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

VANESSA UGARTONDO,† MONTSERRAT MITJANS,† CARLES LOZANO,‡ JOSEP LLUIS TORRES,‡ AND MARIA PILAR VINARDELL*,†

Departament de Fisiologia, Facultat de Farmacia, Universitat de Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Spain, and Institute for Chemical and Environmental Research (IIQAB-CSIC), Jordi Girona 18-26, 08034 Barcelona, Spain

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-

* To whom correspondence should be addressed. Telephone: +34924024505. Fax: +34934035901. E-mail: mpvinardellmh@ub.edu.
† Universitat de Barcelona.
‡ Institute for Chemical and Environmental Research (IIQAB-CSIC).

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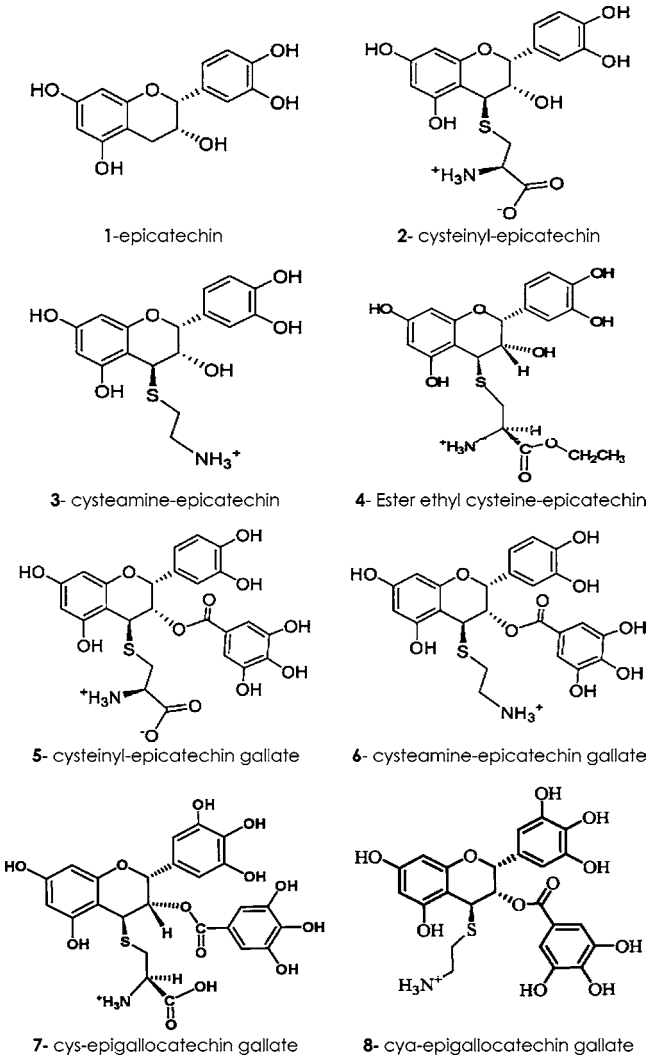


**Figure 1.** Chemical structures of epicatechin and epicatechin derivatives.

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**), 4$\beta$-(*S*-cysteinyl)epicatechin (**2**), 4$\beta$-(2-aminoethylthiol)epicatechin (**3**), 4$\beta$-[*S*-(*O*-ethylcysteinyl)]epicatechin (**4**), 4$\beta$-(*S*-cysteinyl)epicatechin 3-*O*-gallate (**5**), 4$\beta$-(2-aminoethylthiol)epicatechin 3-*O*-gallate (**6**), 4$\beta$-(*S*-cysteinyl)epigallocatechin 3-*O*-gallate (**7**), and 4$\beta$-(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 $8 \times 10^9$ 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 $\mu$L 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 $\mu$M, 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 $\mu$g/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 $10 \times 10^4$ cells/mL for HaCaT and $8.5 \times 10^4$ cells/mL for 3T3 for a 24 h exposure, $6.5 \times 10^4$ and $2.5 \times 10^4$ cells/mL, respectively for a 48 h exposure, and $5.5 \times 10^4$ and $1.5 \times 10^4$ 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 $\mu$M 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 $\mu$L of NR solution (50 $\mu$g/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 $\mu$L/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 | $IC_{50}$ ($\mu$M) | product | $IC_{50}$ ($\mu$M) |
|---|---|---|---|
| **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 |

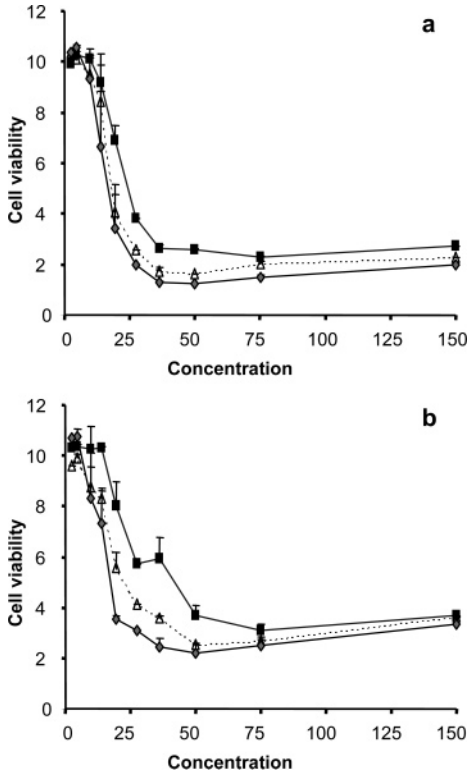


**Figure 2.** Comparative cytotoxicity of compounds toward proliferation of 3T3 (**a**) and HaCaT (**b**) cells after exposure for 24 (■), 48 (△), and 72 h (◆). The data are presented as the mean percentages of the control ± the standard error of the mean.

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

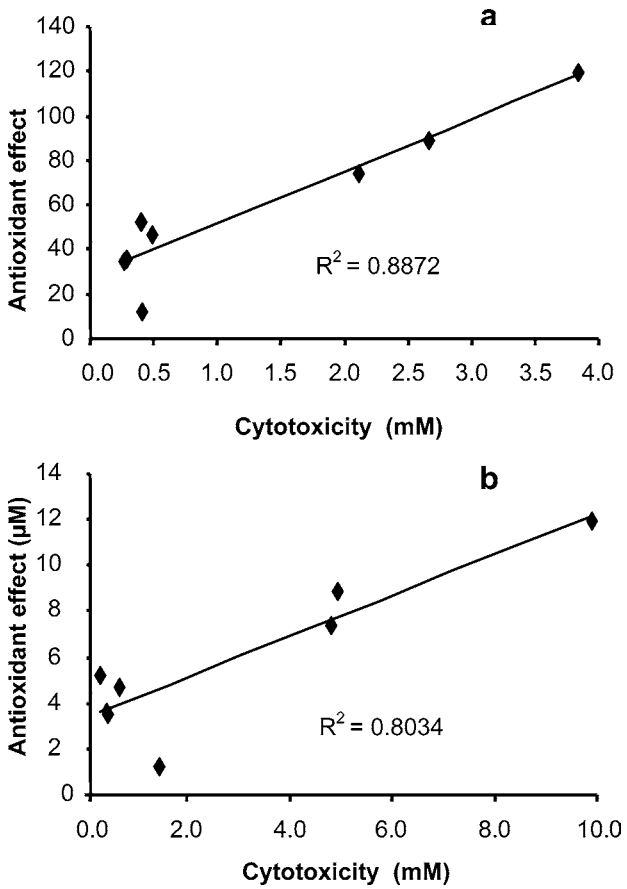


**Figure 3.** Relationship between the antioxidant activity expressed as the concentration of the product that causes 50% inhibition of hemolysis induced by AAPH ($IC_{50}$) in micromolar and the cytotoxicity expressed as the concentration of the product that causes 50% inhibition of growth or death 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-1$\beta$ 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 $\beta$-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 $\beta$-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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