometa (Figura 40).

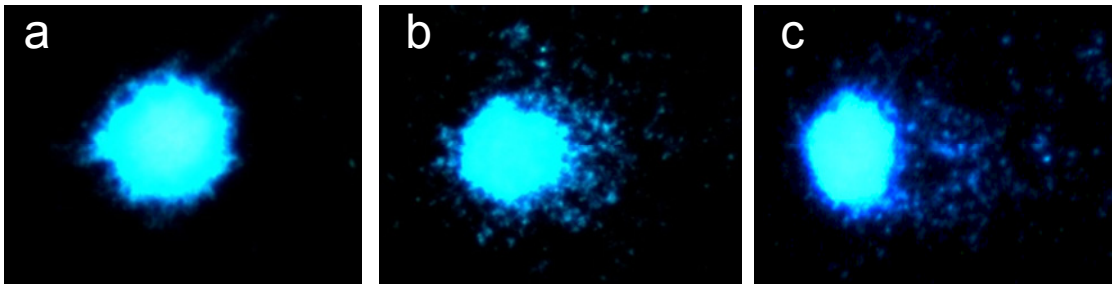


Figura 40. Fotomicrografías representativas de imágenes del cometa. (a) célula control no dañada; (b)(c) células con el material genético dañado.

Está descrito que las sustancias polifenólicas y, entre ellas los flavonoides, sufren diversos procesos de metabolización en el organismo *in vivo* (Manach y col., 2004), razón por la cual se ha intentado reproducir en nuestros ensayos dichos procesos. De esta manera se puede determinar si los metabolitos resultantes, en el caso de que nuestros compuestos fueran metabolizados, producen efectos diferentes a los compuestos originales, provocando mayor o menor citotoxicidad y/o genotoxicidad. Para ello las células han sido expuestas a los productos ensayados en presencia y ausencia de un sistema de activación metabólica adecuado. El sistema más común utilizado es una fracción (S9) postmitocondrial suplementado con cofactores, preparada a partir de hígados de roedores y tratados con agentes inductores enzimáticos. Este activador metabólico se añade al medio de cultivo DMEM sin suero fetal bovino (FBS), para evitar la reacción entre el activador S9 y el suero que resultaría citotóxica para las células.

En una primera serie de ensayos se incubaron los fibroblastos 3T3 con concentraciones crecientes de Epicatequina (1, 3, 5 y 7 mM) en presencia y ausencia del activador metabólico para determinar la capacidad de rotura de las moléculas de ADN de este compuesto. Inicialmente se optó por un rango de concentraciones tan elevadas, porque los ensayos previos de citotoxicidad (Ugartondo y col., 2006) realizados con la Ec en esta línea celular a 24 horas habían mostrado unas CI_{50} (concentración que inhibe el 50% de la viabilidad celular) dentro de este rango.

En paralelo a los estudios de genotoxicidad se realizaron estudios de actividad citotóxica que proporcionan datos relacionados con la toxicidad de los compuestos ensayados y son indispensables para acotar el rango de concentraciones seguro de trabajo. En estos ensayos de citotoxicidad se calcularon las CI_{50} (concentración de producto que causa el 50% de muerte celular) para la epicatequina en medio con y sin activador metabólico, siendo, respectivamente, 2,03 mM y 1,96 mM (Figura 41). No se apreciaron diferencias significativas entre las CI_{50} obtenidas para la Epicatequina en medio DMEM con y sin fracción S9, lo que nos indica que, en el caso de metabolizarse la epicatequina, los metabolitos que se forman no alteran la capacidad citotóxica de este flavanol. Además, se obtuvieron valores similares de viabilidad con los controles de células incubadas con DMEM al 5% de FBS y DMEM sin FBS pero con la fracción S9, lo que indica que el activador metabólico durante el período de incubación realizado no resultó citotóxico para las células.

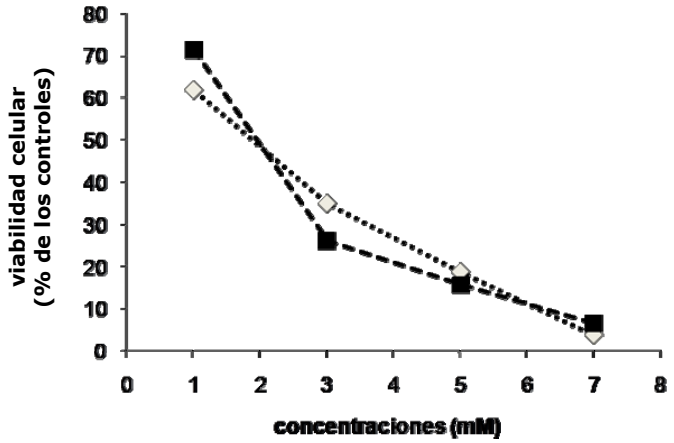


Figura 41. Citotoxicidad celular de la Epicatequina (Ec) en fibroblastos 3T3 después de 15 h de exposición. (- -■- -) Ec en medio DMEM suplementado con 5% de FBS. (···◇···) Ec en medio DMEM sin FBS y con Fracción S9.

Respecto a los resultados de actividad genotóxica, el parámetro que se utiliza para determinar el daño en el ADN es la intensidad de fluorescencia de la cola del cometa. En estos ensayos se incluyeron como controles células incubadas sólo con medio de cultivo, en presencia y ausencia del activador metabólico FS9, células incubadas con ciclofosfamida (CPh) como compuesto sólo con efecto genotóxico al sufrir metabolización y, peróxido de hidrógeno (H_2O_2), compuesto con actividad genotóxica con y sin sufrir proceso de metabolización. La epicatequina a concentraciones iguales o superiores a 1 mM presentó ya cierto efecto genotóxico. Este efecto resultó ser concentración dependiente, a pesar de que entre las concentraciones 3 mM y 5 mM no se apreciaron diferencias significativas (Figura 42). El daño ejercido sobre el ADN por las diferentes concentraciones de Ec fue similar en presencia y ausencia del activador metabólico FS9, sin ser significativa la diferencia encontrada, lo que nos indicaría que la posible metabolización de la epicatequina en nuestras condiciones de ensayo, no repercutiría en sus características genotóxicas. El Departamento de Química de Péptidos y Proteínas del Instituto de Química Avanzada de Cataluña (IQAC-CSIC), con el cual colaboramos en el marco de este trabajo, realizó la caracterización de los posibles metabolitos en las muestras tratadas con el activador metabólico FS9 mediante cromatografía HPLC concluyendo que la Ec, en nuestras condiciones de ensayo e incubación, no se metabolizaba.

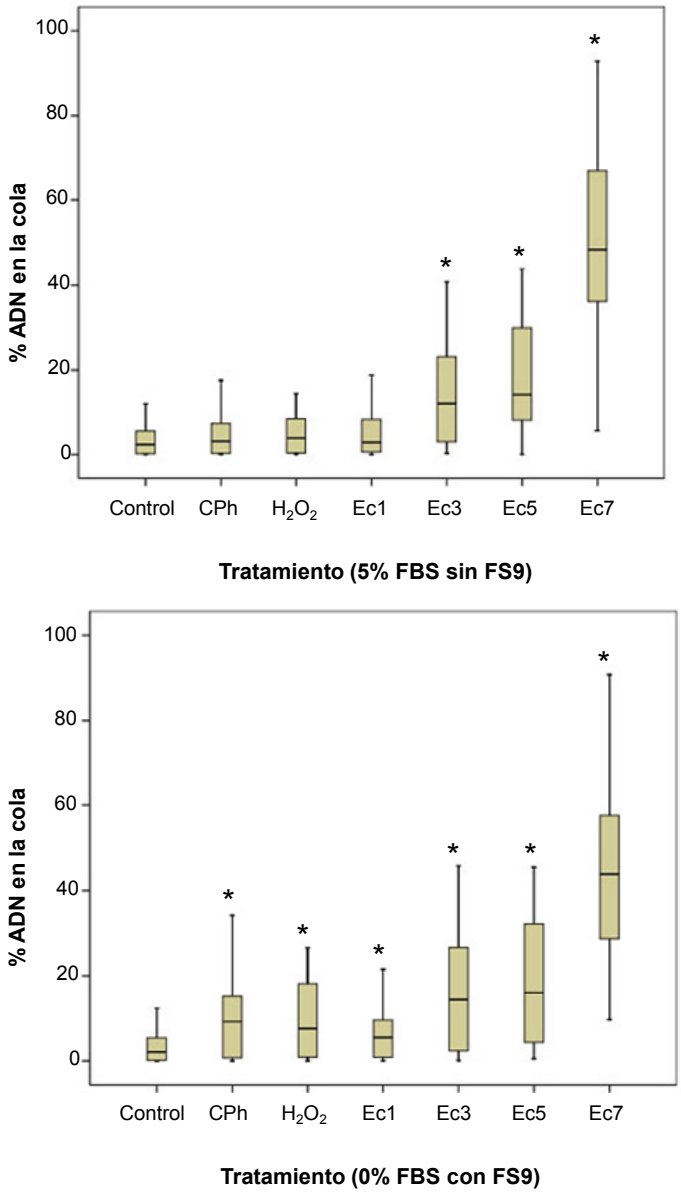


Figura 42. Evaluación de la genotoxicidad de la Epicatequina. Control: células incubadas sólo con medio de cultivo; CPh, ciclofosfamida; H_2O_2, peróxido de hidrógeno; Ec1, Ec2, Ec3, Ec4, epicatequina a concentración 1, 3, 5 y 7 mM respectivamente. (5% FBS sin FS9) medio sin activador metabólico; (0% FBS con FS9) medio con activador metabólico. *diferencias significativas respecto a las células control sólo incubadas con medio ($p<0,05$).

Los resultados indican que la epicatequina por si sola es capaz de dañar el ADN de los fibroblastos 3T3, pero hay que tener en cuenta que las concentraciones de producto a las que se está trabajando son muy elevadas y que en ningún caso podrán alcanzarse a nivel fisiológico. Igualmente, otros autores han demostrado efectos genotóxicos de la Epicatequina a concentraciones inferiores (< 500 µM) en otros tipos celulares (Savi y col., 2006), dato que respalda los resultados obtenidos en nuestros ensayos.

A partir de los datos obtenidos para la epicatequina, decidimos realizar otro ensayo a una concentración única más baja (300 μM), pero con epicatequina y tres de sus derivados con cisteína a nivel comparativo. Dado que las diferencias estructurales de las moléculas podrían influir en la capacidad genotóxica de estos compuestos, se quiso comprobar cómo afectaba la parte no fenólica y el contenido en grupos galato en el potencial efecto genotóxico de estos compuestos.

En los ensayos de citotoxicidad se pudo comprobar de nuevo que la viabilidad no se veía afectada por la adición del activador metabólico al medio de cultivo, dado que los controles sin tratamiento con y sin la FS9 dieron valores similares de viabilidad. También se observó que los derivados con cisteína y grupos galato en su estructura eran más citotóxicos que los compuestos sin galatos. Estos resultados corroboran los ensayos previos de citotoxicidad realizados por nuestro grupo en la línea celular de fibroblastos 3T3, ya que la concentración ensayada de 300 μM se aproxima a las CI_{50} obtenidas para la Cys-EcG y la Cys-EgcG en dichos ensayos (Ugartondo y col., 2006). De nuevo, no se observó que la adición del activador metabólico FS9 modificara la actividad citotóxica de estos compuestos, por lo tanto, o estos compuestos no sufren metabolización en nuestras condiciones de ensayo o si la sufren, los nuevos metabolitos no afectarían a su toxicidad (Figura 43). Como control positivo de citotoxicidad se utilizó lauril sulfato sódico (SDS).

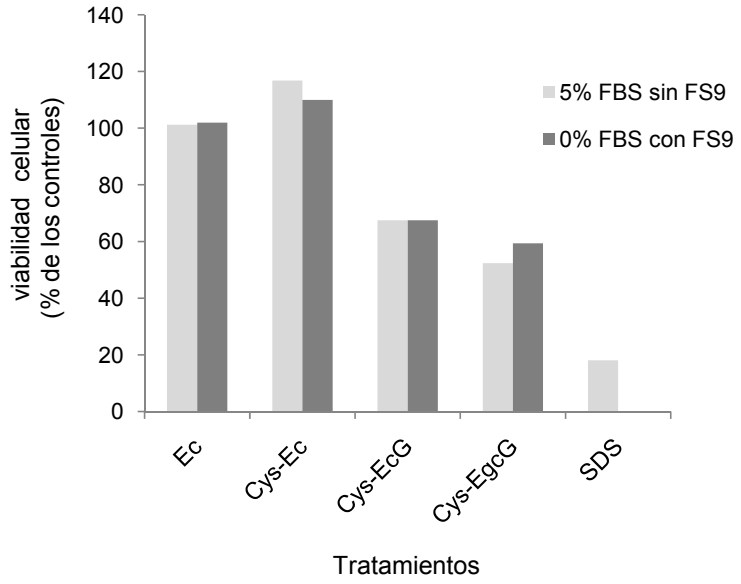


Figura 43. Citotoxicidad celular de la Epicatequina (Ec) y de sus tiol derivados en fibroblastos 3T3 después de 15 h de exposición. FBS, suero fetal bovino; FS9, activador metabólico. Los resultados se expresan en relación a los valores de las células control no tratadas.

Respecto a los ensayos de genotoxicidad, los resultados obtenidos muestran que la Epicatequina a la concentración de 300 μM no presenta actividad genotóxica, mientras que los derivados con cisteína sí que muestran cierta capacidad de dañar el ADN a esta concentración, aunque no de forma significativa en todos los casos (Figura 44). De nuevo, el daño ejercido sobre el material genético por los diferentes compuestos fue similar en presencia y ausencia del activador metabólico FS9, sin encontrarse diferencias significativas, lo que sugeriría que la posible

metabolización, en caso de darse, de la epicatequina y sus derivados en nuestras condiciones de ensayo, no repercutiría en sus características genotóxicas.

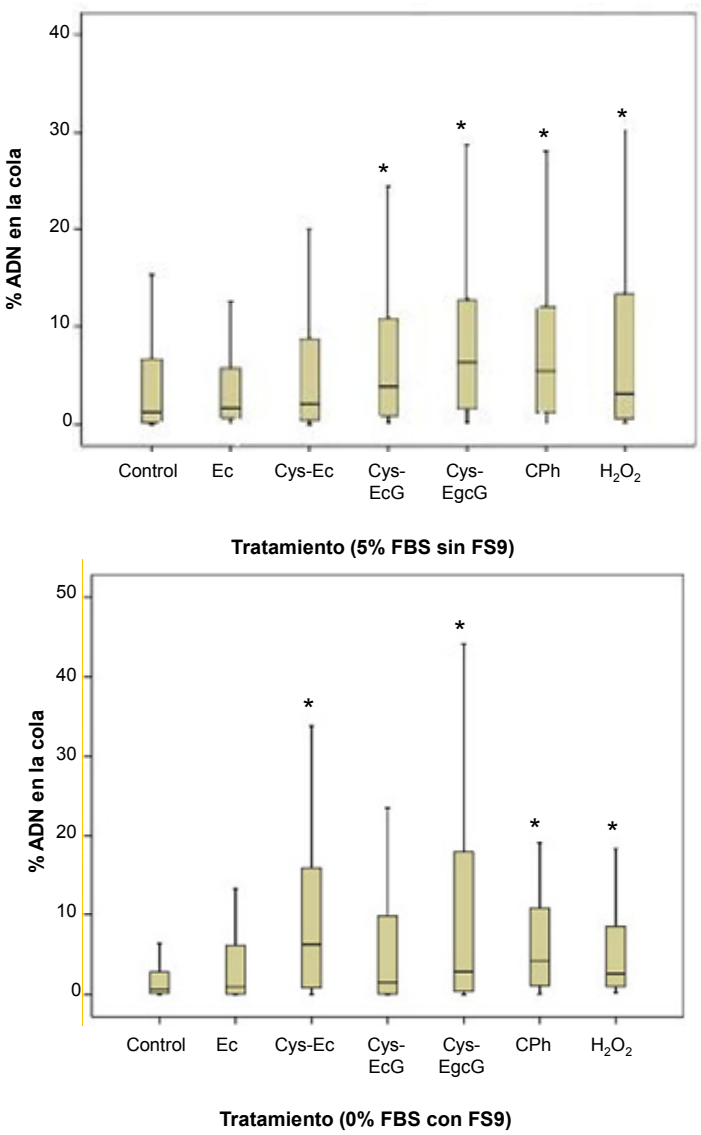


Figura 44. Evaluación de la genotoxicidad de la Epicatequina y sus tiol derivados. Control: células incubadas sólo con medio de cultivo; CPh, ciclofosfamida; H_2O_2, peróxido de hidrógeno; Ec, Cys-Ec, Cys-EcG, Cys-EgcG, compuestos a concentración 300 µM. (5% FBS sin FS9) medio sin activador metabólico; (0% FBS con FS9) medio con activador metabólico. *diferencias significativas respecto a las células control ($p<0,05$).

Los resultados de este estudio preliminar no permiten establecer una clara relación entre la estructura de los compuestos y su capacidad genotóxica. La presencia del grupo tiol parece ser la responsable del incremento del efecto tóxico de la epicatequina, ya que el daño en el ADN provocado por la Cys-Ec es superior al inducido por la Ec (Figura 45). Este efecto más pronunciado de los compuestos con cisteína podría estar relacionado con la mayor capacidad de penetración e interacción con las membranas celulares, que se ha visto que los grupos no flavonoides conferían a las catequinas (Alonso y col., 2004; Lázaro y col., 2007) y que permitiría un mayor acceso al material genético de la célula. La presencia de grupos galatos en las moléculas también parece ejercer cierto efecto en la actividad

genotóxica, resultando el compuesto Cys-EgcG más dañino que el Cys-EcG. Este resultado podría explicarse por el efecto prooxidante que se ha atribuido al grupo pirogalol del anillo B presente en las galocatequinas que puede favorecer la producción del anión superóxido ($O_2^{\cdot -}$) y, por consiguiente, de peróxido de hidrógeno, muy reactivo con el ADN (Kondo y col., 1999a; Lozano y col., 2006). Por el contrario, el grupo galato, que contiene tres estructuras hidroxilo acopladas al grupo carbonilo, no produce anión superóxido debido a la estabilidad de su radical.

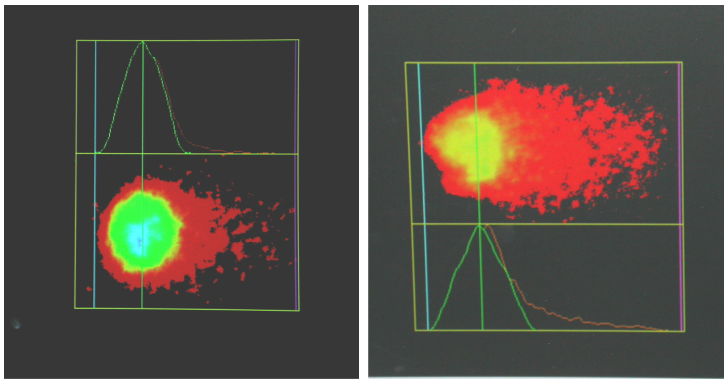


Figura 45. Análisis del daño inducido por el derivado Cys-Epicatequina en el material genético de células 3T3. En amarillo, ADN intacto, en rojo, ADN fragmentado.

Los datos obtenidos en estos ensayos preliminares, coinciden con observaciones previas de otros autores de la toxicidad sobre el material genético que producen las sustancias polifenólicas, por ejemplo la epicatequina, a elevadas concentraciones (Gupta y col., 2002); Fan y Lou, 2004 demostraron que la catequina a concentraciones superiores a 150 μM inducía daño en el ADN por sí misma, datos respaldados por Savi y col., 2006 que también demostraron que la catequina, la epicatequina y algunos de sus derivados galoizados presentaban efectos genotóxicos.

Los bioflavonoides pueden comportarse como antioxidantes y prooxidantes y su actividad dependerá de varios factores como la línea celular o la concentración ensayada. Diversos estudios de protección frente al daño oxidativo del ADN por agentes oxidantes realizados con compuestos de naturaleza flavonoide, mostraron que el efecto protector no se incrementaba al aumentar la concentración utilizada. Este fenómeno podría ser explicado si se tiene en cuenta el efecto prooxidante atribuido a los compuestos polifenólicos (Fan y Lou, 2004). El daño en el ADN podría ser el resultado de la acción prooxidante de estos compuestos, que a elevadas concentraciones generarían ROS, mientras que a concentraciones bajas no producirían cambios en los niveles intracelulares de ROS. Está bien documentado que los ROS inducen efectos genotóxicos y citotóxicos, y esta podría ser la razón del daño genético observado (Rucinska y col., 2007). Este efecto prooxidante de los fenoles podría estar relacionado con su reacción con cationes metálicos, por ejemplo podrían reducir Fe^{3+} y catalizar la formación del radical hidroxilo. La generación de radicales hidroxilo en las proximidades del ADN se conoce con certeza que causa escisión de las hebras del ADN (Azmi y col., 2006).

En general, el daño en el ADN detectado para las concentraciones de cada producto ensayado podría explicarse por dos hipótesis. La primera, es posible que el daño se deba a la formación del radical hidroxilo. La segunda, se podrían liberar cationes metálicos en la célula como resultado del estrés oxidativo y entonces unirse al ADN (Savi y col., 2006). Los mecanismos de toxicidad de estos compuestos aún deben ser clarificados, pero uno de los posibles mecanismos de toxicidad propuestos es la habilidad de estas sustancias para autooxidarse. Los productos resultantes de este proceso (quinonas y ROS como peróxido de hidrógeno y radicales hidroxilo) pueden ser señalados como los factores que contribuyen al daño oxidativo. Los radicales hidroxilo también pueden ser generados cuando el H_2O_2 entra en contacto con ciertos cationes de metales de transición, especialmente el hierro y el cobre. Por lo tanto el H_2O_2 cruza las membranas biológicas y puede penetrar en el núcleo celular y reaccionar con los cationes hierro y cobre para formar $^{\cdot}OH$ que fácilmente dañará el ADN (Labieniec y Gabryelak, 2005).

De cualquier forma, los mecanismos de acción de estos compuestos aún no se comprenden del todo y por ello son necesarios más estudios sobre su modo de acción ya que podrían explicar el por qué de su dualidad de acción antioxidante-prooxidante que sigue siendo aún enigmática.

CONCLUSIONES

Los estudios de genotoxicidad de potenciales compuestos antioxidantes son muy importantes para determinar las asociaciones riesgo-beneficio de dichos compuestos (Rao y col., 1997).

El principal interés de estos estudios preliminares de genotoxicidad realizados con nuestros derivados flavanoides, ha sido proporcionar evidencias de que compuestos con potencial antioxidante reconocido, pueden actuar como prooxidantes. Pero, a pesar de que los resultados obtenidos indican que estos compuestos polifenólicos pueden tener efectos tóxicos en nuestro sistema de ensayo, no significa que no puedan ser tratados como prometedoras sustancias antioxidantes y antimutagénicas. Además existe la hipótesis de que el efecto genotóxico podría ser una manera de ejercer un efecto antioxidante por parte de los compuestos flavonoides: inducir apoptosis y activación de enzimas reparadoras del daño del ADN por generación de ROS intracelulares y aumentar así el potencial antioxidante de la célula (Chen y col., 2003b).

Estos estudios son la base para plantear la caracterización futura de la capacidad genotóxica del resto de derivados de epicatequina obtenidos por nuestro grupo, así como realizar estudios de capacidad protectora de estos compuestos a concentraciones que no sean dañinas para el ADN, frente a los efectos genotóxicos de compuestos oxidantes. El objetivo final es demostrar que sustancias fitoquímicas de naturaleza polifenólica obtenidas de fuentes naturales como la uva, además de mostrar una prometedora actividad antioxidante *in vitro*, también pueden ejercer una protección significativa contra el daño oxidativo en el ADN en un rango de concentraciones seguro para el organismo. El cribado de sustancias bioactivas, sintéticas o de fuentes naturales, y la caracterización de su relación estructura-

actividad, en combinación con los avances tecnológicos, son necesarios para el desarrollo de nuevos productos más efectivos y seguros que los disponibles en el mercado (Savi y col., 2006).

3.2. Estudio de la actividad biológica de fracciones polifenólicas de origen natural

Bloque I. Actividad antioxidante y citotoxicidad

ARTÍCULO 3

Comparación del efecto antioxidante y citotóxico de fracciones de procianidinas procedentes de uva y de pino

Comparative antioxidant and cytotoxic effect of procyanidin fractions from grape and pine

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y María Pilar Vinardell

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Resumen

La industria agroalimentaria produce grandes cantidades de residuos cuya gestión supone un elevado gasto económico y un potencial riesgo medioambiental. Actualmente, muchos esfuerzos científico-técnicos se centran en conseguir reciclar estos residuos mediante la búsqueda de nuevas aplicaciones. Por este motivo, existe un gran interés en la caracterización de las propiedades biológicas de compuestos naturales obtenidos de plantas y de sustancias activas presentes en los productos de desecho agrícolas, especialmente de los polifenoles y entre ellos, los flavonoides. En el presente trabajo se han estudiado las relaciones entre la estructura y la actividad citotóxica de un conjunto de fracciones polifenólicas obtenidas de dos fuentes naturales diferentes, bagazo de uva y corteza de pino. Estas fracciones presentan un contenido similar de flavonoides polimerizados (procianidinas), pero difieren en el porcentaje de galoización, lo que les confiere diferentes propiedades biológicas. La citotoxicidad de este grupo de fracciones se evaluó en dos líneas celulares, queratinocitos HaCaT y fibroblastos de ratón 3T3, después de 24, 48 y 72 horas de exposición. También se ha evaluado la actividad antioxidante de las fracciones midiendo la inhibición de la hemólisis causada por el iniciador de radicales peroxilo, AAPH, en eritrocitos humanos. Los resultados obtenidos muestran que las fracciones polifenólicas estudiadas presentan una elevada capacidad antioxidante en un rango de concentraciones que no es dañino para las células normales. Las fracciones de pino presentaron una actividad antioxidante ligeramente inferior que las fracciones de uva, pero también fueron menos citotóxicas, propiedades probablemente relacionadas con el menor contenido en grupos galato en su composición. Este dato proporciona información útil para ayudar al diseño de productos antioxidantes seguros que actúen sin alterar las funciones críticas de la célula.

Comparative Antioxidant and Cytotoxic Effect of Procyanidin Fractions from Grape and Pine

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There is a great interest in characterizing the biological properties of natural compounds obtained from plants, especially polyphenols. We studied the structure–activity–cytotoxicity relationships of polyphenolic fractions obtained from grape pomace and pine bark. These fractions contained similar polymerised flavonoids but different percentages of pyrogallol groups that confer on them different biological properties. The human keratinocyte cell line HaCaT and the mouse fibroblast cell line 3T3 were used to study the cytotoxicity of the different fractions after 24, 48, and 72 h of exposure. Antioxidant activity of the fractions was evaluated by measuring the inhibition of hemolysis mediated by AAPH. Our results demonstrate that the polyphenolic fractions studied show high antioxidant capacity in a concentration range that is not harmful to normal human cells. Pine fractions presented slightly lower antioxidant activity than grape fractions but are less cytotoxic. This data provides useful information to help design safe antioxidant products that act without altering critical cell functions.

Introduction

The efficient use of natural resources is currently the focus of many efforts in both science and technology. The management of agriculture and forestry must be sustainable from both economic and environmental viewpoints. The agrifood industries produce a large volume of waste each year (*1*). Because of the high economic cost of disposal and the potential environmental risk associated with an excess of biomass, the possibility of recycling by finding new applications for these wastes has great potential.

Plant residues from food and forestry industries contain considerable amounts of potentially interesting compounds, but the value of the products obtained must compensate for the cost of their recovery. For this reason, it is essential both to improve the extraction processes and to substantiate the activity and safety claims (*2*) of the new products.

Among the biologically active species present in agricultural by-products, polyphenols and particularly flavonoids are widely appreciated for their putative health-promoting properties. The best-described property of flavonoids is their capacity to act as free radical scavengers (*3*). They also show other properties that may or may not be related to their scavenging potential. These include, but are not limited to, antiproliferation of carcinogenic cells, cell cycle regulation, induction of apoptosis, inhibition of platelet aggregation, and antibacterial and antiallergic properties (*4–8*). Therefore, it is assumed that flavonoids play a relevant role in the prevention of degenerative diseases such as cancer and cardiovascular diseases and that it may be wise to include in our diet vegetables, fruits, and moderate amounts of plant-derived products such as tea, wine, and chocolate, which are rich in polyphenols (*9, 10*).

Polyphenolic mixtures have already been proposed as food antioxidants and preventive agents against skin irritation and cancer (*4, 11*). In accordance with the scientific and market interest in polyphenols as chemopreventive agents, our group is investigating the possible applications of plant proanthocyanidins in the fields of food preservation, skin protection, and cancer with particular emphasis on their structure–activity relationships and safety profiles.

From white grape (*Vitis vinifera*) pomace and pine (*Pinus pinaster*) bark polyphenolic extracts, we generated fractions containing different amounts of monomeric catechins and oligomeric procyanidins (*12–14*). Figure 1 summarizes the structure of the procyanidins found in both sources. We previously carried out several assays to assess the structure/function relationships of these fractions. We determined their efficiency as antioxidants under different experimental setups, including free radical scavenging in solution and inhibition of lipid peroxidation in both pure oil and oil-in-water emulsion. Furthermore, we investigated the influence of these compounds on the proliferation of different tumoral cell lines and their capacity to induce apoptosis (*13, 14*).

The fractions from the two sources are highly homologous in terms of mean molecular size; they are mainly differentiated by their galloylation (presence of gallate esters). Grape pomance (skin, seeds, and a small amount of stems) are galloylated to some extent, but pine bark appears to contain only procyanidins with no measurable galloylation (*4, 15*). Because the gallate group both provides high antiradical power and appears to interfere with crucial cell functions, galloylation appears to be a crucial structural feature defining the activity and toxicity of phenolic mixtures.

The aim of this study is to take a step forward in the characterization of the biological properties of procyanidins by using a set of grape and pine fractions with different mean size and galloylation. We report the protective antioxidant potential

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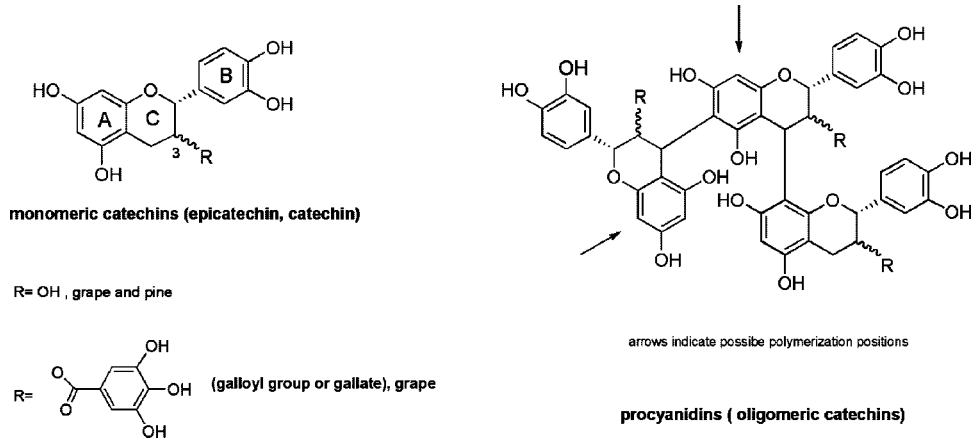

Figure 1. Structures of the procyanidins found in grape and pine fractions.

Table 1. Size and Composition of Polyphenolic Fractions from Parellada White Grape Pomace and Pine Bark (*11, 12*)

fraction	mDP	mMW	galloylation (%)
OWG	1.7	552	15
IVG	2.7	880	25
VIIIG	3.4	1160	34
XIG	3.7	1232	31
OWP	2.1	601	
IVP	2.9	833	
VIIIP	3.0	876	
XIP	3.4	999	

control	Mdp	Mmw	galloylation (%)
(−)-Epicatechin	1.0	290	0

in a biological system, namely, the inhibition of red blood cell lysis after the addition of AAPH (2,2′-azobis(amidinopropane)dihydrochloride), a peroxil radical initiator, and the estimation of possible toxic effects by using cell culture assays. We evaluated the relationship between the potential cytotoxic properties and the antioxidant activity of these polyphenolic fractions and how their structure (polymerization degree and percentage of galloylation) may influence their behaviour. The characterization of these biological properties will permit us to better define the possible applications of phenolics and to study their potential health benefits and risks in depth.

Experimental Procedures

Materials. 1. Grape Fractions. The total extract, **OWG**, was obtained from Parellada grape (*Vitis vinifera*) pomace following the procedure described by Torres and Bobet (*12*). **OWG** contained monomeric cathechins, oligomeric catechins (procyanidins), and, in lower proportion, flavonols, mainly glycosylated (*16*). Isolated procyanidins with variable galloylation, which we labeled **IVG**, **VIIIG**, **XIG**, were obtained by application of size-exclusion chromatography to **OWG**, as described previously (*13*). Procyanidin size and composition were estimated by thiolysis with cysteamine, and glycosylated flavonols were detected by analytical RP-HPLC at 365 nm. The qualitative composition of the fractions, the mean molecular weight (mMW), the degree of polymerization (mDP), and the percentage of galloylation previously described by our group (*13*) are summarized in Table 1. The fractions contained mostly procyanidins. Molar concentrations of these procyanidins were calculated using the mean molecular weight of the mixtures, which was estimated by thiolysis with cysteamine as described (*14*).

2. Pine Bark Fractions. The polyphenolic total extract, **OWP**, was obtained essentially as described for grape pomace (*12*), with some extraction modification (*14*)**OWP** contained monomeric and oligomeric catechins and other monomeric flavonoids. From this mixture, our group generated fractions homologous to those obtained from grape pomace, differing in composition and procyanidin structure (Table 1). The procyanidin oligomers **IVP**, **VIIIP**, and **XIP** were obtained using a combination of chromatographic techniques. 2,2′-Azobis(amidinopropane)dihydrochloride (AAPH) and (−)-Epicatechin (Ec) were purchased from Sigma (ST Louis, MO).

Blood Samples and Preparation of Red Blood Cells and AAPH. Blood samples were obtained from healthy donors by venipuncture (Blood Bank of Hospital Vall d'Hebrón, Barcelona, Spain), following the ethical guidelines of the Hospital, and collected in citrated tubes. Blood was centrifuged at 1000*g* for 10 min, and the plasma and buffy coat were removed. Red blood cells (RBCs) were washed three times in phosphate buffer isotonic saline (PBS) containing 22.2 mM Na2HPO4, 5.6 mM KH2PO4, 123.3 mM NaCl, and glucose 10.0 mM in distilled water (pH 7.4). The cells were then resuspended in isotonic saline solution to get the desired cellular density (8×10^9 cells/mL). An AAPH solution was prepared at the moment of its use using the same buffer and protected from the light.

Antioxidant Activity. We measured the hemolysis of RBCs mediated by AAPH using a modification of the method described previously (*17*). The addition of AAPH (a peroxyl radical initiator) to the suspension of RBCs induces the oxidation of cell membrane lipids and proteins, thereby resulting in hemolysis. The erythrocyte suspension (250 μL) was incubated in the presence of AAPH at a final concentration of 100 mM for 150 min in a shaker at 37 °C to achieve 100% hemolysis. Hemolysis was assessed by reading the absorbance of the hemoglobin released at 540 nm in a Shimadzu spectrophotometer.

The antihemolytic activity of fractions from different sources was tested by adding several concentrations of the compounds solved in PBS, ranging from 12.5 to 200 μg/mL, to the RBC suspension in the presence of 100 mM AAPH at 37 °C for 2.5 h. A blood sample incubated at the same conditions but without AAPH or fractions was included as a control for the spontaneous hemolysis. The IC_{50} or concentration inducing 50% inhibition of the hemolysis induced by **AAPH** was determined for each compound.

Culture of Cell Lines and Experimental Treatments. We used the spontaneously immortalized human keratinocyte cell line, HaCaT, and the mouse fibroblast cell line, 3T3 from "Banco de Células Eucariotas", Barcelona (Spain). Cells were grown in Dulbeccos's modified Eagle's medium (DMEM) (4.5 g/L glucose) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 mM Hepes buffer, and 1% penicillin (10,000 U/mL)/streptomycin (10,000 μg/mL) and maintained in a humidified atmosphere with 5% CO_2 at 37 °C. When 75 cm^2 culture flasks were approximately 80% confluent, the cells were seeded into the central 60 wells of 96-well plates as follows: for HaCaT, at densities of 10×10^4 cells/mL, 6.5×10^4 cells/mL, and 5.5×10^4 cells/mL for 24, 48, and 72 h of exposure, respectively, and for 3T3 at densities of 8.5×10^4 cells/mL, 2.5×10^4 cells/mL, and 1.5×10^4 cells/mL for 24, 48, and 72 h of exposure, respectively (*18*).

Plates were incubated at 37 °C, 5% CO_2 for 24 h. Triplicate runs were performed with different passage cells.

After 1 day of incubation, the growth medium was removed and replaced with exposure medium (DMEM medium supplemented with 5% FBS, 2 mM L-glutamine, 10 mM Hepes buffer, and 1% antibiotic mixture), with or without the polyphenolic fractions at concentrations ranging from 500 μg/mL to 7 μg/mL previously sterilized by filtration. Controls, containing culture medium only, were included in each plate. Cells were then incubated at 37 °C and 5% CO_2 for 24, 48, or 72 h.

NRU Assay. The NRU assay was performed as described by Borenfreund and Puerner (*19*) and modified to remove the use of formaldehyde (*20*). After the treatments, the medium was aspirated and replaced with 100 μL per well of NR solution (50 μg/mL in RPMI medium without phenol red and serum). After 3 h of incubation at 37 °C and 5% CO_2, the medium was aspirated, the cells were washed twice with PBS, and a solution containing 50% ethanol and absolute 1% acetic acid in distilled water was added (100 μL per well) to release into the supernatant the dye that had been absorbed into the viable cells. After 10 min on a microtitre-plate shaker, the absorbance of neutral red was measured at a wavelength of 550 nm in a Bio-Rad 550 microplate reader.

Statistical Analysis. Each experiment was performed at least three times using three replicates for each concentration assayed. Results were expressed as the mean ± SE.

The cytotoxicity of each fraction was expressed as the percentage of viability compared to control wells (the mean optical density of untreated cells was set to 100% viability) in terms of its IC_{50} (concentration of product that causes 50% inhibition of growth or death of the cell population); IC_{50} was calculated from the dose–response curves by linear regression analysis. NRU assay results were expressed as the percentage of uptake of neutral red dye by the lysosomes.

Statistical significance was determined by Student's *t*-test and one-way analysis of variance (ANOVA) using the SPSS software (SPSS Inc., Chicago, IL, USA). Statistical significance was considered $P < 0.05$.

Results and Discussion

Antioxidant Activity. Because of the basic chemical structure of their components (monomeric and oligomeric catechins), the most obvious feature of polyphenolic fraction mixtures is their strong antioxidant activity (*4*). By means of chemical methods (DPPH, HNTTM, and ABTS Assays), previous studies have demonstrated that extracts from pine and grape appear to be efficient antioxidant agents (*13, 14, 21*). It is known that flavonoids can display antioxidant activity in numerous biological systems; therefore, we considered it appropriate to evaluate the antioxidant potential of these fractions using a biological method. Because of their susceptibility to peroxidation, red blood cells (RBCs) have been used as a model to investigate oxidative damage in biomembranes. We therefore chose to investigate the oxidation of RBCs induced by AAPH, a well-known peroxyl radical initiator that causes hemolysis by means of membrane lipid and protein oxidation, and the extent of protection offered by the polyphenolic fractions in order to compare their efficacy as antioxidants.

Dose–response curves were analyzed, and IC_{50} values were obtained (concentration inducing 50% inhibition of hemolysis induced by AAPH). These values are represented in Figure 2, together with that for (−)-Epicatechin, a known antioxidant flavonoid present in grapes and tea (*22, 23*).

All of the fractions tested showed an inhibition of the in vitro AAPH-induced red blood cell hemolysis in a dose-dependent manner (data not shown), and all of them were more effective than (−)-Epicatechin, showing significant differences in all cases.

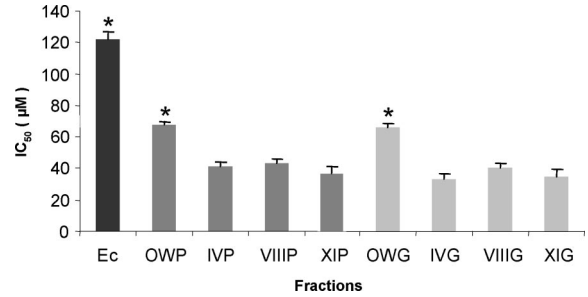

Figure 2. Antioxidant activity of the fractions from different sources and (−)-Epicatechin by the AAPH assay in red blood cells. Results are expressed as IC_{50} or the concentration inducing 50% inhibition of the hemolysis induced by AAPH (mean ± SE). (*) Marked compounds are statistically different to the rest. $P < 0.05$ was considered to denote statistically significant differences.

Among grape fractions, the highest antioxidant power corresponded to the mixtures of compounds with the highest degree of polymerization and galloylation and no glycosylated flavonols. The presence of glycosylated flavonols, which are less efficient scavengers than the aglycons, lowered the overall antiradical power of fractions such as the total fraction **OWG** (*13*).

The most efficient grape fraction was **IVG**, although **VIIIG** and **XIG** gave similar results. (No statistical differences were noted for IC_{50} values.) At equal galloylation (**VIIIG** and **XIG**) antioxidant capacity was proportional to mDP. These observations corroborate other studies in which it is described that antioxidant activity depends on polymerization and increases with galloylation (*24*).

Pine bark fractions also showed good antioxidant activity against oxidation of RBCs. In this case, the most potent antioxidant was fraction **XIP** with an antioxidant efficiency 3 times higher than that obtained for (−)-Epicatechin. We also found a very strong correlation between antioxidant activity and the degree of polymerization ($r = 0.967$) of pine bark fractions, i.e., the higher the mDP, the better the capacity to inhibit AAPH-induced oxidation. Total fraction **OWP** was the least effective, possibly because of its higher levels of monomeric catechins, which reduce antioxidant capacity.

When comparing homologous fractions, pine polyphenols were slightly less potent antioxidants than those from grape (although no statistically significant differences were recorded). These data agree with the results previously obtained by our group (*14*). This less effective antioxidant activity may be attributed to the absence of galloyl esters in their structure, which confer extra antioxidant capacity as reported (*25, 26*).

Studies have suggested that prehemolytic damage caused by AAPH is mediated mainly through lipid peroxidation and to a lesser extent by the oxidation of proteins located in the hydrophobic region of the membrane (*27*). Then, fractions according to their antihemolytic effect should prevent lipid peroxidation and protein oxidation.

Several studies have tried to discover a structure–activity relationship responsible for the biological activity of catechins and other flavonoids, but no conclusive evidence has been found so far (*28*). Several investigations have shown that flavonoids such as (−)-Epicatechin, (+)-catechin, and their related procyanidins can adsorb to membranes through associations with the polar headgroups of phospholipids, generating an environment rich in flavonoids. Such a flavonoid coat would provide protection against oxidants as well as other external aggressors by limiting the access of oxidants to the bilayer and/or controlling the rate of propagation of free radical chain reactions

occurring in the hydrophobic core membranes (*29*). Particularly, galloylated catechins could affect the membrane configuration by forming more compact structures that limit the access of pro-oxidants (*16*). This could be one of the reasons why the grape fractions were in general more active antioxidants. However, it is known that gallate groups influence intracellular events (cell cycle, apoptosis) as reported elsewhere (*13, 30–32*); therefore, it may be preferable, in some cases, to use fractions composed of nongalloylated catechins for applications related to food and skin protection.

In conclusion, all of these polyphenolic fractions are effective antioxidants that can protect human red blood cells from free radical induced oxidative hemolysis (*33*). We have demonstrated that pine fractions although slightly less potent than grape fractions showed effective antioxidant properties (especially those with high mDP (**XIP** and **IVP**)) and that for this reason they are an interesting option for the design of safe products that exert antioxidant protection without influencing normal cell functions.

Cytotoxicity Evaluation. The natural antioxidant properties shown by the polyphenolic fractions suggested that potential applications in different areas can be explored, but we have to guarantee that these new fractions are safe, that is, that the possible concentration range employed does not result in unacceptable damage to normal body cells (*34*). We think it is reasonable to use, as a primary screening stage, in vitro toxicity assays to select the least toxic compounds from among the most actives ones. Use of simple and reproducible in vitro tests consisting of cultures of submerged monolayers of epidermal keratinocytes and dermal fibroblast will allow us to predict adverse effects including potential toxicity and to define safe application concentrations for future formulations (*35*). In this study, we determined cytotoxicity through the neutral red uptake assay in human keratinocyte HaCaT and murine fibroblast 3T3 cell lines and clarified the in vitro cell toxicity effects of our new polyphenolic fractions. We selected the 3T3 cell line because 3T3 neutral red uptake assay is recommended by the U.S. National Institute of Environmental Health Science (NIEHS) Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). The use of the HaCaT, nontumorigenic, spontaneously immortalized keratinocyte cell line provides an almost unlimited supply of identical cells, ensuring high intra and interlaboratory reproducibility (*34*).

We exposed cell cultures to the test compounds for 24, 48, and 72 h, and typical concentration–response curves were recorded to calculate the IC_{50} or dose of compound that inhibits viability to 50%. These IC_{50} values are represented in Figure 3 and Figure 4 for HaCaT and 3T3.

All the fractions showed a certain degree of toxicity as indicated by the decrease in the rate of neutral red uptake. After 48 h of exposure, there was an increase in the cytotoxicity induced by all the fractions, but no significant differences were recorded as compared to the cytotoxicity after 72 h.

The responses of fibroblast and keratinocytes to the given fractions were different. The 3T3 cell line was in general the most sensitive to both pine and grape fractions, although grape fractions showed more significant effects. This different sensitivity of the cells has been previously reported (*36*), and is related to morphologic and physiologic differences between the cell types, especially the differing ability to resist oxidative stress.

Cytotoxicity of grape fractions showed a strong correlation with the degree of polymerization ($r = 0.968$ and 0.978 for 72 h to HaCaT and 3T3, respectively) and the percentage of galloylation ($r = 0.973$ and 0.966 for 72 h to HaCaT and 3T3, respectively) i.e., the fractions with the highest degree of polymerization and galloylation (**XIG** and **VIIIG**) exerted the most toxic effect on the cell cultures. This result is in agreement with those of other authors who also attribute the greater level of cytotoxicity to polyphenolic compounds with these characteristics (*32, 37*).

Pine fractions exhibited lower cytotoxicity, but toxicity increased with the degree of polymerization ($r = 0.897$ and

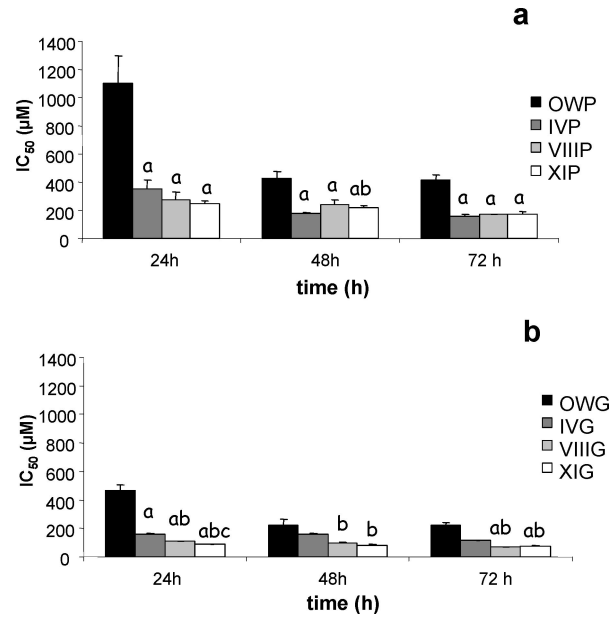

Figure 3. Comparative cytotoxicity of pine fractions (a) and grape fractions (b) toward proliferation of **HaCaT**cells after 24, 48, and 72 h of exposure. Data are presented as IC_{50} or dose inhibiting viability to 50% (mean ± SE). (a) a, statistically different from OWP; b, statistically different from IVP. (b) a, statistically different from OWG; b, statistically different from IVG; c, statistically different from VIIIG. $P < 0.05$ was considered to denote statistically significant differences.

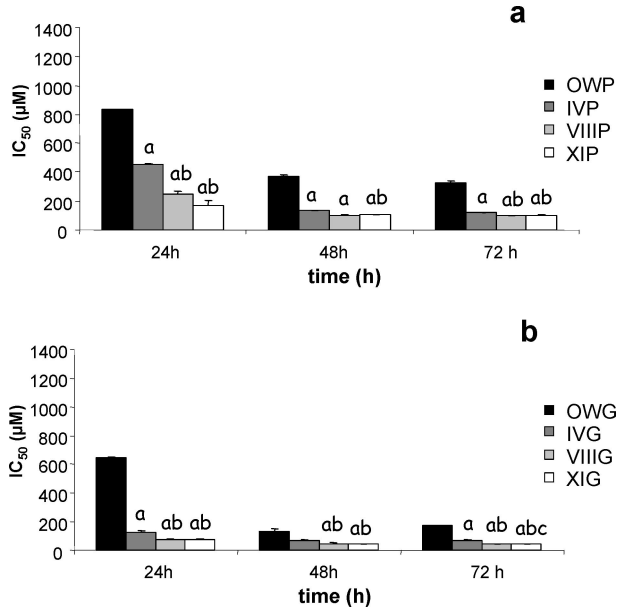

Figure 4. Comparative cytotoxicity of pine fractions (a) and grape fractions (b) toward proliferation of **3T3**cells after 24, 48, and 72 h of exposure. The data are presented as IC_{50} or dose inhibiting viability to 50% (mean ± SE). (a) a, statistically different from OWP; b, statistically different from IVP. (b) a, statistically different from OWG; b, statistically different from IVG; c, statistically different from VIIIG. $P < 0.05$ was considered to denote statistically significant differences.

$r = 0.932$ for 72 h to HaCaT and 3T3, respectively). The lower toxic effect of these fractions was not an unexpected result because their composition is devoid of galloyl esters.

When comparing homologous fractions, those from grape were more cytotoxic in all cases, except for the pair **IVG–IVP**, for which we did not find significant differences. As galloylation is the main difference between grape and pine fractions, these results confirmed the influence of gallate groups in cell viability, and their role in cell cycle regulation. Previous studies in melanoma cells have detected higher antiproliferative and apoptotic effects of galloylated catechins than nongalloylated ones (*26, 38*), but in some cases, especially for applications not related to anticancer drugs such as food or skin protection, it is preferable to use compounds that do not alter normal cell functions.

Although all the fractions tested in this study have shown more cytotoxicity than (−)-Epicatechin (data not shown) (*34*), they exhibited antioxidant activities at concentrations nontoxic to cells. We found a strong correlation between antioxidant and cytotoxic activities for all fractions and for all exposure conditions. The best antioxidant fraction was also the most toxic to cells. To find out if we can work in a safe range of concentrations with these fractions, we calculated the relationship between the cytotoxicity index (IC_{50}) at 72 h in 3T3 and the antioxidant potential. We found that while antioxidant concentration of the more effective pine fractions, **XIP** and **VIIIP**, was approximately 2.5-fold lower than the cytotoxic concentration, in the case of homologous grape fractions, **XIG** and **VIIIG**, it was only 1.3-fold lower. From all of this, we can conclude that an effective antioxidant activity of procyanidin mixtures can be obtained at a concentration range not toxic for the cell lines studied. This is especially true in the case of pine fractions, which present an effective antioxidant capacity with low cytotoxicity due to their lack of gallate groups.

Summary and Conclusions

Plant phenolics from agrifood byproducts are being increasingly used as nutraceuticals. To explore the structure–activity–toxicity relationships of antioxidant procyanidins present in plant extracts, we used a collection of polyphenolic fractions from two different sources (grape and pine). The results obtained in this study show that we can get effective antioxidant activity from these compounds in a concentration range that is safe for normal cells.

Although grape fractions presented slightly higher antioxidant capacity, the observation that pine fractions such as **VIIIP** and **XIP** that are rich in nongalloylated procyanidins with moderate mDP showed an efficient antihemolysis activity with relatively low cytotoxicity provides useful information for the design of safe antioxidant products that exert their protection without altering crucial cell functions.

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References

(1) Laufenberg, G., Kunz, B., and Nystroem, M. (2003) Transformation of vegetable waste into value added products: (A) the upgrading concept; (B) practical implementations. *Bioresour. Technol. 87*, 167–198.

(2) Torres, J. L., Selga, A., and Cascante, M. (2003) Bioactive products from by-products and wastes. *Electron. J. Environ. Agric. Food. Chem. 2*, 211–214.

(3) Rice-Evans, C. (2001) Flavonoid antioxidants. *Curr. Med. Chem. 8*, 797–807.

(4) Packer, L., Rimbach, G., and Virgili, F. (1999) Antioxidant activity and biologic properties of a procyanidin-rich extract from pine (Pinus maritima) bark, pycnogenol. *Free Radical Biol. Med. 27*, 704–724.

(5) Hara-Kudo, Y., Yamasaki, A., Sasaki, M., Okubo, T., Minai, Y., Haga, M., Kondo, K., and Sugita-Konishi, Y. (2005) Antibacterial action on pathogenic bacterial spore by green tea catechins. *J. Sci. Food Agric. 85*, 2354–2361.

(6) Toda, M., Okubo, S., Hiyoshi, R., and Shimamura, T. (1989) The Bactericidal Activity of Tea and Coffee. *Lett. Appl. Microbiol. 8*, 123–125.

(7) Hayakawa, S., Kimura, T., Saeki, K., Koyama, Y., Aoyagi, Y., Noro, T., Nakamura, Y., and Isemura, M. (2001) Apoptosis-inducing activity of high molecular weight fractions of tea extracts. *Biosci. Biotechnol. Biochem. 65*, 459–462.

(8) Ruf, J. C. (1999) Wine and polyphenols related to platelet aggregation and atherothrombosis. *Drugs Exp. Clin. Res. 25*, 125–131.

(9) Katiyar, S. K., and Mukhtar, H. (1997) Tea antioxidants in cancer chemoprevention. *J. Cell Biochem. Suppl. 27*, 59–67.

(10) Middlenton, E, Jr., Kandaswami, C., and Theoharis, C. (2000) The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol. Rev. 52*, 673–751.

(11) Medina, I., Lois, S., Lizarraga, D., Pazos, M., Touriño, S., Cascante, M., and Torres, J. L. (2006) Functional fatty fish supplemented with grape procyanidins. Antioxidant and proapoptotic properties on colon cell lines. *J. Agric. Food Chem. 54*, 3598–3603.

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

(13) Torres, J. L., Varela, B., García, M. T., Carilla, J., Matito, C., Centelles, J. J., Cascante, M., Sort, X., and Bobet, R. (2002) Valorization of grape (*Vitis vinifera*) byproducts. Antioxidant and biological properties of polyphenolic fractions differing in procyanidin composition and flavonol content. *J. Agric. Food. Chem. 50*, 7548–7555.

(14) Touriño, S., Selga, A., Jiménez, A., Julià, L., Lozano, C., Lizarraga, D., Cascante, M., and Torres, J. L. (2005) Procyanidin fractions from Pine (*Pinus pinaster*) bark: radical scavenging power in solution, antioxidant activity in emulsion, and antiproliferative effect in melanoma cells. *J. Agric. Food Chem. 53*, 4728–4735.

(15) Selga, A., Sort, X., Bobet, R., and Torres, J. L. (2004) Efficient one pot extraction and depolymerization of grape (*Vitis vinifera*) pomace procyanidins for the preparation of antioxidant thio-conjugates. *J. Agric. Food Chem. 52*, 467–473.

(16) Pazos, M., Lois, S., Torres, J. L., and Medina, I. (2006) Inhibition of hemoglobin- and iron-promoted oxidation in fish microsomes by natural phenolics. *J. Agric. Food. Chem. 54*, 4417–4423.

(17) Miki, M., Tamai, H., Mino, M., Yamamoto, Y., and Niké, E. (1987) Free radical chain oxidation of rar red blood cells by molecular oxygen and its inhibition by α-toxopherol. *Arch. Biochem. Biophys. 258*, 373–80.

(18) Babich, H., Krupka, M. E., Nissim, H. A., and Zuckerbraun, H. L. (2005) Differential in vitro cytotoxicity of (−)-epicatechin gallate (ECG) to cancer and normal cells from the human oral cavity. *Toxicol. In Vitro, 19*, 231–42.

(19) Borenfreund, E. P. J. A. (1983) Rapid colorimetric assay to cellular growth and survival: Application to proliferation and cytotoxicity assay. *Toxicol. Lett. 24*, 119–124.

(20) Riddell, R. J., Clothier, R. H., and Balls, M. (1986) An evaluation of three in vitro cytotoxicity assays. *Food Chem. Toxicol. 24*, 469–471.

(21) Vuorela, S., Kreander, K., Karonen, M., Nieminem, R., Hämäläinen, M., Galkin, A., Laitinen, L., Salminen, J., Moilanen, E., Pihlaja, K., Vuorela, H., Vuorela, P., and Heinonen, M. (2005) Preclinical evaluation of rapeseed, raspberry, and pine bark phenolics for health related effects. *J. Agric. Food Chem. 53*, 5922–5931.

(22) Mitjans, M., Del Campo, J., Abajo, C., Martinez, V., Selga, A., Lozano, C., Torres, J. L., and Vinardell, M. P. (2004) Immunomodulatory activity of a new family of antioxidants obtained from grape polyphenols. *J. Agric. Food Chem. 52*, 7297–7299.

(23) Kitawa, S., Sakamoto, H., and Tano, H. (2004) Inhibitory effects of flavonoids on free-radical-induced hemolysis and their oxidative effects on hemoglobin. *Chem. Pharm. Bull. 52*, 999–1001.

(24) Plumb, G. W., De Pascual-Teresa, S., Santos-Buelga, C., Cheynier, V., and Williamson, G. (1998) Antioxidant properties of catechins and proanthocyanidins: effect of polymerisation, galloylation and glycosylation. *Free Radical Res. 29*, 351–358.

(25) Sroka, Z. (2005) Antioxidative and antiradical properties of plant phenolics. *Z. Naturforsch., C: Biosci. 60*, 833–843.

(26) Torres, J. L., Lozano, C., Julià, L., Sánchez, F. J., Anglada, J., Centelles, J. J., and Cascante, M. (2002) Cysteiniyl-flavan-3-ol conjugates from grape procyanidins. Antioxidant and antiproliferative properties. *Bioorg. Med. Chem. 10*, 2497–2509.
(27) Simao, A., Suzukawa, A., Casado, M. F., Oliveira, R., Guarnier, F., and Cecchini, R. (2006) Genistein abrogates pre-hemolytic and oxidative stress damage induced by 2,2′-azobis(amidinopropane). *Life Sci. 78*, 1202–1210.
(28) Caturla, N., Vera-Samper, E., Villalaín, J., Mateo, C., and Micol, V. (2003) The relationship between the antioxidant and the antibacterial preperties of galloylated catechins and the structure of phospholipid model membranes. *Free Radical Biol. Med. 34*, 648–662.
(29) Erlejman, A.G., Verstraeten, S. V., Fraga, C. G., and Oteiza, P. I. (2004) The interaction of flavonoids with membranes: Potential determination of flavonoid antioxidant effects. *Free Radical Res. 38*, 1311–1320.
(30) Na, H. K., and Surh, Y. J. (2006) Intracellular signaling network as a prime chemopreventive target of (−)-epigallocatechin gallate. *Mol. Nutr. Food Res. 50*, 152–159.
(31) Tan, X. H., Hu, D. R., Li, S. R., Han, Y., Zhanc, Y. L., and Zhou, D. Y. (2000) Differences of four cathechins in cell cycle arrest and induction of apoptosis in LoVo cells. *Cancer Lett. 158*, 1–6.
(32) Matito, C., Mastorakou, F., Centelles, J. J., Torres, J. L., and Cascante, M. (2003) Antiproliferative effect of antioxidant polyphenols from grape in murine Hepa-1c1c7. *Eur. J. Nutr. 42*, 43–49.
(33) Dai, F., Miao, Q., Zhou, B., Yang, L., and Liu, Z. (2006) Protective effects of flavonols and theirglycosides against free radical-induced oxidative hemolysis of red blood cells. *Life Sci. 78*, 2488–2493.
(34) Ugartondo, V., Mitjans, M., Lozano, C., Torres, J. L., and Vinardell, M. P. (2006) Comparative study of the cytotoxicity induced by antioxidant epicatechin conjugates obtained from grape. *J. Agric. Food Chem. 54*, 6945–6950.
(35) Benavides, T., Mitjans, M., Martínez, V., Clapés, P., Infante, M. R., Clothier, R. H., and Vinardell, M. P. (2004) Assessment of primary eye and skin irritants by in vitro cytotoxicity and phototoxicity models: an in vitro approach of nex arginine-based surfactants-induced irritation. *Toxicology 197*, 229–237.
(36) Clothier, R., Willshaw, A., and Cox, H. (1999) The use of human keratynocytes in the EU/COLIPA international in vitro phototoxicity test validation study and the ECVAM/COLIPA study on UV filter chemicals. ATLA. *Altern. Lab. Anim. 27*, 247–259.
(37) Schmidt, M., Schmitz, H. J., Baumgart, A., Guédon, D., Netsch, M. I., Kreuter, M. H., Schmidlin, C. B., and Schrenk, D. (2005) Toxicity of green tea extracts and their constituents in rat hepatocytes in primary culture. *Food Chem. Toxicol, 43*, 307–314.
(38) Lozano, C., Torres, J., Julià, L., Jiménez, A., Centelles, J. J., and Cascante, M. (2005) Effect of new antioxidant cysteinyl-flavanol conjugates on skin cancer cells. *FEBS Lett. 579*, 4219–4225.

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ARTÍCULO 4

Fracciones de taninos con elevado grado de galoización procedentes de corteza de hamamelis (*Hamamelis virginiana*): capacidad de transferencia de electrones, actividad antioxidante *in vitro* y efecto sobre células de la piel

Highly galloylated tannin fractions from witch hazel (*Hamamelis virginiana*) bark: electron transfer capacity, in vitro antioxidant activity, and effects on skin-related cells.

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Resumen

La corteza del arbusto hamamelis (*Hamamelis virginiana*) es una rica fuente de taninos oligoméricos tanto condensados como hidrolizables. A partir de un extracto polifenólico soluble en acetato de etilo y en agua, se ha generado un conjunto de fracciones ricas en polifenoles con elevado contenido en grupos pirogalol (proantocianidinas, galotaninos y galatos). Las mezclas se mostraron muy activas como secuestradoras de radicales libres contra los radicales ABTS, DPPH (mecanismo de acción implicado es la donación de átomos de hidrógeno y la transferencia de electrones) y HNTTM (sólo transferencia de electrones). También fueron capaces de reducir el nuevo radical TNPTM, lo que significa que estos compuestos incluyen algunos componentes altamente activos. Los fenoles de hamamelis presentaron una eficaz protección de los eritrocitos frente a la hemólisis inducida por radicales libres y mostraron una citotoxicidad media hacia las líneas celulares de fibroblastos 3T3 y queratinocitos HaCaT. Además, inhibieron la proliferación de células tumorales de melanoma SK-Mel 28 en un rango de concentraciones inferior al obtenido para las procianidinas de uva y de pino. El elevado contenido en grupos pirogalol podría ser el responsable del efecto de los polifenoles de hamamelis sobre las células de la piel. Debido a que las mezclas más citotóxicas y antiproliferativas fueron también las más eficientes como agentes transferidores de electrones, se sugiere que el supuesto efecto antioxidante de los polifenoles podría atribuirse, en parte, a la estimulación de los sistemas de defensa mediante leves ataques prooxidantes causados por especies reactivas del oxígeno generadas por los compuestos fenólicos a través del ciclo redox.

Highly Galloylated Tannin Fractions from Witch Hazel (*Hamamelis virginiana*) Bark: Electron Transfer Capacity, In Vitro Antioxidant Activity, and Effects on Skin-Related Cells

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Witch hazel (*Hammamelis virginiana*) bark is a rich source of both condensed and hydrolizable oligomeric tannins. From a polyphenolic extract soluble in both ethyl acetate and water, we have generated fractions rich in pyrogallol-containing polyphenols (proanthocyanidins, gallotannins, and gallates). The mixtures were highly active as free radical scavengers against ABTS, DPPH (hydrogen donation and electron transfer), and HNTTM (electron transfer). They were also able to reduce the newly introduced TNPTM radical, meaning that they included some highly reactive components. Witch hazel phenolics protected red blood cells from free radical-induced hemolysis and were mildly cytotoxic to 3T3 fibroblasts and HaCat keratinocytes. They also inhibited the proliferation of tumoral SK-Mel 28 melanoma cells at lower concentrations than grape and pine procyanidins. The high content in pyrogallol moieties may be behind the effect of witch hazel phenolics on skin cells. Because the most cytotoxic and antiproliferative mixtures were also the most efficient as electron transfer agents, we hypothesize that the final putative antioxidant effect of polyphenols may be in part attributed to the stimulation of defense systems by mild prooxidant challenges provided by reactive oxygen species generated through redox cycling.

Introduction

Phenolics from plants are appreciated for their putative health-promoting properties (*1, 2*). The antioxidant activity, taken in a broad sense, is believed to be responsible for the preventative properties of flavonoids. The main mechanisms behind this antioxidant activity are direct free radical scavenging (*3, 4*), transition metal chelation (*5, 6*), and maintenance of endogenous antioxidants such as the glutathione and superoxide dismutase systems (*7*). Interestingly, polyphenols may be antioxidant and prooxidant at the same time (*8, 9*). While all phenolics are scavengers of reactive oxygen species (ROS), strongly reducing species such as pyrogallol (three adjacent phenol groups) containing (−)-epigallocatechin (EGC)[1] and (−)-epigallocatechingallate (EGCG) are able to form the superoxide radical from molecular oxygen (*10, 11*) (Figure 1). Moreover, the *ortho*-quinones formed by the loss of two electrons from pyrogallol and catechol moieties may participate in enzymatic redox cycling with the formation of superoxide and other ROS (*12, 13*). Apart from their participation in redox-related events, tannins may modify cell functions by substrate−receptor interactions (e.g., kinase inhibition), which may or may not involve redox reactions (*14*). This ensemble of activities influence cell proliferation, cell cycle regulation, and apoptosis, and the pyrogallol moieties, both on ring B and as gallate ester at C-3, appear to play a pivotal role (*15, 16*).

Whether all of these effects detected in vitro have any significance in vivo is controversial. Because polyphenols are extensively metabolized into less reactive species (*17*) and the cell redox system is too carefully regulated to be influenced by low concentrations of scavengers, it has been argued that polyphenols may not exert any significant effect on the cell redox status of complex organisms (*18, 19*). However, it can also be argued that polyphenols, particularly the less metabolized oligomeric species, may still have a significant influence on organs such as the skin and the intestinal tract (*20, 21*). In any case, flavonoid-containing nutritional supplements and over the counter drugs have become so popular and available that people risk overdosing. This is why it is important to examine the action of these plant actives from different angles and to evaluate their putative benefits and risks. Most of the information available in the literature about the antioxidant/prooxidant activities and substrate−receptor interactions of phenolics relates to monomeric EGCG and quercetin (*13, 14, 22*). Because the redox and binding properties of phenolics are affected by polymerization

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[1] Abbreviations: AAPH, 2,2′-azobis(amidinopropane)dihydrochloride; ARC, antiradical capacity; C, catechin; Cya, cysteamine; EC, epicatechin; ECG, epicatechin-gallate; EGC, epigallocatechin; EGCG, epigallocatechin-gallate; GC, gallocatechin; DMEM, Dulbecco's modified Eagle's medium; DPPH, 1,1-diphenyl-2-picrylhydrazyl free radical; FCS, fetal calf serum; HNTTM, Tris(2,3,6-trichloro-3,5-dinitrophenyl)methyl; HPLC-DAD, high-performance liquid chromatography with diode array detection; MTT, 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide; NRU, neutral red uptake; RP-HPLC, reversed-phase high-performance liquid chromatography; RCBs, red blood cells; TFA, trifluoroacetic acid; Trolox, 2,5,7,8-tetramethylchroman-2-carboxylic acid; TNPTM, tris(2,3,5,6-tetrachloro-4-nitrophenyl)-methyl.

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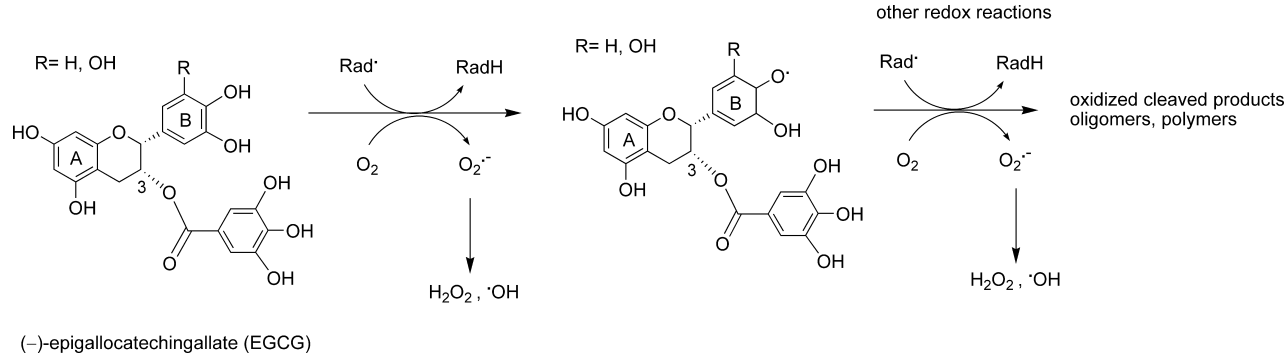


Figure 1. Scavenging of ROS and superoxide formation by catechins.

(*23, 24*), it is of great interest to evaluate the activity of the oligomers (e.g., proanthocyanidins) on cells. We have previously reported the extraction and fractionation of phenolics from grape pomace and pine bark and the evaluation of their free radical scavenging capacity, antioxidant activity in vitro, cytotoxicity on nontumoral 3T3 fibroblasts and keratinocites, and antiproliferative activity on melanoma cells (*25–27*). Polyphenols from grape and pine were essentially procyanidins (oligomeric catechins with only two hydroxyls on ring B, catechol moiety) with low gallate content or no galloylation at all, respectively. The fractions were effective free radical scavenger antiproliferative agents against skin and colon tumoral cells and weakly cytotoxic. To test the behavior of phenolics with high pyrogallol content, we have now prepared and evaluated a homologous series of fractions from witch hazel (*Hamamelis virginiana*) bark, which contains gallocatechins and prodelphinidins (monomeric and oligomeric catechins with three hydroxyls on ring B) with a high proportion of gallates. The gallates come from both condensed and hydrolyzable tannins. As compared to grape and pine, phenolics from hamamelis showed higher electron transfer capacity, cytotoxicity, and antiproliferative activity against skin-related cell lines.

Experimental Procedures

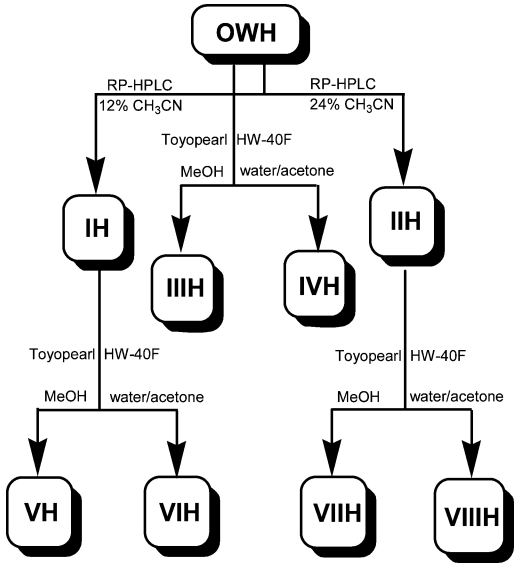
The starting material was witch hazel (*H. virginiana*) chopped stems provided by Martin Bauer GMBH (Alveslohe, Germany). The sample was stored in the dark at room temperature.

Solvents and Reagents. For extraction, deionized water, bulk EtOH (Momplet y Esteban, Barcelona, Spain), bulk acetone (Quimivita, Sant Adrià del Besòs, Spain), and bulk hexane (Quimivita) were used for polyphenol extraction. For purification, deionized water, analytical grade MeOH (Panreac, Montcada i Reixac, Spain), and analytical grade acetone (Carlo Erba, Milano, Italy) and preparative grade CH_3CN (E. Merck, Darmstadt, Germany) were used for semipreparative and preparative chromatography; milli-Q water and HPLC grade CH_3CN (E. Merck) were used for analytical reversed-phase high-performance liquid chromatography (RP-HPLC). Analytical grade MeOH (Panreac) was used for thioacidolysis and free radical scavenging assays, and analytical grade CH_3Cl (Panreac) was used for the electron transfer assays. Trifluoroacetic acid (TFA, Fluorochem, Derbyshire, United Kingdom) biotech grade was distilled in-house. Cysteamine hydrochloride was from Sigma-Aldrich Chemical (Steinheim, Germany), and 37% HCl and acetic acid were from E. Merck. Triethylamine (E. Merck) was of buffer grade. Deuterated solvents for nuclear magnetic resonance (NMR) were from SDS (Peypin, France). 1,1-Diphenyl-2-picrylhydrazyl free radical (DPPH) (95%) was from Aldrich (Gillingham-Dorset, United Kingdom), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) (97%) was from Aldrich (Milwaukee, WI), and standards of (−)-epicatechin, (+)-catechin, (+)-gallocatechin (−)-epigallocatechin, (−)-epigallocatechin 3-*O*-gallate, gallic acid, methyl gallate, and hamamelitannin were purchased from Sigma Chemical (St. Louis, MO). 4-β-(2-Aminoethylthio)catechin, 4-β-(2-aminoethylthio)epicatechin, β-(2-aminoethylthio)epicatechin-3-*O*-gallate, 4-β-(2-aminoethylthio)epigallocatechin, and β-(2-aminoethylthio)epigallocatechin-3-*O*-gallate were synthesized and purified from grape and witch hazel extracts essentially as described (*28*). 2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) crystallized diammonium salt, horseradish peroxidase type IV (RZ A403/A275 <3) and 2,2′-azobis(amidinopropane)dihydrochloride (AAPH) were obtained from Sigma Chemical. Hydrogen peroxide (3% v/v) was from Sigma Chemical. Tris-(2,4,6-trichloro-3,5-dinitrophenyl)-methyl (HNTTM) and tris(2,3,5,6-tetrachloro-4-nitrophenyl)methyl (TNPTM) radicals were synthesized as described (*29, 30*). Dulbecco's modified Eagle's medium (DMEM) and Dulbecco's phosphate buffer saline were from Gibco-BRL (Eggenstein, Germany), fetal calf serum (FCS) was from Invitrogen (Carlsbad, CA), and trypsin EDTA solution C (0.05% trypsin–0.02% EDTA) was from Biological Industries (Kibbutz Beit Haemet, Israel). 3-[4,5-Dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT) was from Sigma Chemical.

Extraction and Solvent Fractionation. The preparation of the crude extract was performed using already described methodology (*25, 31, 32*). Brief, witch hazel chopped stems (3 kg) were incubated with an acetone–water mixture (7:3, 10.5 L) for a period of 24 h at room temperature, with occasional shaking. The solid was filtered off, and the acetone was evaporated at reduced pressure. The remaining solution was defatted with *n*-hexane (3 × 300 mL), and the oligomeric fraction was extracted with ethyl acetate (3 × 600 mL). This organic phase was dried under vacuum, the pellet was dissolved in deionized water, and the solution was filtered through a porous plate. The dry fraction OWH (28.5 g), soluble in both ethyl acetate and water, was obtained by lyophilization.

Chromatographic Fractionation. Fractions IH (hydrophilic) and IIH (hydrophobic) were obtained by preparative RP-HPLC essentially as described before (*26, 33*). The rest of the fractions were generated from these two or directly from OWH by semipreparative chromatography on Toyopearl TSK HW-40F (TosoHass, Tokyo, Japan) following a protocol previously described by the authors (*25, 26*) (Scheme 1). The phenolics were eluted with MeOH and water/acetone 1:1, evaporated almost to dryness, redissolved in 100 mL of Milli-Q water, and freeze-dried; from OWH, 315 mg of IIIH and 573 mg of IVH; from fraction IH, 235 mg of VH and 336 mg of VIH; and from fraction IIH, 126 mg of VIIH and 468 mg of VIIIH. The fractions were analyzed by high-performance liquid chromatography with diode array detection (HPLC-DAD) using a Hitachi (San Jose, CA) Lachrom Elite HPLC system equipped with a quaternary pump, autosampler, in-line degassing unit, temperature control unit, photodiode array UV detector, and fitted with an analytical column Kromasil C18 (Teknokroma, Barcelona, Spain) (25 cm × 0.4 cm i.d., 100 Å, 5 μm particle size). Acquisitions were made using EZChrom Elite 3.1.3 from Scientific Software Inc. (Pleasanton, CA). Load, 40 μL, 10 μg; elution, (A) 0.1% (v/v) aqueous TFA and (B) 0.08% (v/v) TFA in water/CH_3CN 1:4,

Scheme 1



gradient 12−30% B over 30 min at a flow rate of 1 mL/min. DAD detection was performed from 210 to 380 nm. Data were acquired in triplicate.

Characterization by Thiolysis with Cysteamine and RP-HPLC. The size and composition of the proanthocyanidins within the fractions were estimated from the HPLC analysis of acid-catalyzed degradation of proanthocyanidins in the presence of cysteamine, followed by RP-HPLC as described (*34*). Briefly, the terminal flavan-3-ols units were released as such by acid cleavage in the presence of cysteamine whereas the extension moieties were released as the cysteamine derivatives on the fourth position of the flavanoid system. The resulting mixtures were submitted to analytical RP-HPLC using the same conditions described above for the intact samples, and the molar amount (nanomoles) of all of the released moieties was calculated from the peak areas and calibration curves obtained with pure samples. Terminal units: (+)-gallocatechin (GC), (−)-EGC, (+)-C, (−)-epicatechin (EC), (−)-EGCG, and (−)-ECG; extension units: cysteamine (Cya)-C, Cya-EC, Cya-EGC, Cya-EGCG, and Cya-ECG. Mean degree of polymerization (mDP) = total nmol/nmol terminal units.

Characterization by Chromatography Coupled to Mass Spectrometry. Liquid chromatography–mass spectrometry (LC-MS-MS) was used for the identification of gallotannins and gallates within the fractions. The analyses were carried out on an Agilent 1100 (Waldrom, Germany) coupled to an API 3000 triple quadrupole mass spectrometer (Perkin-Elmer Sciex, Concord, ON, Canada) at the Parc Cientific de la Universitat de Barcelona. Mass scan (MS) and daughter (MS/MS) spectra were measured from *m*/*z* 100 to 1500. Mass spectrometry data were acquired in the negative ionization mode.

ABTS Radical Cation Decolorization Assay. The method is based on the capacity of a sample to scavenge the ABTS radical cation ($ABTS^{\bullet+}$) as compared to a standard antioxidant (Trolox). $ABTS^{\bullet+}$was generated from ABTS as described (*35*) with some modifications (*26*). To prepare the initial $ABTS^{\bullet+}$ solution, 3‰ H_2O_2 (45μL) was added to a reaction mixture containing ABTS (54.9 mg, 1 mM) and horseradish peroxidase (HRP, 1.1 mg, 0.25 μM) in 50 mM gly HCl buffer, pH 4.5 (100 mL). The reaction mixture was left to stand at room temperature for 15 min in the dark. Then, the polyphenolic solutions (50 μL) at concentrations of 0.3, 0.2, 0.15, 0.10, and 0.05 mg/mL in MeOH were added to the $ABTS^{\bullet+}$ solution (1950 μL). The total time needed to carry out each assay was 20 min, including ABTS radical generation, addition of antioxidant, and acquisition of final absorbance value. The decrease of absorbance at 734 nm with respect to the 1 mM solution of $ABTS^{\bullet+}$ was recorded on a UV spectrophotometer Cary 300-Bio (Varian, Palo Alto, CA). The assay was performed in triplicate. The dose–response curves obtained with the antioxidant mixtures and Trolox were plotted as the percentage of absorbance decrease against the amount of antioxidants expressed as μg/mL. The total antioxidant activity (TAA) of the fractions was expressed in mmol Trolox equiv/g of OWH.

DPPH Assay. The antiradical efficiency of the fractions was evaluated by the DPPH stable radical method (*36, 37*). The samples (0.1 mL) were added to aliquots (3.9 mL) of a solution made up with DPPH (4.8 mg) in MeOH (200 mL), and the mixture was incubated for 1 h at room temperature in the dark. The initial concentration of DPPH, approximately 60 μM, was calculated for every experiment from a calibration curve made by measuring the absorbance at 517 nm of standard samples of DPPH at different concentrations. The equation of the curve was $Abs_{517nm} = 11345 \times C_{DPPH}$ as determined by linear regression. The results were plotted as the percentage of absorbance disappearance at 517 nm $[(1 - A/A_0) \times 100]$ against the amount of sample divided by the initial concentration of DPPH. Each point was acquired in triplicate. A dose–response curve was obtained for every fraction. ED_{50} corresponds to micrograms of fraction able to consume half the amount of free radical divided by micromoles of initial DPPH. The results were expressed as antiradical capacity (ARC), which is the inverse of ED_{50}. UV measurements were made on a UV spectrophotometer Cary 300-Bio (Varian).

Electron Transfer Capacity against HNTTM and TNPTM. The fractions were dissolved in CH_3Cl/MeOH (2:1) at different concentrations. Aliquots (1 mL) were added to a solution (1 mL) of HNTTM [120 μM in CH_3Cl/MeOH (2:1)] (*29*), and the mixture was incubated for 7 h. The exact initial concentration of radical, around 60 μM, was calculated for every experiment from calibration curves made by measuring the absorbance (A_0) at 385 nm of standard samples of the radical at different concentrations. The equations of the curve was $A_0 = 21170 \times C_{radical}$. The results were plotted as the percentage of absorbance disappearance $[(1 - A/A_0) \times 100]$ against the amount of sample divided by the initial micromoles of the radical as described for DPPH. Each point was acquired in triplicate. A dose–response curve was obtained for every fraction. The results were expressed as the efficient dose ED_{50} given as micromoles of fraction able to consume half the amount of free radical divided by micromoles of initial HNTTM.

The working conditions with TNPTM were essentially those described for HNTTM with some differences. The incubation time was 48 h, and the absorbance was measured at 378 nm. The equation for the calibration curve was $A_0 = 17153 \times C_{radical}$. The results were plotted as described for HNTTM. UV measurements were made on a UV spectrophotometer Cary 300-Bio (Varian). A solution of pyrogallol [60 μM in CH_3Cl/MeOH (2:1)] was stable for 48 h as ascertained by RP-HPLC under the elution conditions described before for the analysis of the fractions.

Antioxidant Activity on Red Blood Cells by the AAPH Assay. Blood samples were obtained from healthy donors by venipuncture (Blood Bank of Hospital Vall d'Ebron, Barcelona, Spain) following the ethical guidelines of the hospital and collected in citrated tubes. Red blood cells (RBCs) were separated from plasma and buffy coat by centrifugation at 1000*g* for 10 min. The erythrocyte layer was washed three times in phosphate buffer 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 isotonic saline solution at a density of 8×10^9 cells/mL. We measured the hemolysis of RBCs mediated by AAPH using a modification of the method described previously (*38*). The addition of AAPH (a peroxyl radical initiator) to the suspension of RBCs induces the oxidation of cell membrane lipids and proteins, thereby resulting in hemolysis. The erythrocyte suspension (250 μL) was incubated in the presence of AAPH at a final concentration of 100 mM for 150 min at 37 °C to achieve 100% hemolysis. Hemolysis was assessed by measuring the absorbance of the supernatant fraction, that is, the hemoglobin release, at 540 nm in a Shimadzu spectrophotometer (Shimadzu, Japan). The antihemolytic activity of the fractions was studied by adding the compounds at several concentrations (10−150 μg/mL)

to the RBCs suspension in the presence of 100 mM AAPH at 37 °C for 2.5 h. The IC_{50} (sample concentration causing 50% protection) of the hemolysis induced by AAPH was determined for each compound.

Cytotoxicity on Keratinocytes and Fibroblasts. To evaluate the cytotoxicity on nontumoral cells, we used the spontaneously immortalized human keratinocyte cell line HaCaT and the mouse fibroblast cell line 3T3. Cells were grown in DMEM (4.5 g/L glucose) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 mM Hepes buffer, and 1% penicillin (10000 U/mL) streptomycin (10000 μg/mL) maintained in a humidified atmosphere with 5% CO_2 at 37 °C. When 75 cm^2 culture flasks were approximately 80% confluent, the cells were seeded into the central 60 wells of 96-well plates as described previously (*39*) at a density of 5.5×10^4 cells/mL for HaCaT and 1.5×10^4 cells/mL for 3T3 (*40*). Plates were incubated at 37 °C and 5% CO_2 for 24 h. Triplicate runs were undertaken with different passage cells. After 1 day of incubation, the growth medium was removed and replaced with exposure medium (DMEM medium supplemented with 5% FBS, 2 mM L-glutamine, 10 mM Hepes buffer, and 1% antibiotic mixture), with or without the polyphenolic mixtures, which were previously sterilized by filtration. Controls, containing culture medium only, were included in each plate. Cells were then incubated at 37 °C and 5% CO_2 for 72 h.

The cell viability was assessed by the neutral red uptake (NRU) assay and performed as described (*41*) and modified to avoid the use of formaldehyde (*42*). After the treatments, medium was aspired and replaced with 100 μL/well of NR solution (50 μg/mL in RPMI medium without phenol red and serum). After 3 h of incubation at 37 °C and 5% CO_2, the medium was aspirated, the cells were washed twice with PBS, and a solution containing 50% ethanol absolute and 1% acetic acid in distilled water was added (100 μL/well) to release the dye incorporated into the viable cells into the supernatant. After 10 min on a microtiter plate shaker, the absorbance of the neutral red was measured at a wavelength of 550 nm in a Bio-Rad 550 microplate reader (Bio-Rad Laboratories, Hercules, CA).

The cytotoxicity of each fraction was expressed as a percentage of viability as compared to control wells (the mean optical density of untreated cells was set to 100% viability) in terms of its IC_{50} (concentration of product that causes 50% inhibition of growth or death of the cell population), calculated from the dose–response curves by linear regression analysis. NRU assay results were expressed as the percentage of uptake of neutral red dye by the lysosomes. Each experiment was performed at least three times using three replicates for each concentration assayed. The results were expressed as means ± SEM. Statistical significance was determined by Student's *t* test and one-way analysis of variance (ANOVA) using the SPSS software (SPSS Inc., Chicago, IL). Statistical significance was considered at $P < 0.05$.

Antiproliferative Activity on SK-Mel-28 Human Melanoma Cells. SK-MEL-28 adherent cells (ATCC #HTB-72) were grown in DMEM supplemented with 10% (v/v) heat-inactivated FCS in the presence of 0.1% (v/v) antibiotics (10000 U/mL penicillin and 10000 μg/mL streptomycin) at 37 °C in a humidified environment with 5% CO_2. The cells were split (ratio 1:2 to 1:5) by mild trypsinization every 4–5 days, and the medium was changed every 2–3 days. The cell culture used in this study was free of mycoplasm infection as shown by the EZ-PCR Mycoplasm test kit (Biological Industries) prior to the treatment with the samples. The cell viability was determined using the Mosmann assay (*43*) with some modifications. Cells were seeded into 96-well plates at 1×10^4 cells/mL density, 200 μL/well, and incubated for 24 h in the culture medium prior to addition of the samples dissolved in DMEM. Control wells were treated with equal volumes of DMEM as the test cultures. After 72 h of culture, the supernatant was aspirated and 100 μL of sterile-filtered MTT (0.5 mg/mL in DMEM) was added to each well. The plates were incubated at 37 °C and 5% CO_2 for 1 h. The supernatant was removed, the blue MTT formazan that precipitated was dissolved in DMSO (100 μL), and the optical density (OD) was measured at 550 nm on a multiwell reader (Merck ELISA System MIOS).

The inhibitory effect of the fractions at each concentration was expressed as a percentage [(mean OD treated cells after 72 h of incubation with the product/mean OD of control cells after 72 h of incubation with extra medium instead of product) × 100]. The IC_{50} or sample concentration causing a 50% reduction in the mean OD value relative to the control at 72 h of incubation was estimated using GraFit 3.00 (Data Analysis and Graphics Program, Erithacus Software Ltd. Microsoft Corp., Surrey, United Kingdom) curve option: IC_{50} curve − start at 0.

Results and Discussion

Fractionation of Witch Hazel Oligomeric Tannins. Following a combination of already described methods (*25, 28, 44*), a polyphenolic mixture of monomeric and oligomeric tannins soluble in both ethyl acetate and water (OWH) was obtained from witch hazel bark. First, a sugar free mixture was obtained by extraction with water/acetone (3:7). After the acetone was evaporated, the lipid soluble material was eliminated with hexane, and the resulting aqueous phase was extracted with ethyl acetate. The organic solvent was eliminated to yield a crude polyphenolic mixture (28.5 g from 3 kg of dry stems, ca. 1% yield). Witch hazel contained more small and medium-sized phenolics (OWH) than grape pomace (OWG, yield ca. 0.1%) (*28*) or pine bark (OWP, yield >0.1%) (*26*). This crude mixture was fractionated (Scheme 1) into eight fractions by a combination of two chromatographic techniques, namely, reversed-phase and size discrimination using the same strategy applied to grape and pine extracts (*25, 26*). RP-HPLC retains solutes by hydrophobicity while Toyopearl HW-40 has been shown to separate flavonoids in order of increasing sizes (*45*). In this way, we generated a collection of mixtures containing hydrolyzable tannins and oligomeric pronthocyanidins of different mean degrees of polymerization, galloylation, and prodelphinidin contents.

Characterization of the Fractions. The structures of significant compounds found in OWH and its fractions are depicted in Figure 2. In accordance with the literature (*44, 46*), the mixtures contained flavanol (catechin) monomers, proanthocyanidins, and hydrolyzable tannins such as hamamelitannin. Some of the mixtures also contained methyl gallate and pentagalloyl glucose. Tables 1 and 2 summarize the results obtained from the HPLC analysis after thioacidolysis (condensed tannins) and direct HPLC-DAD analysis (hamamelitannin, gallic acid, methyl gallate, and pentagalloylglucose). The mean degree of polymerization and composition in constituent monomers of the condensed tannin portion (monomers + proanthocyanidins) were estimated by thioacidolysis in the presence of cysteamine as described in the Experimental Procedures. This procedure, which uses cysteamine hydrochloride as an alternative reagent to thiol-α-toluene, was originally applied to procyanidins (catechol-containing condensed tannins). Now, we have extended the method to prodelphinidins. The appropriate pyrogallol containing new standards, namely, 4-β(2-aminoethylthio) epigallocatechin (Cya-EGC) and 4-β(2-aminoethylthio) epigallocatechin 3-*O*-gallate (Cya-EGCG), have been obtained from the polymeric fraction insoluble in ethyl acetate following essentially the procedures described before (*28*). All of the fractions contained condensed tannins, both monomers and oligomers. Interestingly, the more retained mixture on reversed-phase HPLC (IIH) contained less condensed tannins (34.7%) than IH (79.1%). Small condensed tannins from witch hazel are markedly hydrophilic as compared to phenolics from pine and grape. This is due to the presence of the pyrogallol moiety on ring B,

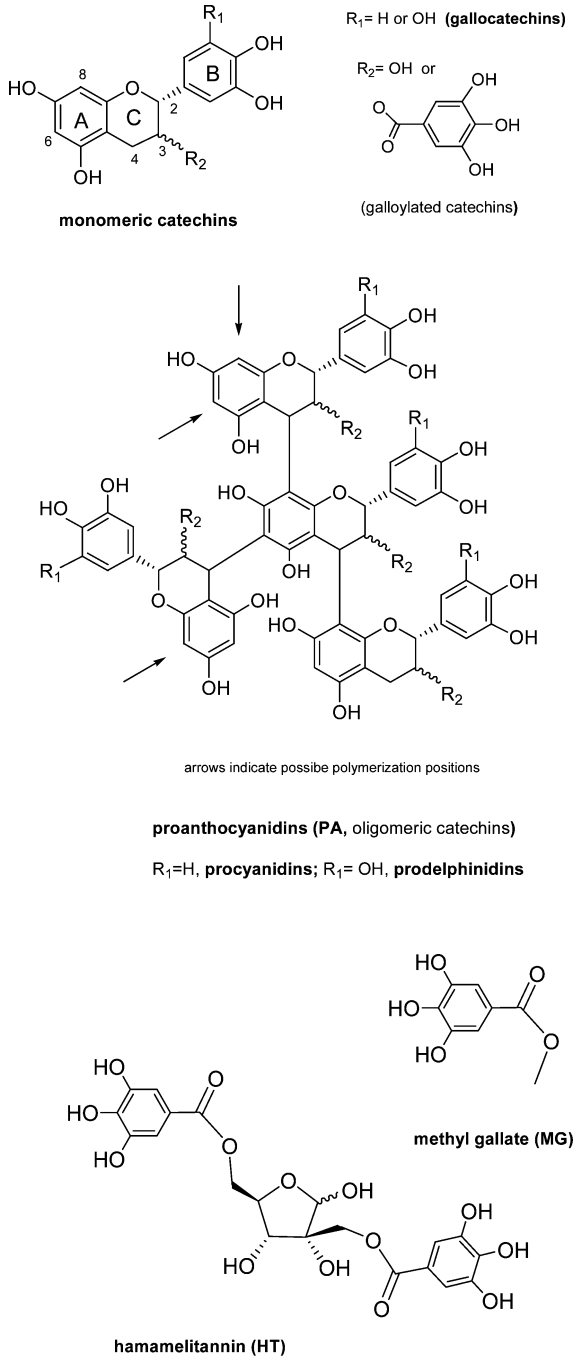


Figure 2. Structures of polyphenolics in *H. virginiana* bark extract.

which is absent in pine and very sparse in grape. Data from Table 2 show that the hydrophobic fractions IIH, VIIH, and VIIIH were low in gallocatechins, which were mainly found in IH, VIH, and IVH. The higher amounts of gallocatechins in the more retained fractions on Toyopearl indicate that they are mainly included in oligomeric structures (prodelphinidins) in contrast with tea gallocatechins, which are monomeric (*47*). This is in agreement with the composition of fraction VH, which was mainly monomeric and low in gallocatechins. The extract and fractions also contained the so-called galloylhamameloses, hydrolyzable tannins that were identified by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Di- and trigalloyl hamamelofuranoses have been described before (*46, 48*). We have identified hamamelitannin (2′,5′ di-*O*-galloyl hamamelose HT *m/z* [M − H]$^-$483, Figure 2) and a pentagalloyl glucose (PGG, *m/z* [M − H]$^-$ 939) as the two main galloyltannins in OWH and derived fractions. Gallic acid (GA) and methyl gallate (MG *m/z* [M − H]$^-$ 183) were detected in some of the fractions. The pentagalloyl glucose content was particularly high in fractions IIH and VIIIH.

Table 1. Polyphenolic Composition of Fractions from Witch Hazel Bark[a]

fraction	% M + PA[b]	mDP[c] M + PA	% HT[d]	% GA[d]	% MG[d]	% PGG[e]
OWH	62.7	1.2	7.9	21.9		7.4
IH	79.1	1.1	10.9	4.1		5.8
IIH	34.7	1.7	2.5	14.7		48.2
IIIH	62.3	1.0	2.1	35.4		0.6
IVH	62.7	1.6	15.0	2.3		19.9
VH	78.8	1.1	2.0	12.1	7.0	
VIH	41.9	2.6	16.1	1.8	18.2	21.7
VIIH	7.9	1.0	0.6	43.3	30.4	17.8
VIIIH	4.3	1.1	0.5	2.4	31.8	61.0

[a] Molar percentages in the total measured phenolics. [b] M + PA, monomeric catechins and proanthocyanidins estimated from the thioacidolysis experiment. [c] mDP, mean degree of polymerization. Mean of three independent thioacidolysis experiments with three RP-HPLC replicate injections. [d] HT, hamamelitannin; GA, gallic acid; and MG, methyl gallate, estimated by HPLC and standards. [e] PGG, pentagalloylglucose, expressed as HT equivalents.

Table 2. Composition of the Condensed Tannins in Polyphenolic Fractions from Witch Hazel Bark[a]

fraction	% GC	% EGC	% C	% EC	% EGCG	% ECG
OWH	14.1	2.0	67.4	5.8	3.6	7.1
IH	11.4	1.5	73.3	7.0	1.6	5.2
IIH	2.8	0.6	70.7	6.2	2.7	16.9
IIIH	4.6	0.4	86.5	6.2	0.3	1.9
IVH	24.9	4.3	38.4	9.3	5.7	17.4
VH	2.1	0.3	89.4	6.5	0.4	1.3
VIH	29.2	4.1	32.9	10.9	4.4	18.4
VIIH	0.0	0.9	75.7	5.7	1.4	16.3
VIIIH	1.5	2.4	44.1	14.1	4.5	3.4

[a] Molar percentage. Mean of three independent thioacidolysis experiments with three RP-HPLC replicate injections.

Briefly, all of the mixtures derived from witch hazel bark presented high amounts of galloylated species pertaining to both condensed and hydrolyzable types of tannins. Some of the fractions, particularly IVH and VIH, also contained the pyrogallol moiety on ring B of their condensed tannins (gallocatechins and prodelphinidins). Because all of the fractions were rich in heavily hydroxylated phenolic molecules, we expected to obtain high free radical-scavenging activities.

Total Antioxidant Activity TAA. The total antioxidant activity of the polyphenolic mixture OWH and its fractions was measured by the ABTS cation radical method, which is a widely used assay for the evaluation of natural antioxidant mixtures such as extracts, juices, and wine (*49, 50*). OWH contained 6 mmol of Trolox equiv/g. In general agreement with the number of hydroxyls per molecule, the OWH extract showed a total antioxidant activity 70% higher than OWP (homologous extract from pine). TAA for the fractions generated from OWH are summarized in Figure 3. As compared to pine bark (*26*), hamamelis was a richer source of free radical-scavenging phenolics. The fractions retained on Toyopearl (IVH, VIH, and VIIIH), which contain bulky galloylated species, concentrated most of the activity, followed by fraction VH. To obtain information on the scavenging efficiency of the phenolics in every fraction, we then turned to the use of stable radicals.

Free Radical Scavenging and Electron Transfer Capacity. The extract and fractions were evaluated as free radical scavengers using different stable radicals, namely, DPPH and the newly introduced HNTTM and TNPTM. DPPH reacts with

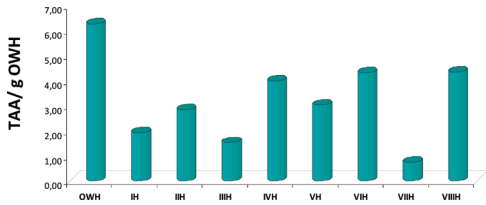


Figure 3. Total antioxidant activity (TAA) of the fractions by the ABTS cation radical method. TAA expressed as mmol Trolox equiv obtained per g of OWH.

Table 3. Hydrogen Donation and Electron Transfer Capacity of Polyphenolic Fractions from Witch Hazel Bark

	DPPH		HNTTM		TNPTM	
fractions	ED_{50}[a]	ARC[b]	ED_{50}	ARC[b]	ED_{50}[a]	ARC[b]
OWH	42.4	23.6	49.8	20.1	1225.5	0.8
IH	44.6	22.4	38.2	26.2	922.5	1.1
IIH	26.1	38.3	60.3	16.6	1592.4	0.6
IIIH	57.9	17.3	86.2	11.6	1059.3	0.9
IVH	28.6	35.0	57.2	17.5	956.0	1.0
VH	58.8	17.0	69.4	14.4	605.3	1.6
VIH	29.5	33.9	45.5	22.0	1488.1	0.7
VIIH	52.8	18.9	77.5	12.9	534.2	1.9
VIIIH	26.4	37.9	49.1	20.4	1265.8	0.8
			control			
Ec	49.3	20.3	60.9	16.4	NR	NR

[a] ED_{50} μg of fraction/μmol of radical, mean of three experiments. [b] ARC, $(1/ED_{50}) \times 10^3$; NR, no reaction.

polyphenols by mechanisms that may include both hydrogen donation and electron transfer (*37, 51*), while the new radicals are only sensitive to electron transfer (*30, 52*). Interestingly, HNTTM reacts with both catechol and pyrogallol moieties, while TNPTM will react only with the most reducing positions, namely, the pyrogallol group on ring B of condensed tannins, while being inactive against cathecols and gallates (*30*). By comparing the results generated with the three radicals, we gained information about the combined hydrogen donation and electron transfer capacity (DPPH), global electron transfer capacity (HNTTM), and the presence of highly reactive electron transfer positions (Figure 1). The results with HNTTM and particularly TNPTM may provide valuable information about the ability of some components to engage in putatively prooxidant/toxic effects involving electron transfer to oxygen. Table 3 summarizes the results obtained with the three radicals. As expected, witch hazel fractions were more potent (1.5−3-fold) scavengers than the homologous pine bark fractions. Again, fractions VIH and VIIIH, rich in bulky galloylated phenolics, were particularly efficient hydrogen donors and electron transfer agents (DPPH and HNTTM assays). Interestingly, fractions IVH (mainly condensed tannins) and VIIIH (mainly hydrolyzable tannins) were equally effective. Because the common structural feature of both fractions is the pyrogallol/gallate group, our results underscore the relevance of the trihydroxybenzene moiety for the scavenging activity of tannins. All of the fractions were active against the TNPTM radical, meaning that they contained highly reactive species. Because the ARCs were low, these reactive species are probably present as minor components. Interestingly, the most effective mixtures (VH and VIIH) were not those with the highest global electron transfer capacity. Both fractions, excluded from the Toyopearl column, were low in proanthocyanidins. Fraction VH contained monomeric catechin as the major component, and both included gallic acid and methyl gallate. None of these single molecules reacted with TNPTM when tested alone. This suggests that the mixtures might contain other reactive species. Alternatively, because all of the fractions were reactive to some extent, it may be that under the test conditions, highly reactive species are formed from otherwise inert precursors. These hypotheses are currently being tested in our laboratory.

Antioxidant Protection of Red Blood Cells. To evaluate the antioxidant protective effect of hamamelis fractions on cells submitted to oxidative stress, we used red blood cells (RBCs). Because of their susceptibility to peroxidation, RBCs have been used as a model to investigate oxidative damage in biomembranes. We investigated the oxidation of RBCs induced by AAPH, a well-known peroxyl radical initiator that causes hemolysis by means of membrane lipid and protein oxidation. Dose–response curves were analyzed, and IC_{50}(concentration triggering 50% inhibition of APPH induced hemolysis) values were obtained for some significant fractions. All of the fractions tested showed an inhibition of the in vitro AAPH-induced RBC hemolysis in a dose-dependent manner (data not shown). The IC_{50} values were 21.5 ± 1.6 (OWH), 22.6 ± 1.7 (IVH), and 24.5 ± 0.8 μg/mL (VIIIH), and all of them were more effective than the homologous fractions from grape and pine (*27*).

Cytotoxicity on Keratinocytes and Fibroblasts. To gain preliminary information about the cytotoxicity of the fractions as compared to their pine and grape homologues, the mixtures were tested on nontumoral HaCat keratinocytes and 3T3 fibroblasts. We selected the 3T3 neutral red uptake assay because this test is recommended by the U.S. National Institute of Environmental Health Science (NIEHS) Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). Nontumorigenic HaCat, a spontaneously immortalized keratinocyte cell line, provides an almost unlimited supply of identical cells, ensuring high intra- and interlaboratory reproducibility. Selected fractions showed some capacity to inhibit the proliferation of nontumoral skin cells with IC_{50} values of 41 ± 2 (OWH), 38 ± 3 (IVH), and 68 ± 10 μg/mL (VIIIH) on HaCat keratinocytes; and 51 ± 3 (OWH), 51 ± 1 (IVH), and 33 ± 3 μg/mL (VIIIH) on 3T3 fibroblasts. The cytotoxicity of the mixtures was relatively low. To visualize how safe the mixtures were for skin cells at their antioxidant active concentration, we calculated the relationship between the cytotoxicity index (IC_{50}) at 72 h in 3T3 and the antioxidant potential. We found that antioxidant concentrations were approximately 1.4−2.4-fold lower than the cytotoxic concentrations. We can conclude that an effective antioxidant activity of the fractions can be obtained at a concentration range not toxic for the nontumoral cell lines studied.

Antiproliferation of SK-Mel 28 Human Melanoma Cells. We and others have shown that plant phenolics influence the viability of eukaryotic cells by arresting the cell cycle and inducing cell death by apoptosis or necrosis (*53–55*). These effects appear to relate to the number and position of phenolic hydroxyls and, consequently, to the free radical scavenging and electron transfer capacity of the active species (*8, 55, 56*). To test the effect of the hamamelis phenolics on skin cancer cells, selected fractions (OWH, IVH, VH, VIH, and VIIIH) homologous to those from grape pomace and pine bark tested before (*25, 26*) were assayed for their influence on the proliferation of SK-Mel 28 human melanoma cells. All of the fractions showed some activity at relatively high concentrations. The IC_{50} values obtained were 26 ± 2 (OWH), 29 ± 2 (IVH), 32 ± 2 (VH), 28 ± 2 (VIH), and 39 ± 2 μg/mL (VIIIH). Interestingly again, the phenolics from witch hazel bark fractions were more efficient antiproliferative agents than those from grape and pine on this tumoral cell line. Particularly, hamamelis phenolics were between 4- and 6-fold more potent than pine bark procyanidins.

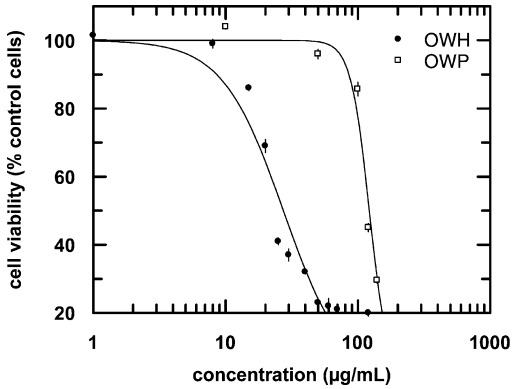


Figure 4. Percentage of proliferation of SK-Mel 28 human melanoma cells as a function of polyphenolic mixture concentration. Samples shown are the crude mixtures soluble in both ethyl acetate and water OWH (□) and OWP (●). Cells were incubated for 72 h with medium alone (control) or containing the polyphenols. $IC_{50} = 26 \pm 2$ μg/mL (OWH) and 122 ± 5 μg/mL (OWP). Data are given as the mean value ± SEM; experiments were performed in triplicate.

Figure 4 depicts the dose–response curve corresponding to the crude extract OWH as compared to the homologous mixture from pine bark (OWP). In agreement with the results on scavenging capacity, fractions IVH and VIIIH, which differed in percentage of condensed and hydrolyzable tannins but had in common a high content in trihydroxybenzene moieties (pyrogallol/gallates), were equally effective against cell proliferation.

In general agreement with the literature (*8, 27, 53, 55*), our comparative results show that the most efficient scavengers (hamamelis phenolics as compared to pine and grape components) as measured with both DPPH and HNTTM stable radicals were also the most cytotoxic/antiproliferative agents. This could be due to the so-called pro-oxidant effect of polyphenols. The generation of the superoxide radical and other ROS by EGCG, quercetin, and other phenolics in a variety of experimental setups have been reported before (*11–13, 22*) and might be behind the mild effect of phenolics on cell growth and apoptotic/necrotic death. The common structural feature mainly responsible for the high activity of witch hazel fractions appears to be the pyrogallol group both on ring B of gallocatechins/prodelphinidins and on galloyl moieties (gallates). Interestingly, fraction VH showed lower global electron transfer capacity than VIH or VIIIH as measured with HNTTM but higher electron transfer capacity as measured by TNPTM. The fact that all three fractions were equally antiproliferative against melanoma cells is in agreement with the presence in VH of the highly reactive species suggested above. The new radical TNPTM may have picked up on some relevant information by detecting the presence of putative cytotoxic species through a simple chemical test. Alternatively or complementarily, the gallate group may be interacting with relevant domains for cell replication (e.g., kinase domains of phosphorylating factors).

Concluding Remarks

Natural plant polyphenols appear to exert their action on living organisms by a combination of redox reactions and receptor–ligand interactions (*14*). They are considered antioxidants and perceived popularly as beneficial agents for the prevention of many diseases. However, what do we really mean by antioxidants? The concept is usually linked to free radical scavenging since it has been accepted that the underlying cause of cell damage is the production of ROS by mitochondrial metabolism and that ROS are essentially harmful and should be eliminated. However, ROS may not be always harmful. First, ROS as well as reactive nitrogen species (RNS) are key agents in the regulation of cell functions by acting as secondary messengers in intracellular signaling cascades (*8, 57*). Second, moderate generation of ROS may end up producing an antioxidant effect by fostering the endogenous defenses. It is becoming evident that mild prooxidant challenges such as physical exercise trigger mild transient oxidative stress with subsequent stimulation of antioxidant detoxifying defenses (*58*). Polyphenols may, at least in part, exert their activity in a similar way by providing mild prooxidant challenges through electron transfer reactions leading to moderate formation of ROS. The so-called prooxidant effect of some polyphenols may be in fact the real antioxidant activity. The results presented here on witch hazel bark phenolics, together with our previous studies with homologous fractions from pine and grape (*25–27, 55*), show that the higher the percentage of pyrogallols in the mixtures is, the higher the antiproliferative potency on epithelial cells is. Because the most cytotoxic/antiproliferative mixtures were also those with the highest electron transfer capacity, we hypothesize that tannins may provide the cell with a mild prooxidative challenge through the formation of the superoxide radical and redox cycling to oxidative species, which may stimulate the endogenous detoxifying systems. The prooxidant activity may be, at least in part, responsible for the alleged antioxidant effect of plant phenolics. The new stable radicals HNTTM and TNPTM, which are sensitive only to electron transfer and possess different redox potentials, may help to define the prooxidant and cytotoxic profile of phenolics. The abundance of pyrogallol groups appears to play a major role in the antioxidant/prooxidant effects of hamamelis phenolics.

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Supporting Information Available: RP-HPLC chromatograms obtained for all of the fractions before and after thioacidolysis and dose–response curves from the SK-Mel 28 proliferation assay. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Rusznyák, S. P., and Szent-Györgyi, A. (1936) Vitamin P: Flavonols as vitamins. *Nature 138*, 27.
(2) Ross, J. A., and Kasum, C. M. (2002) Dietary flavonoids: Bioavailability, metabolic effects, and safety. *Annu. Rev. Nutr. 22*, 19–34.
(3) Rice-Evans, C. A., Miller, N. J., and Paganga, G. (1996) Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med. 20*, 933–956.
(4) Yokozawa, T., Chen, C. P., Dong, E., Tanaka, T., Nonaka, G.-I., and Nishioka, I. (1998) Study on the inhibitory effect of tannins and flavonoids against the 1,1-diphenyl-2-picrylhydrazyl radical. *Biochem. Pharmacol. 56*, 213–222.
(5) Brown, J. E., Khodr, H., Hider, R. C., and Rice-Evans, C. A. (1998) Structural dependence of flavonoid interactions with Cu2+ ions: Implications for their antioxidant properties. *Biochem. J. 330*, 1173–1178.
(6) Hider, R. C., Liu, Z. D., and Khodr, H. H. (2001) Metal chelation of polyphenols. In *Flavonoids and Other Polyphenols* (Packer, L., Ed.) pp 190–203, Academic Press Inc., San Diego, CA.
(7) Ishige, K., Schubert, D., and Sagara, Y. (2001) Flavonoids protect neuronal cells from oxidative stress by three distinct mechanisms. *Free Radical Biol. Med. 30*, 433–446.
(8) Alanko, J., Riutta, A., Holm, P., Mucha, I., Vapaatalo, H., and Metsa-Ketela, T. (1999) Modulation of arachidonic acid metabolism by phenols: Relation to their structure and antioxidant/prooxidant properties. *Free Radical Biol. Med. 26*, 193–201.

(9) Lambert, J. D., Sang, S., and Yang, C. S. (2007) Possible controversy over dietary polyphenols: Benefits vs risks. *Chem. Res. Toxicol. 20*, 583–585.
(10) Kondo, K., Kurihara, M., Miyata, N., Suzuki, T., and Toyoda, M. (1999) Mechanistic studies of catechins as antioxidants against radical oxidation. *Archiv. Biochem. Biophys. 362*, 79–86.
(11) Kondo, K., Kurihara, M., Miyata, N., Suzuki, T., and Toyoda, M. (1999) Scavenging mechanisms of (−)-epigallocatechin gallate and (−)-epicatechin gallate on peroxyl radicals and formation of superoxide during the inhibitory action. *Free Radical Biol. Med. 27*, 855–863.
(12) Nutter, L. M., Wu, Y. Y., Ngo, E. O., Sierra, E. E., Gutierrez, P. L., and Abul-Hajj, Y. J. (1994) An o-quinone form of estrogen produces free radicals in human breast cancer cells: Correlation with DNA damage. *Chem. Res. Toxicol. 7*, 23–28.
(13) Boots, A. W., Li, H., Schins, R. P. F., Duffin, R., Heemskerk, J. W. M., Bast, A., and Haenen, G. (2007) The quercetin paradox. *Toxicol. Appl. Pharmacol. 222*, 89–96.
(14) Sang, S., Hou, Z., Lambert, J. D., and Yang, C. S. (2005) Redox properties of tea polyphenols and related biological activities. *Antioxid. Redox Signaling 7*, 1704–1714.
(15) Liang, Y. C., Lin-shiau, S. Y., Chen, C. F., and Lin, J. K. (1997) Suppression of extracellular signals and cell proliferation through EGF receptor binding by (−)-epigallocatechin gallate in human A431 epidermoid carcinoma cells. *J. Cell Biochem. 67*, 55–65.
(16) Ahmad, N., Cheng, P. Y., and Mukhtar, H. (2000) Cell cycle dysregulation by green tea polyphenol epigallocatechin-3-gallate. *Biochem. Biophys. Res. Commun. 275*, 328–334.
(17) Scalbert, A., Morand, C., Manach, C., and Remesy, C. (2002) Absorption and metabolism of polyphenols in the gut and impact on health. *Biomed. Pharmacother. 56*, 276–282.
(18) Halliwell, B. (2006) Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. *Plant Physiol. 141*, 312–322.
(19) Linnane, A. W., Kios, M., and Vitetta, L. (2007) Healthy aging: regulation of the metabolome by cellular redox modulation and prooxidant signaling systems: The essential roles of superoxide anion and hydrogen peroxide. *Biogerontology 8*, 445–467.
(20) Halliwell, B., Zhao, K., and Whiteman, M. (2000) The gastrointestinal tract: A major site of antioxidant action? *Free Radical Res. 33*, 819–830.
(21) Cross, C. E., van der Vliet, A., Louie, S., Thiele, J. J., and Halliwell, B. (1998) Oxidative stress and antioxidants at biosurfaces: Plants, skin, and respiratory tract surfaces. *Environ. Health Perspect. 106* (Suppl. 5), 1241–1251.
(22) Azam, S., Hadi, N., Khan, N. U., and Hadi, S. M. (2004) Prooxidant property of green tea polyphenols epicatechin and epigallocatechin-3-gallate: Implications for anticancer properties. *Toxicol. in Vitro 18*, 555–561.
(23) Plumb, G. W., De Pascual-Teresa, S., Santos-Buelga, C., Cheynier, V., and Williamson, G. (1998) Antioxidant properties of catechins and proanthocyanidins: Effect of polymerization, galloylation and glycosylation. *Free Radical Res. 29*, 351–358.
(24) Saint-Cricq de Gaulejac, N., Vivas, N., Freitas, V., and Bourgeois, G. (1999) The influence of various phenolic compounds on scavenging activity assessed by an enzymatic method. *J. Sci. Food Agric. 79*, 1081–1090.
(25) Torres, J. L., Varela, B., García, M. T., Carilla, J., Matito, C., Centelles, J. J., Cascante, C., Sort, X., and Bobet, R. (2002) Valorization of grape (*Vitis vinifera*) byproducts. Antioxidant and biological properties of polyphenolic fractions differing in procyanidin composition and flavonol content. *J. Agric. Food Chem. 50*, 7548–7555.
(26) Touriño, S., Selga, A., Jiménez, A., Juliá, L., Lozano, C., Lizárraga, D., Cascante, C., and Torres, J. L. (2005) Procyanidin fractions from pine (*Pinus pinaster*) bark: Radical scavenging power in solution, antioxidant activity in emulsion and antiproliferative effect in melanoma cells. *J. Agric. Food Chem. 53*, 4728–4735.
(27) Ugartondo, V., Mitjans, M., Touriño, S., Torres, J. L., and Vinardell, M. P. (2007) Comparative antioxidant and cytotoxic effect of procyanidin fractions from grape and pine. *Chem. Res. Toxicol. 20*, 1543–1548.
(28) Torres, J. L., and Bobet, R. (2001) New flavanol-derivatives from grape (*Vitis vinifera*) byproducts. Antioxidant aminoethylthio-flavan-3ol conjugates from a polymeric waste fraction used as a source of flavanols. *J. Agric. Food Chem. 49*, 4627–4634.
(29) Torres, J. L., Varela, B., Brillas, E., and Juliá, L. (2003) Tris(2,4,6Trichloro-3,5-dinitrophenyl)methyl radical: A new stable coloured magnetic species as a chemosensor for natural polyphenols. *Chem. Commun.* 74–75.
(30) Torres, J. L., Carreras, A., Jiménez, A., Brillas, E., Torrelles, X., Rius, J., and Juliá, L. (2007) Reducing power of simple polyphenols by electron-transfer reactions using a new stable radical of the PTM series, tris(2,3,5,6-tetrachloro-4-nitrophenyl)methyl radical. *J. Org. Chem. 72*, 3750–3756.
(31) Dauer, A., Metzner, P., and Schimmer, O. (1998) Proanthocyanidins from the bark of *Hamamelis virginiana* exibit antimutagenic properties against nitroaromatic compounds. *Planta Med. 64*, 324–327.
(32) Deters, A., Dauer, A., Schnetz, E., Fartasch, M., and Hensel, A. (2001) High molecular compounds (polysaccharides and proanthocyanidins) from *Hamamelis virginiana* bark: Influence on human skin keratinocyte proliferation and differentiation and influence on irritated skin. *Phytochemistry 58*, 949–958.
(33) Matito, C., Mastorakou, F., Centelles, J. J., Torres, J. L., and Cascante, M. (2003) Antiproliferative effect of antioxidant polyphenols from grape in murine Hepa-1c1c7. *Eur. J. Nutr. 42*, 43–49.
(34) Torres, J. L., and Selga, A. (2003) Procyanidin size and composition by thiolysis with cysteamine hydrochloride and chromatography. *Chromatographia 57*, 441–445.
(35) Alcolea, J. F., Cano, A., Acosta, M., and Arnao, M. B. (2002) Hydrophilic and lipofilic antioxidant activities of grapes. *Nahrung 46*, 353–356.
(36) Blois, M. S. (1958) Antioxidant determinations by the use of stable free radical. *Nature 181*, 1199–1200.
(37) Brand-Williams, W., Cuvelier, M. E., and Berset, C. (1995) Use of a free radical method to evaluate antioxidant activity. *Lebensm.-Wiss. Technol. 28*, 25–30.
(38) Miki, M., Tamai, H., Mino, M., Yamamoto, Y., and Niké, E. (1987) Free radical chain oxidation of rat red blood cells by molecular oxygen and its inhibition by α-tocopherol. *Arch. Biochem. Biophys. 258*, 373–380.
(39) Ugartondo, V., Mitjans, M., Lozano, C., Torres, J. L., and Vinardell, M. P. (2006) Comparative study of the cytotoxicity induced by antioxidant epicatechin conjugates obtained from grape. *J. Agric. Food Chem. 54*, 6945–6950.
(40) Babich, H., Krupka, M. E., Nissim, H. A., and Zuckerbraun, H. L. (2005) Differential in vitro cytotoxicity of (−)-epicatechin gallate (ECG) to cancer and normal cells from the human oral cavity. *Toxicol. in Vitro 19*, 231–242.
(41) Borenfreund, E. P., and Rapad, J. (1983) A colorimetric assay to cellular growth and survival: Application to proliferation and cytotoxicity assay. *Toxicol. Lett. 24*, 119–124.
(42) Riddell, R. J., Clothier, R. H., and Ball, M. (1986) An evaluation of three in vitro cytotoxicity assays. *Food Chem. Toxicol. 24*, 469–471.
(43) Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* 55–63.
(44) Dauer, A., Rimpler, H., and Hensel, A. (2003) Polymeric proanthocyanidins from the bark of *Hamamelis virginiana*. *Planta Med. 69*, 89–91.
(45) Sun, B. S., Belchior, G. P., Ricardo da Silva, J. M., and Spranger, M. I. (1999) Isolation and purification of dimeric and trimeric procyanidins from grape seeds. *J. Chromatogr. A 841*, 115–121.
(46) Hartisch, C., and Kolodziej, H. (1996) Galloylhamameloses and proanthocyanidins from *Hamamelis virginiana*. *Phytochemistry 42*, 191–198.
(47) Harbowy, M. E., and Balentine, D. A. (1997) Tea chemistry. *Crit. Rev. Plant Sci. 16*, 415–480.
(48) Haberland, C., and Kolodziej, H. (1994) Novel galloylhamameloses from *Hamamelis virginiana*. *Planta Med. 60*, 464–466.
(49) Miller, N. J., and Rice Evans, C. (1997) The relative contributions of ascorbic acid and phenolic antioxidants to the total antioxidant activity of orange and apple fruit juices and blackcurrant drink. *Food Chem. 60*, 331–337.
(50) Pietta, P., Simonetti, P., and Mauri, P. (1998) Antioxidant activity of selected medicinal plants. *J. Agric. Food Chem. 46*, 4487–4490.
(51) Dangles, O., Fargeix, G., and Dufour, C. (2000) Antioxidant properties of anthocyanins and tannins: A mechanistic investigation with catechin and the 3′,4′,7-trihydroxyflavylium ion. *J. Chem. Soc., Perkin Trans. 2* 1653–1666.
(52) Jiménez, A., Selga, A., Torres, J. L., and Juliá, L. (2004) Reducing activity of polyphenols with stable radicals of the TTM series. Electron transfer versus H-abstraction reactions in flavan-3-ols. *Org. Lett. 6*, 4583–4586.
(53) Tan, X. H., Hu, D. R., Li, S. R., Han, Y., Zhang, Y. L., and Zhou, D. Y. (2000) Differences of four catechins in cell cycle arrest and induction of apoptosis in LoVo cells. *Cancer Lett. 158*, 1–6.
(54) Kozikowski, A. P., Tuckmantel, W., Bottcher, G., and Romanczyk, L. J., Jr. (2003) Studies in polyphenol chemistry and bioactivity. 4. (1) Synthesis of trimeric, tetrameric, pentameric, and higher oligomeric epicatechin-derived procyanidins having all-4β,8-interflavan connectivity and their inhibition of cancer cell growth through cell cycle arrest. *J. Org. Chem. 68*, 1641–1658.
(55) Lizárraga, D., Lozano, C., Briede, J. J., van Delft, J. H., Touriño, S., Centelles, J. J., Torres, J. L., and Cascante, M. (2007) The importance of polymerization and galloylation for the antiproliferative properties of procyanidin-rich natural extracts. *FEBS J. 274*, 4802–4811.

(56) Lozano, C., Torres, J. L., Juliá, L., Jiménez, A., Centelles, J. J., and Cascante, M. (2005) Effect of new antioxidant cysteinyl-flavanol conjugates on skin cancer cells. *FEBS Lett. 579*, 4219–4225.
(57) Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T. D., Mazur, M., and Telser, J. (2007) Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol. 39*, 44–84.
(58) Ascensao, A., Magalhaes, J. F., Soares, J. M., Ferreira, R. M., Neuparth, M. J., Appell, H. J., and Duarte, J. A. (2005) Cardiac mitochondrial respiratory function and oxidative stress: The role of exercise. *Int. J. Sports Med. 26*, 258–267.

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Bloque II. Actividad protectora frente al estrés oxidativo inducido por el H_2O_2

ARTÍCULO 5 (MANUSCRITO)

Protección contra el daño oxidativo inducido por peróxido de hidrógeno en eritrocitos humanos y la línea celular de fibroblastos 3T3 por fracciones polifenólicas procedentes de diferentes fuentes

Protection against hydrogen peroxide-induced oxidative damage in human erythrocytes and the 3T3 fibroblast cell line by polyphenolic fractions from different sources.

Vanessa Ugartondo, María Pilar Vinardell, Sonia Touriño, Josep Lluís Torres y Montserrat Mitjans

Enviado a Chemical Research in Toxicology

Resumen

Las especies reactivas del oxígeno (ROS) reaccionan con los ácidos nucleicos, las proteínas y lípidos, provocando daños en su estructura y función. Cada vez hay más evidencias que sugieren que concentraciones excesivas de ROS en el cuerpo humano estarían relacionadas con ciertos eventos patológicos y que el daño oxidativo de los componentes celulares jugaría un importante papel fisiopatológico en diversas enfermedades. Por lo tanto, hay un creciente interés en el estudio de los efectos beneficiosos de sustancias antioxidantes procedentes de fuentes naturales para obtener nuevos compuestos capaces de proteger a las células del daño producido por los radicales libres y otros compuestos oxidantes. En este estudio se han examinado el potencial efecto inhibitorio de la peroxidación lipídica y la capacidad protectora contra la citotoxicidad que presentaban una serie de fracciones polifenólicas procedentes de diferentes fuentes como el bagazo de uva blanca, corteza de pino y corteza del arbusto hamamelis. El estudio se diseñó para evaluar si las fracciones polifenólicas eran capaces de prevenir la peroxidación lipídica y la hemólisis de eritrocitos humanos inducida por el H_2O_2, y evaluar el grado en que estos compuestos podían proteger a la línea celular 3T3 del daño oxidativo inducido por H_2O_2. Las fracciones se mostraron eficaces en la protección contra la peroxidación lipídica y la hemólisis en eritrocitos, así como contra la citotoxicidad causada por el H_2O_2 en células 3T3. Al comparar fracciones homólogas, se observó que los efectos inhibitorios contra la peroxidación lipídica estaban notablemente relacionados con el grado de galoización presente en su estructura. Los resultados obtenidos muestran que el grado de galoización, además de estar estrechamente relacionado con la capacidad antioxidante protectora de los compuestos en la línea celular 3T3, también es responsable del efecto prooxidante que presentan algunos de estos compuestos polifenólicos a altas dosis. Por lo tanto, estos compuestos, que son productos residuales en la industria agroalimentaria, se comportan como agentes protectores a bajas concentraciones, lo que los

convierte en buenas fuentes de suplementos dietéticos y fitonutrientes y en productos con gran potencial para aplicaciones en la industria alimentaria y farmacéutica.

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Protection against hydrogen peroxide-induced oxidative damage in human erythrocytes and the 3T3 fibroblast cell line by polyphenolic fractions from different sources

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Running title: Peroxidative protection by polyphenolic fractions

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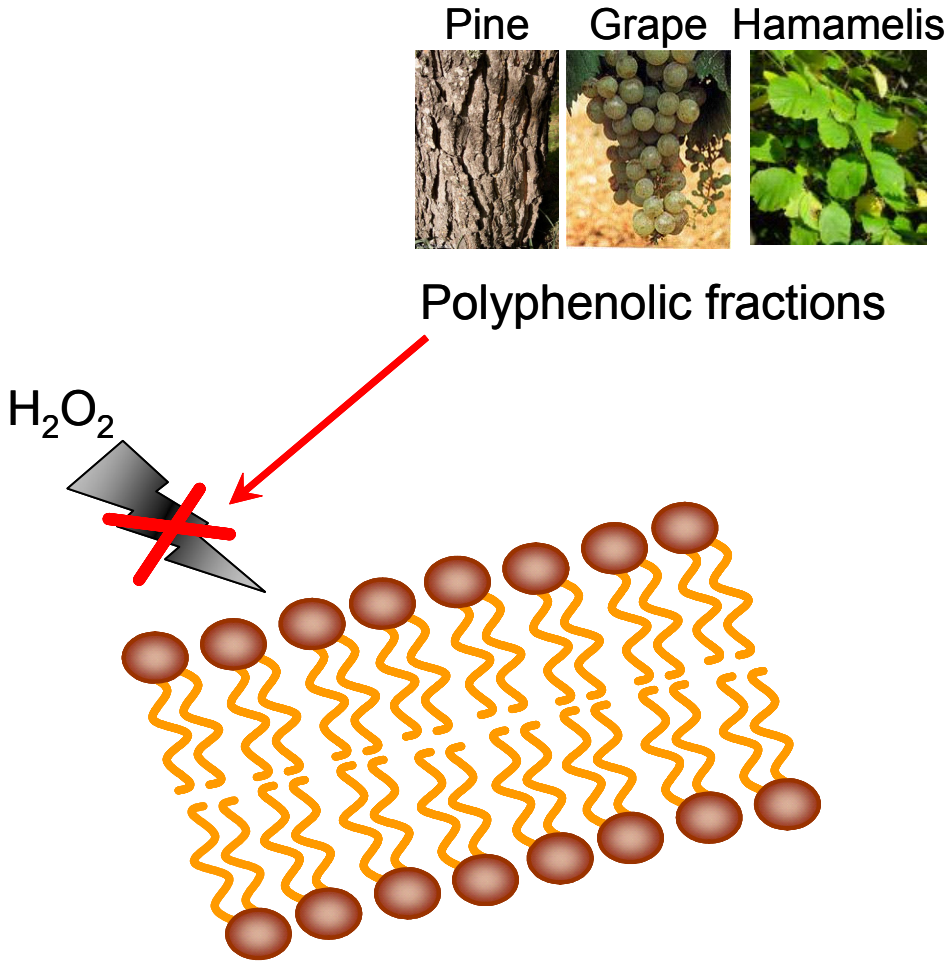
Table of Contents Graphic (TOC)



ABSTRACT

Increasing evidence suggest that excessive concentrations of reactive oxygen species in the human body are involved in a number of pathological events and that oxidative damage to cell components may play an important pathophysiological role in many types of human diseases. Therefore, there is increasing interest in studying the beneficial effects of antioxidants from natural sources.

Here we examined and compared the inhibition of lipid peroxidation and the cytotoxic protection capacity of polyphenolic fractions from different natural sources such as white grape pomace, pine bark and witch hazel bark. The study was designed to evaluate whether polyphenolic fractions prevent H_2O_2-induced lipid peroxidation and hemolysis of human erythrocytes, and to evaluate the extent to which these compounds can protect the 3T3 cell line from an oxidative insult induced by H_2O_2.

Fractions demonstrated protection against erythrocyte lipid peroxidation and hemolysis, as well as against cytotoxicity caused by H_2O_2 in 3T3 cells. When comparing homologous fractions the inhibitory effects against lipid peroxidation were notably related to the degree of galloylation they presented. Our results show that the degree of galloylation was not only strongly related to the antioxidant protective capacity of compounds in the 3T3 cell line but was also responsible for their pro-oxidant effect at high doses. The findings indicated that these fractions, which are by-products in the agro-food industry, are good sources of nutraceuticals and phytonutrients and show great potential for such applications in the food and drug industries.

INTRODUCTION

Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot -}$) and the hydroxyl radical ($HO^{\cdot}$) are generated in biological systems by aerobic metabolism and also by exogenous sources such as drugs, ultraviolet light, ionizing radiation and pollution systems (*1*).

Increasing evidence suggests that excessive concentrations of reactive oxygen species in the human body are involved in a number of pathological events and that oxidative damage to cell components may play an important pathophysiological role in many types of human diseases (*2*, *3*). Reactive oxygen species (ROS) induce a number of molecular alterations in cellular components, leading to changes in cell morphology, viability and function. These changes include DNA damage, protein cross-links and side chain oxidation (*4*). In this regard, hydrogen peroxide (H_2O_2) is one of many compounds that can be injurious to cells (*5*); high concentrations of H_2O_2 induce cell necrosis while low concentrations induce apoptosis.

Many endogenous and exogenous defence mechanisms are available in living organisms to limit the levels of ROS and the damage caused by them (*2*). These defences include antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, as well as, many non-enzymatic antioxidant compounds, for example polyphenols, ascorbic acid and glutathione. It has been reported that unbalanced ROS production and antioxidant cell defences are associated with physiological and pathological conditions such as aging, cancer, rheumatoid arthritis, atherosclerosis and neurodegenerative diseases (*1*).

In this context, there is increasing interest in the protective biochemical function of naturally-occurring antioxidants in biological systems and on their mechanisms of action. Several plant constituents possess considerable free radical scavenging or antioxidant activity (*6*, *7*). Thus, polyphenols extracted from various sources protect against stress (*8*), while flavonoids and other phenolic compounds of plant origin act as scavengers and inhibitors of lipid peroxidation (*3*, *9*).

In previous studies, we evaluated the antioxidant activity of several polyphenolic fractions from different natural sources in various *in vitro* assay systems in order to elucidate their possible applications in the fields of food preservation, skin protection, and cancer prevention. In this regard, we have evaluated phenolic fractions and compounds extracted from grape (*Vitis vinifera*) pomace, pine (*Pinus*

pinaster) bark and witch hazel (*Hamamelis virginiana*) bark, using different methods to demonstrate their radical scavenging and antioxidant activity, their cytotoxicity on non-tumoural 3T3 fibroblasts and human HaCaT keratinocytes, and their antiproliferative activity on melanoma cells (*10-13*). Polyphenols from pine and grape were essentially procyanidins (oligomeric catechins with only two hydroxyls on ring B) with no galloylation or low gallate content, respectively. The homologous series of fractions from witch hazel bark contain gallocatechins and prodelphinins (monomeric and oligomeric catechins with three hydroxyls on ring B) with a high proportion of gallates as well as gallate rich hydrolysable tannins. The pine and grape fractions were effective free radical scavengers, antiproliferative agents against skin and colon tumour cells, and weakly cytotoxic. In comparison, phenolics from *Hamamelis* showed higher electron transfer capacity, higher antioxidant activity in red blood cell systems, moderate cytotoxicity, and more efficient antiproliferative activity against skin-related cells (*13*).

Because the gallate group not only provides high antiradical power but also appears to interfere with crucial cell functions, galloylation seems to be an essential structural feature defining the activity and toxicity of phenolic mixtures (*12*). Our group has placed particular emphasis on the structure-activity relationships of these compounds.

Different models have been employed to detect and understand both the effects of reactive oxygen species and the activity of natural and synthetic scavengers (3, 14). The antioxidant properties of plant-derived phenolic compounds have been extensively studied by using *in vitro* chemical systems. These systems have the advantage of being relatively simple and inexpensive to implement. However, such *in vitro* assays can only rank antioxidant activity for their particular reaction system and their relevance to *in vivo* health-protective activities is uncertain (*15*). It is therefore considered prudent to use more than one antioxidant assay system to measure antioxidant activities, as there may be distinct mechanisms involved, resulting in different outcomes depending on the test method use (*16*).

We therefore considered that the next step in characterizing the biological properties of our polyphenolic fractions was to conduct an in-depth study of their antioxidant properties, but this time analysing their protective capacity against H_2O_2-induced oxidative damage.

H_2O_2 is considered an attractive oxidant model and its cellular actions and reactivity have been well studied (*17*). Although in chemical terms H_2O_2 is poorly reactive, it readily crosses cell membranes and gives rise to the highly reactive

hydroxyl radical ($OH^{\cdot}$), which reacts with macromolecules, including DNA, proteins and lipids, and to ultimately damages the cell (*18*).

The present study aimed to examine and compare the inhibition of lipid peroxidation and the cytotoxic protection capacity afforded by polyphenolic fractions from different natural sources such as white grape pomace, pine bark and witch hazel bark, using different *in vitro* cell models. The study was designed: (1) to evaluate whether polyphenolic fractions prevent H_2O_2-induced lipid peroxidation and hemolysis of human erythrocytes, and (2) to evaluate the extent to which these compounds can protect the 3T3 cell line from an oxidative insult, i.e. against H_2O_2-induced cell damage. The cytoprotective effects of these compounds were measured by the degree of protection against H_2O_2-induced cell damage using cell viability as the endpoint.

EXPERIMENTAL PROCEDURES

Materials. The polyphenolic total extract (OW) was obtained from *Pinus pinaster* bark (OWP), from Parellada grape (*Vitis vinifera*) pomace (OWG), and from chopped witch hazel stems (OWH), following the procedure described by Torres and Bobet (*19*) and Touriño et al. (*11*, *13*). OWP contained monomeric and oligomeric catechins and other monomeric flavonoids; OWG contained monomeric catechins, oligomeric catechins (procyanidins) and, in a lower proportion, flavonols, mainly glycosylated (*12*); OWH contained monomeric and oligomeric tannins (proanthocyanidins and hydrolysable tannins). Witch hazel included more small- and medium-sized phenolics than did grape pomace or pine bark. From these total extracts our group obtained mixtures of variable phenolic composition using a combination of chromatographic techniques, and we then generated a set of homologous fractions of each type, which differed in composition and procyanidin structure. We labelled these homologous fractions OWP, IVP, and XIP for pine; OWG, IVG, and XIG for grape; and OWH, IVH, and XIH for *Hamamelis*. The fractions from the three sources were highly homologous in terms of mean molecular size (data not shown); they were mainly differentiated by their degree of galloylation (presence of gallate esters). Table 1 summarizes the degree of polymerization (mDP) and the percentage of galloylation previously described by our group (*10*, *11*, *13*).

Polyphenols from grape (*Vitis vinifera*) pomace and pine (*Pinus pinaster*) bark were essentially procyanidins with low gallate content or no galloylation at all, respectively. Witch hazel (*Hamamelis virginiana*) bark fractions contained a high proportion of gallates included in both prodelphinidins and hydrolysable tannins (*20*).

2-thiobarbituric acid, hydrogen peroxide 30% (w/w) solution and sodium azide were purchased from Sigma (St Luis, MO); trichloroacetic acid solution 20% w/v, extra pure was purchased from Scharlau (Sentmenat, Spain)

Blood samples and Preparation of Red Blood Cells. Human blood was obtained from the Blood Bank of the Hospital Vall d'Hebrón (Barcelona, Spain) following the ethical guidelines of this institution. The erythrocytes were washed three times in a phosphate buffer solution (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 suspended in isotonic saline solution at a cell density of 8 x 10^9 cells/mL. The final packet cell volume was adjusted to 12.5% under the assay conditions. Sodium azide at 2mM in PBS was added to the cell suspension, which was then the suspension was pre-incubated for 15 minutes in continuous rotation to enable inactivation of erythrocyte catalase by the sodium azide.

***In vitro* assay for the inhibition of human erythrocyte hemolysis.** Hemolysis induced by H_2O_2 was evaluated according to the technique of Grinberg et al., with slight modifications (*21*). The erythrocyte suspension (250 µL) was incubated for 90 min at 37°C with continuous shaking and in the presence of H_2O_2 at a final concentration of 20 mM in order to achieve maximal hemolisys this being based on previous studies in our laboratory (data not shown). The same test was performed to detect the protective effect of polyphenolic fractions, i.e. the anti-hemolytic activity. Several concentrations of the compounds dissolved in PBS were added to the red blood cell (RBC) suspension in the presence of 20 mM H_2O_2 at 37 °C for 90 min. RBC controls were included in all the assays to detect spontaneous hemolysis in the absence of oxidant agent or products. After the incubation period, cells were centrifuged and the percentage of hemolysis was determined spectrophotometrically at 540 nm (release of hemoglobin). We included a 630 nm reading to detect Met-Hemoglobin formation (*22*). The ratio of hemolysis was calculated via the equation:

$$H(\%) = \frac{Ap}{A_{water}} \times 100$$

where H(%) is the hemolysis of erythrocytes incubated with the different compounds, *A*p is the absorbance of sample supernatant and A_{water} is the absorbance after complete hemolysis with distilled water (*23*).

***In vitro* assay for the inhibition of lipid peroxidation on erythrocytes.** Lipid peroxidation mediated by H_2O_2 led to malondialdehyde (MDA) production, which was indirectly measured by spectrophotometrically determining the thiobarbituric acid reactive (TBAR) substances. The principle of this method depends on extraction of MDA from erythrocyte suspension by trichloroacetic acid (TCA) solution and the subsequent reaction of this MDA with thiobarbituric acid (TBA), which yields a pink colored complex (maximum absorption at 532 nm) (*24*). To induce lipid peroxidation, RBCs were incubated under the same conditions as the hemolysis assay (i.e. with H_2O_2 20 mM alone or with different compound concentrations for 90 min at 37 ^{o}C). Following incubation, the RBC suspension was mixed with 1 mL of trichloroacetic acid solution 20% w/v (TCA) to remove potentially interfering substances (*25*). Samples were then centrifuged and 1 mL of supernatant was mixed with 1 mL of 1% 2-thiobarbituric acid (TBA). Finally, samples were heated at 90 ^{o}C for 50 min, cooled and centrifuged before measuring the absorbance of the supernatant 532 nm and 600 nm to eliminate possible impurities. The appropriate blanks and controls were run alongside the test samples. The degree of lipid peroxidation was expressed in arbitrary absorbance units.

3T3 cell line culture. The mouse embryonic fibroblast cell line 3T3 was obtained from the "Banco de Células Eucariotas", Barcelona (Spain). Cells were grown in DMEM medium (4.5 g/L glucose) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 mM Hepes buffer, and a 1% penicillin (10000 U/mL)–streptomycin (10000 µg/mL) mixture and maintained in a humidified incubator at 37 ^{o}C and 5% CO_2. Cells were routinely cultured in 75 cm^2 culture flasks.

When the cells were approximately 80% confluent they were harvested with trypsin/EDTA and seeded at a density of 8.5 x 10^4 cells/mL into the central sixty wells of 96-well plates and then incubated for 24 h at 37 ^{o}C and 5% CO_2.

Cytoprotection against H_2O_2-induced damage. The experimental design consisted of measuring the extent of cytotoxicity and the cytoprotection that antioxidants provided against hydrogen peroxide-induced cell death. In order to assay the ability of the compounds to protect dermal the 3T3 fibroblast cell line against ROS-mediated oxidative stress, cells were pre-incubated overnight (18-20 h) in the presence of the polyphenolic fractions at different concentrations (12.5 µg/mL, 25 µg/mL, 50 µg/mL and 75 µg/mL) in DMEM 5% FBS previously sterilized by filtration. The stock antioxidant solutions were prepared immediately before each set of experiments. After the pre-incubation period the excess of polyphenols was completely removed and the medium was exchanged before adding H_2O_2 as the oxidative stress-inducing agent. This avoided a direct reaction between the polyphenol and the oxidant source in the medium (*4*). Following this, H_2O_2 was added dissolved in DMEM 0% at a final concentration of 2mM to cell culture. Cells were incubated with the oxidant agent for 2 h. The concentration of H_2O_2-induced oxidative stress used during this study was determined from dose and time course studies (data not shown). Controls included cells incubated with basal medium alone, cells treated only with H_2O_2, and cells exposed only to polyphenolic fractions. After incubation with the oxidant agent the medium was eliminated and cells washed with PBS. Finally, cell cultures were analysed for cell viability.

Cell viability assay. Cell viability was determined by the neutral red uptake (NRU) assay, performed as described by Borenfreund and Puerner (*26*) and modified to remove the use of formaldehyde (*27*). Following treatments, the medium was removed and neutral red solution (50µg/mL in RPMI medium without phenol red and serum) was added (100 µL per well). After 3 h of incubation at 37 ^{o}C, 5% CO_2, the 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. After 10 min on a microtitre-plate shaker, the absorbance of neutral red was measured at a wavelength of 550 nm in a Bio-Rad 550 microplate reader. Results are 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 are expressed as mean±SE of at least three independent experiments. Data were analysed by one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test for multiple comparisons between fractions with respect to the H_2O_2 controls, or the Scheffé *post hoc* test to compare

results between extracts, all using the SPSS software (SPSS Inc., Chicago, IL, USA). Statistical differences at $p<0.05$ were considered significant.

RESULTS AND DISCUSSION

In previous studies, our group characterized the antioxidant, anti-tumour and cytotoxic properties of a polyphenolic group of fractions from different natural sources (pine, grape and witch hazel bark, *Hamamelis virginiana*) (*10-13*).

Antioxidant characterization was performed both by chemical methods (DPPH, HNTTM, ABTS, etc.) and by AAPH assay in red blood cells. However, since flavonoids display antioxidant activity in numerous biological systems and against several injurious agents we considered it appropriate to evaluate the antioxidant potential of these fractions using other oxidative models, e.g. H_2O_2-induced RBC oxidative damage and H_2O_2-induced cell cytotoxicity.

Protective effect of polyphenolic fractions against RBC oxidative damage. The excessive peroxidation of biomembranes is accepted as one of the processes by which tissues can be damaged during ischemia/reperfusion, inflammation and aging. Erythrocytes are considered as prime targets for free radical attack due to their being oxygen carriers whose membranes have a high polyunsaturated fatty acid (PUFA) content as well as having a high cell concentration of hemoglobin molecules that are potent promoters of reactive O_2 species (*3*). Because of their great susceptibility to oxidation, erythrocytes have often been used as an excellent cellular model to investigate toxicity *in vitro* and oxidative damage in biomembranes (*8*).

The present study employed H_2O_2 as an oxygen radical generating system. H_2O_2, when present in excess, is one of many compounds that can be injurious to cells and its toxicity is largely due to its ready conversion to the reactive hydroxyl radical (OH^-) and its capacity to cross the RBC membrane and react rapidly with hemoglobin (Hb). Upon reacting with H_2O_2, Hb is converted into oxidized forms such as methemoglobin (met-Hb) and ferrylhemoglobin, which are powerful promoters of oxidative processes that could mediate cell cytotoxicity (*25*, *28*). Moreover, the free hemoglobin exposed to H_2O_2 causes heme-group degradation with the release of iron ions that are catalytically active in initiating free radical reaction and lipid peroxidation (*8*).

Although some studies have reported a higher oxidant capacity for azo compounds than H_2O_2, the latter is considered a key water-soluble oxygen free radical because of its relatively high stability, diffusion, and involvement in cell signalling cascades (*29*). Therefore, we considered H_2O_2-induced RBC damage to be a good oxidative stress model with which to investigate the extent of protection offered by the polyphenolic fractions and thus complete and compare their efficacy as antioxidants.

Oxygen radicals react with polyunsaturated fatty acids and proteins in erythrocytes causing lipid peroxidation and protein degradation. Lipid peroxidation (LPO) produces many pathological events in cells and the human organism (*5*). By causing damage to unsaturated fatty acids it tends to decrease membrane fluidity and leads to polymerization of membrane components that can be transmitted to neighbouring substances such as membrane and cytoskeleton proteins (*30*). These processes can be effectively quenched by plant phenolics. Malondialdehyde (MDA), one of the major products of lipid peroxidation, has been extensively used as both an index of lipid peroxidation and a marker of oxidative stress. MDA has been shown to cross-link erythrocyte phospholipids and proteins and thus impair a variety of membrane-related functions, which ultimately lead to diminished erythrocyte survival (*3*). The reaction of MDA with TBA has been widely adopted as a sensitive assay method for lipid peroxidation.

In the present study, human RBC were treated with 20 mM H_2O_2 with or without antioxidant compounds at 37 ^{o}C for up to 90 min**.** The intracellular level of MDA, which resulted directly from membrane lipid peroxidation, was measured as a marker of H_2O_2-induced injury of RBC. As shown in Figure 1, the level of MDA increased when erythrocytes were treated with H_2O_2, indicating relevant oxidative damage of cell membranes.

The results indicate that lipid peroxidation could be effectively inhibited by the polyphenolic fractions. The addition of procyanidin extracts inhibited H_2O_2-TBAR formation in a dose-dependent manner. This inhibitory effect was statistically significant ($p<0.05$) for all the fractions at the three assayed concentrations, except for pine fractions at 25 µg/mL. Fractions were more effective at the highest concentration, although not significantly so.

It should be noted that a good LPO inhibitory effect is already detected at the lowest concentration assayed (25 µg/ml) in all three cases (35%, 63% and 54% for pine, grape, and witch hazel bark, respectively). When comparing homologous fractions, the hamamelis ones showed the highest inhibitory effects and pine

fractions the lowest, although the differences were not significant. Some authors have attributed a direct relationship between protection against lipid peroxidation and the degree of galloylation of compounds (*1*). In this regard, the hamamelis fractions have the highest gallic-group content, and they also showed the best protection against lipid peroxidation. Moreover, the more modest protection measured for grape and pine fractions correlate well with their low gallate content and no galloylation at all, respectively. Among pine fractions, the strongest LPO inhibitory capacity corresponded to the mixture with the highest degree of polymerization (mDP). In grape and hamamelis mixtures the protection against lipid peroxidation was proportional to mDP and galloylation. Thus, as previously pointed out (*12*, *31*), we conclude that both mDP and galloylation are important chemical features in determining the antioxidant capacity of polyphenolic substances.

The protective effect of polyphenolic fractions was also evaluated by oxidative hemolysis experimentally induced with H_2O_2 in RBC. Under the given conditions, H_2O_2 caused considerable RBC hemolysis (75%) that was significantly inhibited by polyphenolic fractions (Figure 2). All nine extracts exerted an inhibitory effect, which was dose-dependent in some cases. Pine and grape homologous fractions showed similar anti-hemolytic activity, with fraction IV being the most protective in both cases (more than 75% inhibition for the three concentrations assayed). However, hamamelis extracts displayed lower anti-hemolytic capacity (around 40% inhibition for all concentrations tested), as expected given the results obtained against lipid peroxidation. Therefore, we postulate that in the case of H_2O_2-induced hemolysis the degree of polymerization and galloylation of the mixtures does not clearly determine their protective activity.

Free radicals attack erythrocyte membrane components, such as proteins and lipids, causing changes in the structure and function of membranes which may result in hemolysis. The mechanism of free-radicals-induced hemolysis is not completely understood, although a competitive model between lipid and protein oxidation occurring simultaneously is usually proposed as a hypothesis (*32*). Hamamelis fractions almost completely prevented oxidation of polyunsaturated fatty acids but did not completely prevent hemolysis, indicating that factors other than lipid peroxidation are also important in causing lysis of RBC (*33*). Various antioxidants were found to inhibit lipid peroxidation, but no protective effects against protein degradation were recorded. Thus, the mechanism for lipid peroxidation by oxygen radicals must differ significantly from the mechanisms by which intracellular proteins are damaged (*34*).

The molecular mechanisms and structure-activity relationship underlying the antioxidant action of polyphenols have yet to be fully elucidated and remain a matter of considerable debate (*35*). Potential mechanisms by which natural proanthocyanidins and other phenolics may protect erythrocytes from lipid peroxidation and hemolysis include the following: (i) flavanols and procyanidins may function as primary antioxidants by directly reducing the formation of free radicals or peroxyl radicals by the oxidant agent (AAPH, H_2O_2, etc.); (ii) they may spare, maintain, or regenerate α-tocopherol and other antioxidants by donating a hydrogen to the α-tocopherol radicals; and (iii), they may function as chelators and bind redox active metals, including Fe^{2+} and Cu^{2+}, involved in the initiation of free radicals (*36*). In addition to the currently-accepted mechanisms for the protective effects of procyanidins (free radical trapping and metal chelation), these compounds could interact with membranes at two other levels: (a) by increasing membrane fluidity, and thus reducing the rate of lipid peroxidation; and/or (b) by limiting the access of certain molecules that need to reach the hydrophobic region of the membrane to exert their deleterious effects (*37*).

Antioxidant protection against H_2O_2-induced cytotoxicity. The specifically protective effects of our set of polyphenolic fractions against oxidative damage in mouse fibroblast were examined in order to assess their potential role as antioxidant compounds in normal cell culture (*38*). The murine fibroblast 3T3 cell line was chosen for three reasons: (i) because we have previously carried out the cytotoxicity assays of our compounds in this cell line; (ii) the 3T3 neutral red (NR) uptake assay is recommended by the U.S. National Institute of Environmental Health Science (NIEHS) Interagency Coordinating Committe on the Validation of Alternative Methods (ICCVAM); and (iii) because several studies using the mouse embryo fibroblast 3T3 cell line as an assay model have demonstrated that it is a sensitive cell line for the detection of oxidative stress induced by H_2O_2 (*39*).

The 3T3 cell line was treated with polyphenolic fractions at concentrations between 0 and 75 μg/mL and the antioxidant response was then studied. In addition, we established an oxidative condition by exposing cells to 2 mM H_2O_2 for 2 h in order to evaluate the extent to which fractions can protect these cells from an oxidative insult. Compounds were investigated for their potential to protect against H_2O_2–induced damage using the NR uptake assay. As shown in Figure 3 the addition of H_2O_2 to cell cultures resulted in a loss of approximately 85% 3T3 cell viability. Pre-incubation with polyphenolic fractions for 18 h prior to the H_2O_2 insult, resulted in an antioxidant-specific modulation of cell viability. All fractions protected

to some extent against H_2O_2 cytotoxicity, although, significant differences were not always observed.

It should be noted that the protective effect of each compound does not always increase in parallel with its concentration. This behaviour may be explained by considering the pro-oxidant effect of phenolic compounds (*40*). Except for the lowest mDP pine fractions, OWP and IVP, which exerted a dose-dependent protective effect, the remaining fractions lost effectiveness at higher concentrations. Furthermore, grape and hamamelis extracts were toxic to varying degrees at some of the concentrations used in this study, mainly at 50 and 75 µg/mL. This toxic effect was previously reported by our group in cytotoxic assays (*12*, *13*) and is closely related to the degree of galloylation shown by fractions. Thus, it was not possible to inhibit H_2O_2-induced cell damage totally by increasing the fraction concentration, since they assumed the role of pro-oxidants at high concentrations. However, it should be emphasized that some of these highly galloylated fractions (XIG, OWH, IVH and XIH) showed significant biological antioxidant activity against strong ROS-induced oxidative stress at 25 µg/mL, increasing viability up to 5-fold in the case of the XIH fraction. Therefore, our results demonstrate that the degree of galloylation was not only closed related to the antioxidant protective capacity of compounds in this cell model, but also that it was responsible for their pro-oxidant effect at high doses as described for other polyphenolic compounds (*40*, *41*).

Preliminary assays in our laboratory with the flavanol epicatechin showed no protective effect when it was simultaneously added with H_2O_2 (data not shown). There is why, preincubation with fractions before the induction of H_2O_2 injury was preferred. Moreover, previous reports have shown that pre-incubation with polyphenolic compounds was required to achieve protective effects in other cell models, and also that if flavonoids and H_2O_2 were added and incubated simultaneously, the protective effect was lower than when cells were pre-incubated (*4*, *42*, *43*). The fact that recovery from damage was more marked in cells treated with the tested flavonoids before H_2O_2 incubation suggests that simple scavenging of ROS by flavonoids was not the only cause of cell damage protection. There is no clear evidence regarding the intracellular location of polyphenols, and even less evidence in the case of procyanidins, although some studies suggest that they interact with cell membranes and intracellular proteins. Moreover, it has been reported that grape seed procyanidin extracts activate antioxidative enzymes and the glutathione cycle (*44*), so these compounds could enhance the antioxidant potential of cells during pre-incubation.

Spencer et al. (*45*) reported that hydrogen peroxide treatment of fibroblasts leads to cell death involving apoptotic processes. These findings are consistent with observations of other investigators (*46*, *47*), showing an involvement of apoptotic processes in hydrogen peroxide-induced cell death in fibroblast and other cells. Thus, the ability of polyphenolic fractions to provide health benefits may not necessarily be dependent on their acting as scavengers of free radicals or reactive oxygen species, but rather their ability to interact with cell-signalling cascades, to influence the cell at a transcriptional level and to down-regulate pathways leading to cell death. Moreover, polyphenolic compounds could improve cellular antioxidant activity against H_2O_2 stress by modulating the repair potential of damaged DNA, thus functioning as a biological response modifier. However, the mechanism of this enhanced protection against DNA damage seems rather complex.

The location of an antioxidant where oxidation occurs should be considered an important aspect in determining its antioxidant efficiency (*48*). Evidence suggests that several polyphenolic compounds such as tannins and resveratrol are able to traverse cell membranes and may enter the cytoplasmatic or nuclear space (*49*). In addition, polyphenolic structure is essential to determine the activity of these compounds. For example, galloylation appears to be more influential than polymerization as regards biological apoptosis and with respect to the hydroxyl and superoxide anion radical scavenging capacity of fractions. This ability to prevent the imbalance between high-level oxidant exposure and antioxidant capacity, which leads to several pathological processes, may contribute to the chemopreventive effect of the gallic acid derivatives (*50*). The influence of polyphenolic structure on antioxidant activity, protective capacity and particularly, on the mechanisms of action remains open to debate and further study is required.

SUMMARY AND CONCLUSIONS

ROS may potentially react with nucleic acids, lipids and proteins, causing impairment of their structural or enzymatic functions and extensive oxidative damage. A growing number of natural plant constituents are regarded as possible antioxidants with a role in protecting the cell against free radical damage and chemicals that can generate oxidative forms (*51*). However, some of these compounds can behave as both anti- and pro-oxidants and their action is

dependent on the concentration used. Although their antioxidant and toxicological effects have been investigated the mechanisms are yet to be clarified and it is therefore important to develop a better understanding of the health benefits and/or potential toxicity of these plant constituents.

The present study has demonstrated the protective effect of several polyphenolic fractions from different natural sources (pine, grape and witch hazel bark) against the oxidative stress induced by H_2O_2 in human erythrocytes and in the 3T3 cell line. The compounds tested protected against erythrocyte lipid peroxidation and hemolysis, as well as against cytotoxicity caused by H_2O_2 in 3T3 cells. The results indicate that these fractions, which are by-products in the agro-food industry, are good sources of nutraceuticals and phytonutrients and they have great potential for such applications in the food and drug industries. Moreover, the results obtained in the 3T3 cell line support the need for further research into the use of polyphenolic fractions for the chemoprevention of pathological skin disease induced by oxidative stress.

The promise and physiological relevance of the present results lies in the fact that when the tested polyphenols were used at low concentrations they behaved as protective compounds against H_2O_2-induced cell damage. The observation that the protective concentrations for these compounds are in the low range supports the concept that procyanidins can provide significant *in vivo* antioxidant activity.

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References

(1) Ajila, C.M., Prasada Rao, U.J.S. (2008). Protection against hydrogen peroxide induced oxidative damage in rat erythrocytes by *Mangifera indica* L. peel extract. *Food Chem. Toxicol*. *46*(1), 303-309.

(2) Ames, B., Shigenaga, M., Hagen, T. (1993). Oxidants, antioxidants, and the degenerative diseases of aging. *Proc. Natl. Acad. Sci*. USA. *90*, 7915-7922.

(3) Singh, N., Rajini, P.S. (2008). Antioxidant-mediated protective effect of potato peel extract in erythrocytes against oxidative damage. *Chem. Biol. Interact. 173*(2), 97-104.

(4) Park, Y.H., Han, D.W., Suh, H., Ryu, G.H., Hyon, S.H., Cho, B.K., Park, J.C. (2003). Protective effects of green tea polyphenol against reactive oxigen species-induced oxidative stress in cultured rat calvarial osteoblast. *Cell Biol. Toxicol*. *19*, 325-337.

(5) Sroka, Z., Cisowski, W. (2003). Hydrogen peroxide scavenging, antioxidant and anti-radical activity of some phenolic acids. *Food Chem. Toxicol*. *41*, 753-758.

(6) Auddy, B., Ferreira, M., Blasina, F., Lafon, L., Arredondo, F., Dajas, F., Tripathi, P.C., Seal, T., Mukherjee, B. (2003). Screening of antioxidant activity of three Indian medicinal plants, traditionally used for the management of neurodegenerative diseases. *J. Ethnopharmaco. 84*, 131-138.

(7) Medina, I., Lois, S., Lizárraga, D., Pazos, M., Touriño, S., Cascante, M., Torres, J.L. (2006). Functional fatty fish supplemented with grape procyanidins. Antioxidant and proapoptotic properties on colon cell lines. *J. Agric. Food Chem*. *54*, 3598-3603.

(8) Tedesco, I., Russo, M., Russo, P., Iacomino, G., Russo, G.L., Carraturo, A., Faruolo, C., Moio, L., Palumbo, R. (2000). Antioxidant effect of red wine polyphenols on red blood cells. *J. Nutr. Biochem. 11*(2), 114-119.

(9) Erlejman, A.G., Verstraeten, S.V., Fraga, C.G., Oteiza, P.I. (2004). The interaction of flavonoids with membranes: Potential determination of flavonoid antioxidant effects. *Free Radic. Res*. *38*(12), 1311-1320.

(10) Torres, J.L., Varela, B., García, M.T., Carilla, J., Matito, C., Centelles, J.J., Cascante, M., Sort, X., Bobet, R. (2002). Valorization of grape (*Vitis vinifera*) byproducts. Antioxidant and biological properties of polyphenolic fractions differing in procyanidin composition and flavonol content. *J. Agric. Food. Chem. 50*, 7548-7555.

(11) Touriño, S., Selga, A., Jiménez A., Julià L., Lozano, C., Lizárraga, D., Cascante, M., Torres, J.L. (2005). Procyanidin fractions from Pine (*Pinus pinaster*) bark: radical scavenging power in solution, antioxidant activity in emulsion, and antiproliferative effect in melanoma cells. *J. Agric. Food Chem*. *53*, 4728-4735.

(12) Ugartondo, V., Mitjans, M., Touriño, S., Torres, J.L., Vinardell, M.P. (2007). Comparative antioxidant and cytototoxic effect of procyanidin fractions from grape and pine. *Chem. Res. Toxicol*. *20*, 1543-1548.

(13) Touriño, S., Lizárraga, D., Carreras, A., Lorenzo, S., Ugartondo, V., Mitjans, M., Vinardell, M.P., Juliá, Ll., Cascante, M., Torres, J.L. (2008). Highly Galloylated Tannin Fractions from Witch Hazel (*Hamamelis virginiana*) Bark: Electron transfer capacity, *In vitro* antioxidant activity and effects on skin related cells. *Chem. Res. Toxicol*, *21*(3), 696-704.

(14) Magalhaes, L.M., Segundo, M.A., Reis, S., Lima, J.L. (2008). Methodological aspects about *in vitro* evaluation of antioxidant properties. *Anal. Chim. Acta. 613*, 1-19.

(15) Liu, R.H., Finley, J. (2005). Potential cell culture models for antioxidant research. *J. Agric. Food Chem. 53*, 4311-4314.

(16) Zhang, J., Stanley, R., Adaim, A., Melton, L.D., Skinner, M.A. (2006). Free radical scavenging and cytoprotective activities of phenolic antioxidants. *Mol. Nutr. Food Res*. *50*, 996-1005.

(17) Halliwell, B., Clement M.V., Long, L.H. (2000). Hydrogen peroxide in the human body. *FEBS Lett. 486*, 10-13.

(18) Lee, K.J., Jeong, H.G. (2007). Protective effects of kahweol and cafestol against hydrogen peroxide-induced oxidative stress and DNA damage. *Toxicol Lett. 173*(2), 80-87.

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

(20) Touriño, S., Lizárraga, D., Carreras, A., Matito, C., Ugartondo, V., Mitjans, M., Centelles, J.J., Vinardell, M.P., Julià, L., Cascante, M., Torres, J.L. (2008). Antioxidant/Prooxidant effects of bioactive polyphenolics. *EJEAFChe*, *7*(8), 3339-3343.

(21) Grinberg, L.N., Newmark, H., Kitrossky, N., Rahamim, E., Chevion, M., Rachmilewitz, EA. (1997). Protective effects of tea polyphenols against oxidative damage to red blood cells. *Biochem. Pharmacol. 54*(9), 973-978.

(22) Sentürk, U.K., Gündüz, F., Kuru, O., Aktekin, M.R., Kipmen, D., Yalçin, O., Bor-Küçükatay, M., Yeşilkaya, A., Başkurt, O.K. (2001). Exercise-induced oxidative stress affects erythrocytes in sedentary rats but not exercise-trained rats. *J. Appl. Physiol*. *91*(5), 1999-2004.

(23) Bukowska, B., Kowalska, S. (2004). Phenol and catechol induce prehemolytic and hemolytic changes in human erythrocytes. *Toxicol. Lett. 152*, 73-84.

(24) Stocks, J., Dormandy, T.L. (1971). The autooxidation of human red cell lipids induced by hydrogen peroxide. *British J. Haematol. 20*, 95-111.

(25) Srour, M.A., Bilto, Y.Y., Juma, M. (2000). Evaluation of different methods used to measure malondialdehyde in human erythrocytes. *Clin. Hemorheol. Microcirc*. *23*(1), 23-30.

(26) Borenfreund, E., Puerner, J.A. (1985). Toxicity determined in vitro by morphological alterations and neutral red absorption. *Toxicol. Lett. 24*(2/3), 119-124.

(27) Riddell, R.J., Clothier, R.H., Ball, M. (1986). An evaluation of three in vitro cytotoxicity assays. *Food Chem. Toxicol. 24* (6/7), 469-471.

(28) Nagababu, E., Rifkind, J.M. (2000). Heme degradation during autoxidation of oxyhemoglobin. *Biochem. Biophys. Res. Commun*. *273*(3), 839-845.

(29) Shiva Shankar Reddy, C.S., Subramanyam, M.V.V., Vani, R., Asha Devi, S. (2007). *In vitro* models of oxidative stress in rat erythrocytes: Effect of antioxidant supplements. *Toxicol. In Vitro*, *21*, 1355-1364.

(30) Chiu, D., Kuypers, F., Lubin, B. (1989). Lipid peroxidation in human red cells. *Semin. Hematol. 26*(4), 257-276.

(31) Plumb, G.W., De Pascual-Teresa, S., Santos-Buelga, C., Cheynier, V., Williamson G. (1998). Antioxidant properties of catechins and proanthocyanidins: effect of polymerization, galloylation and glycosylation. *Free Radic. Res*. *29*, 351-358.

(32) Zou, C.G., Agar, N.S., Jones, G.L. (2001). Oxidative insult to human red blood cells induced by free radical initiator AAPH and its inhibition by a commercial antioxidant mixture. *Life Sci*. *69*(1), 75-86.

(33) Sato, Y., Kamo, S., Takahashi, T., Suzuki, Y. (1995). Mechanism of Free Radical-Induced Hemolysis of Human Erythrocytes: Hemolysis by Water-Soluble Radical Initiator. *Biochemistry*, *34*(28), 8940-8949.

(34) Davies, K.J., Goldberg, A.L. (1987). Oxygen radicals stimulate intracellular proteolysis and lipid peroxidation by independent mechanisms in erythrocytes. *J. Biol. Chem. 262*(17), 8220-8226.

(35) Caturla, N., Vera-Samper, E., Villalaín, J., Mateo, C., Micol, V. (2003). The relationship between the antioxidant and the antibacterial properties of galloylated catechins and the structure of phospholipid model membranes. *Free Rad. Biol. Med*. *34* (6), 648-662.

(36) Zhu, Q.Y., Holt, R.R., Lazarus, S.A., Orozco, T.J., Keen, C.L. (2002). Inhibitory effects of cocoa flavanols and procyanidin oligomers on free radical-induced erythrocyte hemolysis. *Exp Biol Med (Maywood), 227*(5), 321-329.

(37) Verstraeten, S.V., Oteiza, P.I., Fraga, C.G. (2004). Membrane effects of Cocoa Procyanidins in Liposomes and Jurkat T Cells. *Biol. Res*. *37*(2), 293-300.

(38) Thang, P.T., Patrick, S., Teik, L.S., Yung, C.S. (2001). Anti-oxidant effects of the extracts from leaves of *Chromolaena odorata* on human dermal fibroblast and epidermal keratinocytes against hydrogen peroxide and hypoxanthine-xanthine oxidase induced damage. *Burns 27*, 319-327.

(39) Pool-Zabel, B.L., Adlercreutz, H., Glei, M., Liegibel, UM., Sittlingon, J., Rowland, I., Wähälä, K., Rechkemmer, G. (2000). Isoflavonoids and lignans have different potentials to modulate oxidative genetic damage in human colon cells. *Carcinogenesis*. *21*(6), 1247-1252.

(40) Fan, P., Lou, H. (2004). Effects of polyphenols from grape seeds on oxidative damage to cellular DNA. *Mol. Cell. Biochem*. *267*, 67-74.

(41) Schmidt, M., Schmitz, H.J., Baumgart, A., Guédon, D., Netsch, M.I., Kreuter, M.H., Schmidlin, C.B., Schrenk, D. (2005). Toxicity of green tea extracts and their constituents in rat hepatocytes in primary culture. *Food Chem. Toxicol. 43*, 307-314.

(42) Llópiz, N., Puiggròs, F., Céspedes, E., Arola, Ll., Ardévol, A., Bladé, C., Salvadó, M.J. (2004). Antigenotoxic effect of grape seed procyanidin extract in Fao cells submitted to oxidative stress. *J. Agric. Food Chem. 52*, 1083-1087.

(43) Zhu, Q.X., Shen, T., Tu, D.Y., Ding, R., Liang, Z.Z., Zhang, X.J. (2005) Protective effects of *Ginkgo biloba* leak extracts on trichloroethylene-induced human keratinocyte cytotoxicity and apoptosis. *Skin Pharmacol. Physiol*. *18*, 160-169.

(44) Roig, R., Cascón, E., Arola, L., Bladé, C., Salvadó, M.J. (2002). Procyanidins protect Fao cells against hydrogen peroxide-induced oxidative stress. *Biochim. Biophys. Acta. 1572*(1), 25-30.

(45) Spencer, J., Schroeter, H., Kuhnle, G., Srai, S.K., Tyrrell, R., Hakn, U., Rice-Evans, C. (2001). Epicatechin and its *in vivo* metabolite, 3'-o-methyl epicatechin, protect human fibroblasts from oxidative-stress-induced cell death involving caspase-3 activation. *Biochem. J. 354*, 493-500.

(46) Teramoto, S., Tomita, T., Matsui, H., Ohga, E., Matsuse, T., Ouchi, Y. (1999). Hydrogen peroxide-induced apoptosis and necrosis in human lung fibroblast: protective roles of glutathione. *Jpn. J. Pharmacol. 79*, 33-40.

(47) Lee, S.D., Lee, B.D., Han, J.M., Kim, J.H., Kim, Y., Suh, P.G., Ryu, S.H. (2000). Phospholipasa D2 activity suppresses hydrogen peroxide-induced apoptosis in PC12 cells. *J. Neurochem*. *75*(3), 1053-1059.

(48) Pazos, M., Lois, S., Torres, J.L., Medina, I. (2006). Inhibition of hemoglobin- and iron-promoted oxidation in fish microsomes by natural phenolics. *J. Agric. Food. Chem*. *54*, 4417-4423.

(49) Azmi, A.S., Bhat, S.H., Hanif, S., Hadi, S.M. (2006). Plant polyphenols mobilize endogenous copper in human peripheral lymphocytes leading to oxidative DNA breakage: a putative mechanism for anticancer properties. *FEBS Lett*. *580*(2), 533-538.

(50) Lizárraga, D., Lozano, C., Briedé, J.J., Van Delft, J.H., Touriño, S., Centelles, J.J., Torres, J.L., Cascante, M. (2007). The importance of polymerization and galloylation for the antiproliferative properties of procyanidin-rich natural extracts. *FEBS Journal*, *274*, 4802-4811.

(51) Labieniec, M., Gabryelak, T. (2005). Measurement of DNA damage and protein oxidation after the incubation of B14 Chinese hamster cells with chosen polyphenols. *Toxicol. Lett. 155*, 15-25.

Table 1. Mean degree of polymerization (mDP)* and Percentage of gallate groups of fractions from pine bark (P fractions), grape pomace (G fractions), and Witch hazel (H fractions)† (*10*, *11*, *13*)

Fractions	**mDP***	**galloylation† (%)**
OWP	2.1	-
IVP	2.9	-
XIP	3.4	-
OWG	1.7	15
IVG	2.7	25
XIG	3.7	31
OWH	1.2	44
IVH	1.6	52
XIH	2.7	64

* mDP corresponding to the proanthocyanidins whithin the fractions

†Total galloylation from both proanthocyanidins and hydrolyzable tannins

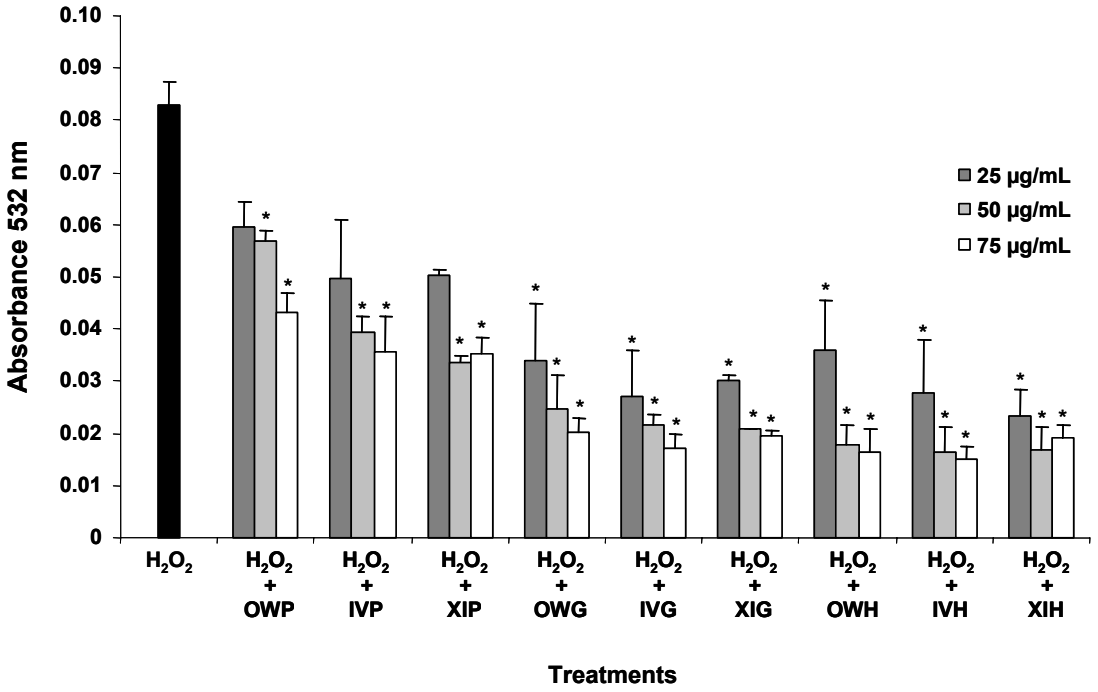

Figure 1. TBAR levels in erythrocytes treated with H_2O_2 20 mM and H_2O_2 20 mM plus polyphenolic fractions from different natural sources. The levels of MDA-TBA complex were indicated as absorbance at 532 nm. Marked compounds are statistically different to the control with H_2O_2 20 mM alone (*$p<0.05$ was considered to denote statistically significant differences).

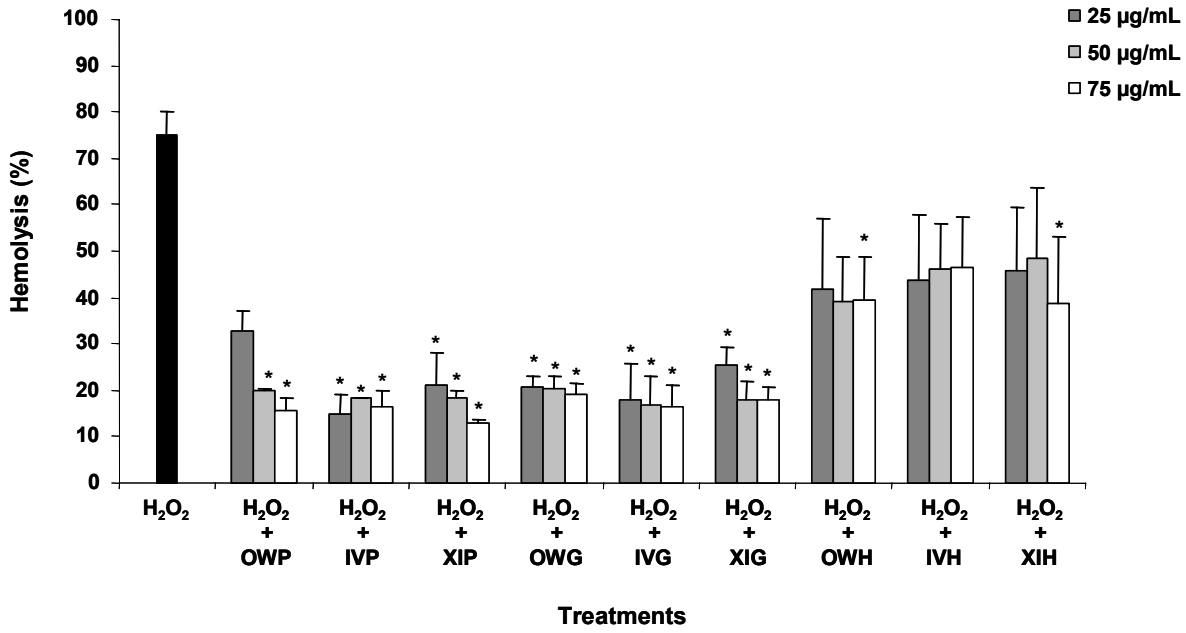

Figure 2. Percentage of hemolysis of erythrocytes incubated with H_2O_2 20 mM alone or H_2O_2 plus polyphenolic fractions extracts at different concentrations; * indicates a significant difference from H_2O_2 group ($p<0.05$).

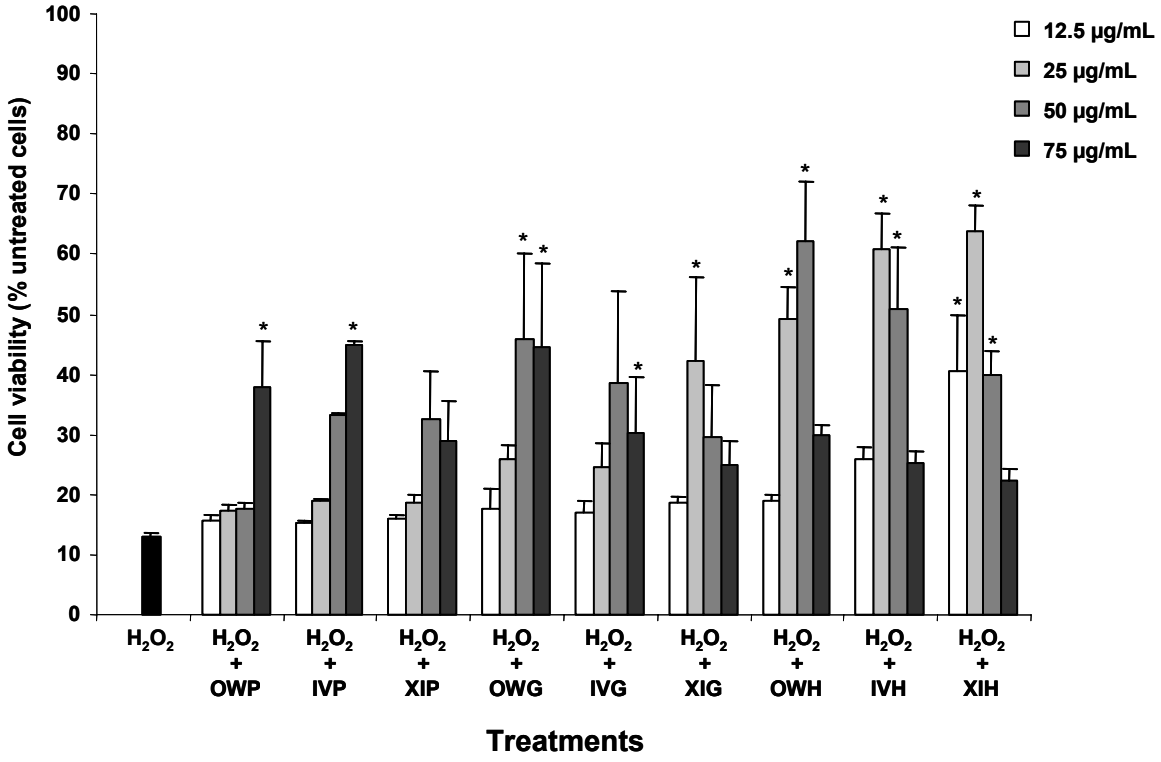

Figure 3. Comparative protective effect of polyphenolic fractions towards H_2O_2-induced cytotoxicity on 3T3 cells. Cells were preincubated overnight with polyphenolic fractions at different concentrations, washed and incubated with 2mM H_2O_2 for 2 h. Cell viability was expressed as percentages of the untreated cells (100%). *$p<0.05$ denoted significant differences with respect 2 mM H_2O_2 control.