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Hamamelitannin from Witch Hazel (*Hamamelis virginiana*) Displays Specific Cytotoxic Activity against Colon Cancer Cells

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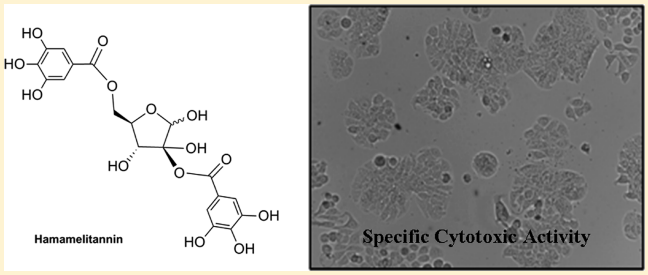
ABSTRACT: *Hamamelis virginiana* (witch hazel) bark is a rich source of condensed and hydrolyzable tannins reported to exert a protective action against colon cancer. The present study characterizes different witch hazel tannins as selective cytotoxic agents against colon cancer. To cover the structural diversity of the tannins that occur in *H. virginiana* bark, the hydrolyzable tannins, hamamelitannin and pentagalloylglucose, together with a proanthocyanidin-rich fraction (F800H4) were selected for the study. Treatment with these compounds reduced tumor viability and induced apoptosis, necrosis, and S-phase arrest in the cell cycle of HT29 cells, with hamamelitannin being the most efficient. Owing to polyphenol-mediated H_2O_2 formation in the incubation media, the antiproliferative effect was determined in the presence and absence of catalase to rule out any such interference. The presence of catalase significantly changed the IC_{50} only for F800H4. Furthermore, at concentrations that inhibit the growth of HT29 cells by 50%, hamamelitannin had no harmful effects on NCM460 normal colonocytes, whereas pentagalloylglucose inhibited both cancerous and normal cell growth. Using the TNPTM assay, we identified a highly reactive phenolic position in hamamelitannin, which may explain its efficacy at inhibiting colon cancer growth.



Several epidemiological studies have indicated that tannins may exert a protective effect against colon cancer, one of the most prevalent neoplastic diseases in the developed world.[1,2] Witch hazel (*Hamamelis virginiana*) bark is a rich source of both proanthocyanidins, or condensed tannins, and hydrolyzable tannins (Figure 1) such as hamamelitannin and pentagalloylglucose,[3] whose capacity to regulate cell proliferation, cell cycle, and apoptosis has attracted much attention.[4] An inverse relation has been reported between proanthocyanidins and colorectal cancer.[5] An in vitro study demonstrated that a grape seed proanthocyanidin extract significantly inhibits cell viability and increases apoptosis in Caco-2 colon cancer cells, but does not alter the viability of the normal colon NCM460 cell line.[6] Other results show that proanthocyanidins from different sources are cytotoxic to human colorectal cells.[7−9] In addition, several in vitro and in vivo studies have shown that hydrolyzable tannins from witch hazel bark exhibit multiple biological activities, which may have potential in the prevention and treatment of cancer. In vivo preclinical studies of pentagalloylglucose, one of the major hydrolyzable tannins in witch hazel, demonstrated inhibition of prostate cancer,[10,11] lung cancer,[12] and sarcoma[13] cells. In vitro inhibition of the growth and invasiveness of breast cancer, leukemia, melanoma, and liver cancer cells has also been reported.[14−17] The other major hydrolyzable tannin in witch hazel, hamamelitannin, inhibits TNF-mediated endothelial cell death and DNA fragmentation in EAhy926 endothelial cells.[18] Since TNFα/TNFR1 signaling may act as a tumor promoter for colon carcinogenesis,[19] the anti-TNF activity of hamamelitannin may indicate a protective effect against colon cancer. Furthermore, hamamelitannin has been described to inhibit 5-lipoxygenase (5-LOX),[20] and given that 5-LOX is an inflammatory enzyme involved in malignant transformation,[21] this inhibition could prevent cancer growth.

Moreover, various studies have analyzed the cytotoxicity and scavenging capacity of *H. virginiana* phenolic compounds. It has been reported that different witch hazel polyphenolic fractions are highly active as free radical scavengers against 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and tris(2,4,6-trichloro-3,5-dinitrophenyl)methyl (HNTTM). They also reduce tris-(2,3,5,6-tetrachloro-4-nitrophenyl)methyl (TNPTM) radical to some extent, which indicates that they contain highly reactive hydroxy groups. In this way, witch hazel fractions protect red blood cells from free radical-induced hemolysis and also inhibit the proliferation of the SK-Mel 28 melanoma tumor cell line.[22] Some of these fractions also inhibited cell proliferation, arrested the cell cycle at the S phase, and induced

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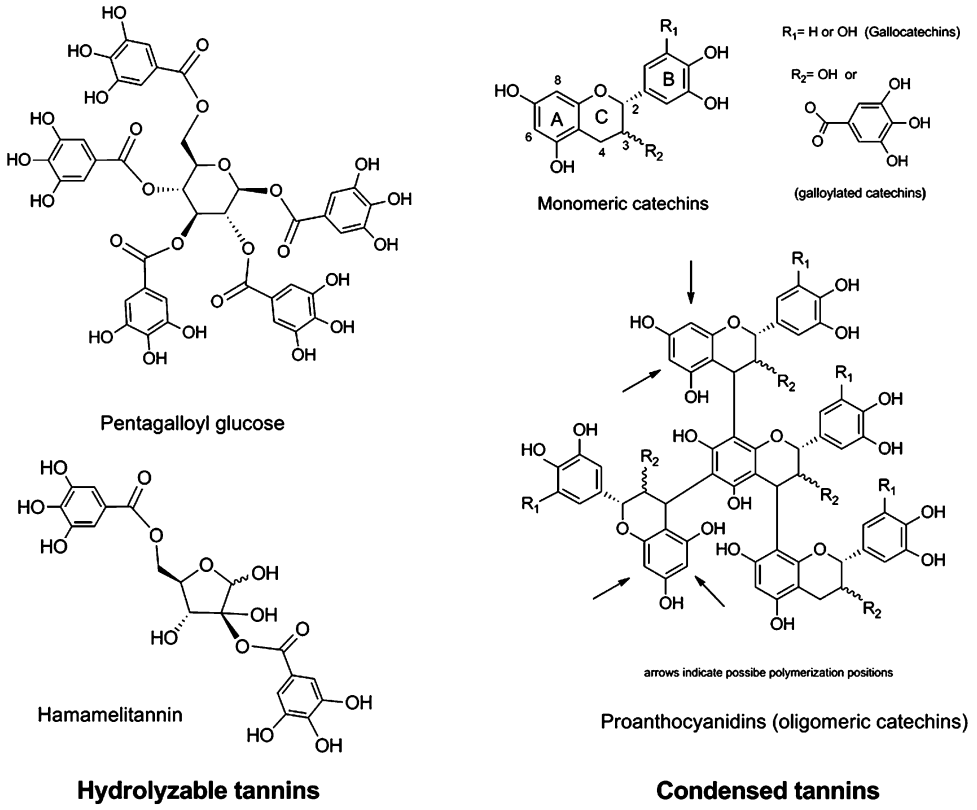

Figure 1. Structures of hydrolyzable and condensed tannins in *Hamamelis virginiana* bark.

apoptosis in HT29 human colon cancer cells.[23] The witch hazel mixtures studied so far include those from highly heterogeneous mixtures containing both hydrolyzable and condensed tannins of low molecular weight, as well as flavan-3-ol monomers;[22,23] however, the activity of oligomeric structures from witch hazel bark has not been evaluated. Furthermore, Masaki et al. reported that hamamelitannin from *H. virginiana* possesses protective activity from cell damage induced by superoxide anion radicals in murine dermal fibroblasts.[24,25]

To advance our understanding of the compounds responsible for the activity of *H. virginiana* bark, we evaluated the behavior of pure hamamelitannin and pentagalloylglucose (hydrolyzable tannins of different size) and a highly purified proanthocyanidin-rich fraction (F800H4). First, we examined the viability, apoptosis, and cell cycle of the human colorectal adenocarcinoma HT29 cell line after treatment with these compounds. To identify products that inhibit cancer cell growth without harming normal cells, the antiproliferative capacity of *Hamamelis* compounds was also measured against the NCM460 cell line (human colonocytes). As several studies have reported that polyphenols can be oxidized under standard cell culture conditions, leading to the production of significant amounts of ROS such as H_2O_2, and that this can modulate cell functions,[26] we supplemented the cell culture medium with catalase, which decomposes polyphenol-generated ROS, thus ruling out this possibility.[27]

RESULTS AND DISCUSSION

Pentagalloylglucose and fraction F800H4 were extracted from the bark of witch hazel, whereas the hydrolyzable tannin hamamelitannin was obtained commercially. Both hydrolyzable tannins presented a purity of 98% or more, as confirmed by HPLC. Once fraction F800H4 was obtained, its polyphenolic composition was characterized to ensure that it possessed a high percentage of condensed tannins. Table 1 summarizes the results of the HPLC analysis after thioacidolysis in the presence of cysteamine (condensed tannins) and direct HPLC analysis (gallic acid, pentagalloylglucose, and hamamelitannin). F800H4 was found to be composed of mostly condensed tannins (83.9% of the total tannins), both monomers and proanthocyanidins [(epi)catechin oligomers and polymers]. It also contained 16.1% hydrolyzable tannins, mainly hamamelitannin. Pentagalloylglucose was not detected in fraction F800H4. The condensed tannins had a mean degree of polymerization (mDP) of 2.6, 35% galloylation and 32% pyrogallol. The total galloylation of the fraction was 45.5%.

Table 1. Polyphenolic Composition of F800H4[a]

Composition of the Condensed Tannins (CTn) 83.9%					
mDP	% G	% P			
2.6	35.0	32.0			
% GC	% EGC	% C	% EC	% EGCG	% ECG
12.4	0.4	29.1	23.0	19.1	15.9
Composition of the Hydrolyzable Tannins (HTn) 16.1%					
% GA		% HT		% PGG	
10.0		90.0		0.0	

[a]mDP, mean degree of polymerization; % G, percentage of galloylation; % P, percentage in pyrogallol; GC, gallocatechin; EGC, epigllocatechin; C, catechin; EC, epicatechin; EGCG, epigallocatechin gallate; ECG, epicatechin gallatel; GA, gallic acid; HT, hamamelitannin; PGG, pentagalloylglucose.

Tannins regulate different cell functions through different actions that may or may not involve redox reactions.[28] Since

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Table 2. Hydrogen Donation and Electron Transfer Capacity

	DPPH			HNTTM			TNPTM		
	EC_{50}[a]	ARP[b]	H/e[c]	EC_{50}[a]	ARP[b]	e[c]	EC_{50}[a]	ARP[b]	e[c]
PGG	23.8	42.0	19.8	54.8	18.2	8.6	2403.9	0.4	0.2
HT	27.8	36.2	8.8	71.2	14.0	3.4	116.2	2.2	1.0
F800H4	39.8	25.1	27.1	66.7	15.0	16.2	1761.6	0.6	0.7

[a]EC_{50}, μg of polyphenol/μmol of radical. [b]ARP, $(1/EC_{50}) \times 10^3$. [c]Number of hydrogen atoms donated or electrons transferred to the stable radical per molecule of polyphenol, calculated as the inverse of 2 × molar EC_{50}.

polyphenols may act as antioxidants and prooxidants, we studied the redox activity of *H. virginiana* compounds and evaluated their free radical scavenging properties using different stable radicals such as DPPH, HNTTM, and TNPTM. DPPH reacts with polyphenols by mechanisms that may include both hydrogen donation and electron transfer,[29] while HNTTM and TNPTM are sensitive only to electron transfer.[30] The reactions with DPPH and HNTTM gave information on the total capacity to scavenge radicals by hydrogen donation or concerted electron proton transfer (DPPH) and by electron transfer (HNTTM). The reaction with TNPTM revealed the presence of highly redox reactive positions. Table 2 summarizes the activities of pentagalloylglucose, hamamelitannin, and the proanthocyanidin fraction F800H4 against the stable free radicals. Overall, pentagalloylglucose, hamamelitannin, and the proanthocyanidin-rich fraction F800H4 showed a similar total scavenging capacity, as their number of phenolic hydroxy groups per unit of mass was similar. Interestingly, differences were detected with TNPTM. While the scavenging capacity of the polyphenols against TNPTM is low because only some of the hydroxy groups are able to donate electrons to this radical, the possible effects of these hydroxy groups may be biologically relevant because they are the most reactive positions. One of the phenolic hydroxy groups in hamamelitannin was reactive enough to transfer its electron to TNPTM, while pentagalloylglucose was much less responsive (Table 2, last column). Hamamelitannin and pentagalloylglucose are structurally similar. In the case of hamamelitannin though, there is a hydroxy moiety geminal to one of the gallate esters, and this might explain the differences detected in the reactivity against the TNPTM radical. The extra hydroxy group might participate in a hydrogen bond with the carbonyl group from the gallate moiety to form a six-membered ring. This could introduce a conformational restriction with loss of planarity and subsequent loss of conjugation within the gallate moiety. The extended conjugation of the carbonyl and aromatic groups is the reason that gallates are less reactive than pyrogallols.[31] The results with TNPTM indicate that hamamelitannin is particularly reactive and may even participate in the formation of ROS through electron transfer to oxygen to form the superoxide radical.

Pentagalloylglucose has been shown to inhibit different malignancies.[10,11,13] Potential mechanisms for its anticancer activity include antiangiogenesis, antiproliferation, S-phase and G1-phase cell cycle arrest, induction of apoptosis, and anti-inflammatory and antioxidative effects . Putative molecular targets include p53, Stat3, Cox-2, VEGFR1, AP-1, SP-1, Nrf-2, and MMP-9. This study reports for the first time the role of pentagalloylglucose in colon cancer. We studied here the viability, the cell cycle, and the apoptosis process in human colorectal adenocarcinoma HT29 cells. In these bioassays, different positive controls were used. Epigallocatechin gallate (EGCG), a major catechin in green tea described to have antitumor activity,[32,33] was used as a standard in the cell viability assays; the cell cycle inhibitor hydroxyurea (HU) was used as a standard in the cell cycle experiments,[34] and staurosporine (ST) was utilized as a positive control in the apoptosis assays.[35] Treatment with pentagalloylglucose reduced the viability of HT29 cells with an IC_{50} value of 28 ± 8.8 μg/mL (Figure 2a) and induced 11% apoptosis compared to control cells, 5% necrosis (Figure 3), and S-phase arrest in the cell cycle with 8% increase in the population of cells in the S phase and a concomitant decrease in the percentage of cells in the G1 and G2 phases (Figure 4). Because pentagalloylglucose inhibits DNA replicative synthesis with greater efficacy than a known DNA polymerase-alpha inhibitor, aphidocolin,[36] this may explain the arrest in the S phase. The antitumor effects of hamamelitannin have not been examined, except for its antigenotoxic action in HepG2 human hepatoma cells reported by Dauer et al.,[37] as well as its anti-TNF[18] and anti-LOX activities.[20] The cellular mechanism that this hydrolyzable tannin induces may be related to the inhibition of the tumor necrosis factor itself and its receptor, which affect apoptosis, necrosis, and cell cycle processes. As a result, after treatment with hamamelitannin, we observed a reduction in the viability of HT29 cells with an IC_{50} of 20 ± 4.5 μg/mL (Figure 2a) and induction of 26% apoptosis, 14% necrosis (Figure 3), and S-phase arrest in the cell cycle with a 16% increase in the population of cells in this phase (Figure 4). With regard to condensed tannins, proanthocyanidins from various sources have been reported to inhibit colon cancer cells.[38,39] Treatment of the human colon adenocarcinoma HT29 cell line with the proanthocyanidin-rich fraction F800H4 extracted from witch hazel bark was less effective at inhibiting cell viability (IC_{50} = 38 ± 4.4 μg/mL; Figure 2a) and inducing apoptosis (9%) and necrosis (6%) (Figure 3) than the same treatment with hydrolyzable tannins. F800H4 had little effect on the normal cell cycle distribution apart from a slight increase in the S and G2 phases (Figure 4).

Overall, the hydrolyzable tannins were more effective than the condensed tannins. Interestingly, hamamelitannin, which includes a highly reactive position, as demonstrated by its reaction with TNPTM (Table 2), showed the strongest inhibition of cell viability, induction of apoptosis and necrosis, and cell cycle arrest in the S phase in HT29 colon cancer cells (Figures 2a, 3, 4). The effect of this reactive position in hamamelitannin may even be prooxidant. The prooxidant effect of some polyphenols has been discussed extensively, and it has been suggested that moderate generation of ROS may produce an antioxidant effect by fostering the endogenous defenses.[40,41] Therefore, in our assays, hamamelitannin may exert its activity, at least in part, by providing mild prooxidant challenges through electron transfer reactions leading to moderate formation of ROS.

On the other hand, since it has been reported that an increase in endogenous ROS levels is required for the transition from the G1 to the S phase of the cell cycle,[42] the cell cycle

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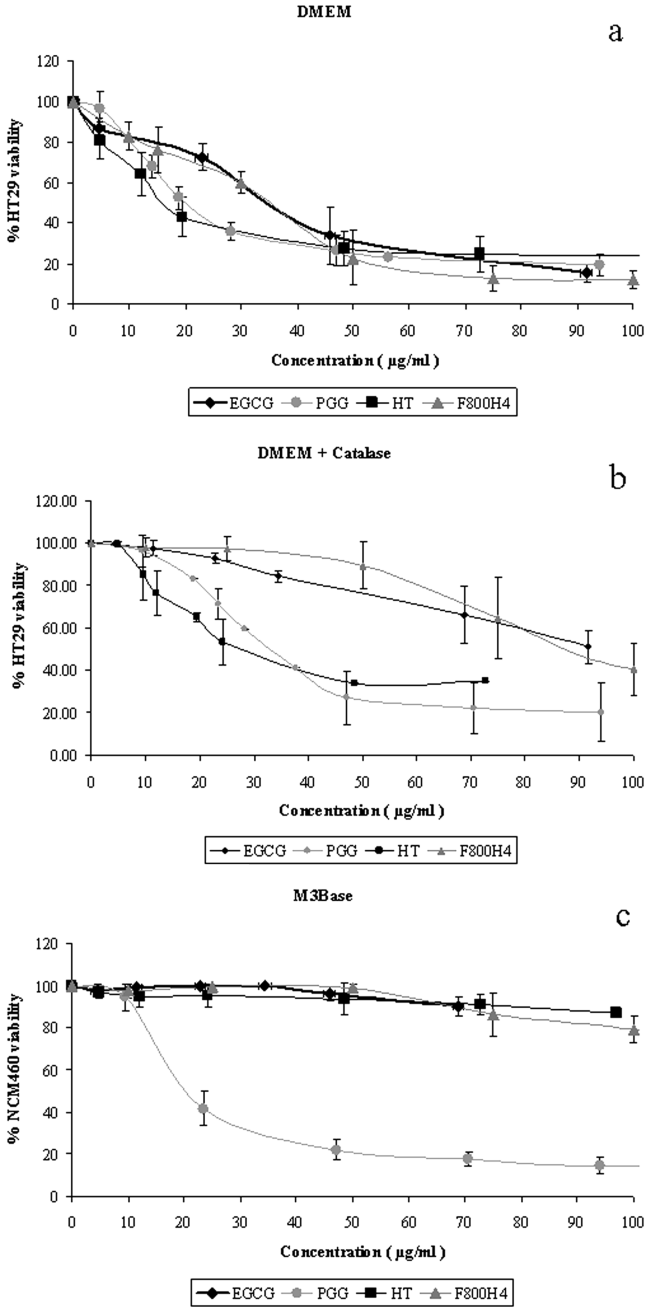

Figure 2. (a) Effect on HT29 cell viability of different concentrations of *Hamamelis virginiana* compounds in DMEM. (b) Effect on HT29 cell viability of witch hazel compounds in DMEM supplemented with catalase (100 U/mL). (c) Effect of Hamamelis products on NCM460 colonocyte growth. In all cases epigallocatechin gallate is used as a standard. Values are represented as mean of percentage of cell viability with respect to control cells ± standard error of three independent experiments.

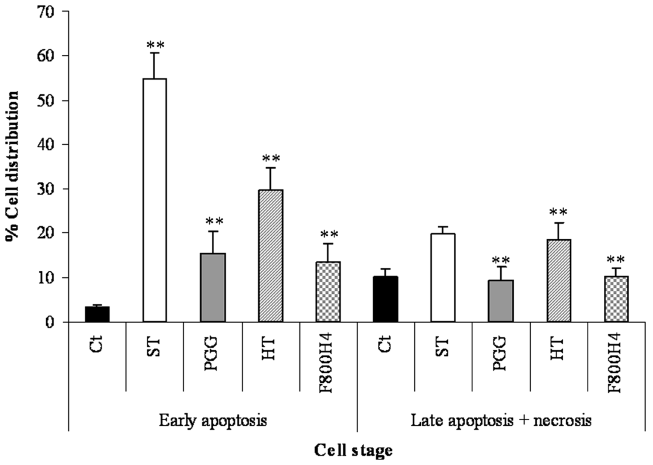

Figure 3. Early apoptotic cells: annexin V+/PI−. Late apoptotic/necrotic cells: annexin V+/PI+ and annexin V−/PI+. Staurosporine is utilized as a positive control. Values are expressed as mean ± standard deviation of three separate experiments. $^{**}p < 0.001$, significant difference with respect to the corresponding value in untreated cells (Ct).

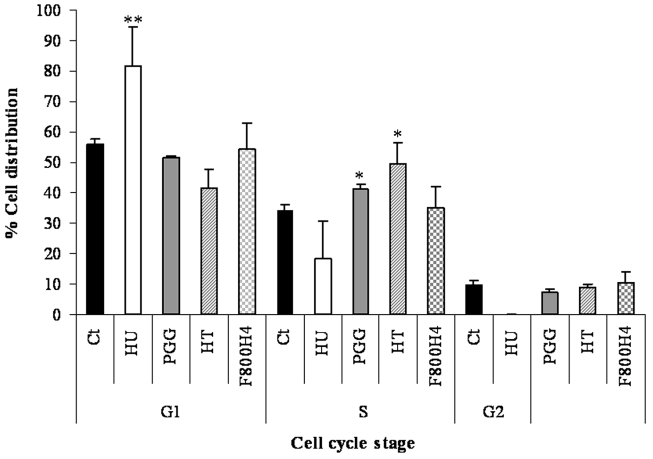

Figure 4. Normalized percentages of cells in different cell stages. Cell phases analyzed: Gl, S, and G2. The cell cycle inhibitor hydroxyurea was used as a standard. Mean ± standard deviation of three separate experiments. $^{*}p < 0.05$; $^{**}p < 0.001$, significant difference with respect to control cells (Ct).

arrest in the S phase induced by witch hazel compounds may be explained to some extent by its ROS scavenging capacity.

In the search for compounds or fractions that inhibit cancer cell growth without harming normal cells, the antiproliferative capacity of pentagalloylglucose, hamamelitannin, and the proanthocyanidin-rich fraction F800H4 was determined in NCM460 human colonocytes. NCM460 are nontumorigenic cells derived from normal colon mucosa that has not been infected or transfected with any genetic information.[43] This is the first comparison of the effects of witch hazel compounds on the growth of nontransformed colonocytes and cancerous colon cells. Our results show that the concentrations of hamamelitannin and F800H4 capable of inducing the death of HT29 cells (Figure 2a) had no harmful effects on normal colon cells (IC_{50} higher than 100 μg/mL for hamamelitannin and F800H4) (Figure 2c), whereas pentagalloylglucose inhibited both cancerous and normal cell growth (Figure 2a, c). Pentagalloylglucose inhibited NCM460 cell viability with an IC_{50} of 23 μg/mL ± 2.4 (Figure 2a, c).

It has been reported that polyphenol-mediated ROS formation in cell culture medium can lead to the artifactual modulation of cytotoxicity attributed to polyphenol exposure. Accordingly, Chai et al. reported that H_2O_2-mediated cytotoxicity, resulting from incubation of PC12 cells with green tea or red wine, was completely prevented by the addition of bovine liver catalase to the culture medium.[44] All *Hamamelis* compounds tested together with the positive

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control used (EGCG)[45,46] generated H_2O_2 in a concentration-dependent manner in DMEM (Figure 5a). Hamamelitannin showed the highest H_2O_2 production, at 100 μg/mL. As expected, supplementing the cell culture medium with 100 U/mL catalase resulted in almost complete decomposition of polyphenol-generated H_2O_2 in all cases (Figure 5b). The next step was to study the antiproliferative capacity of *H. virginiana* polyphenolics by co-incubating with catalase. This enzyme had little effect on HT29 cells incubated with hydrolyzable tannins (IC_{50} in DMEM = 28 μg/mL ± 8.8 (Figure 2a)/IC_{50} in DMEM with catalase = 34 μg/mL ± 1.2 (Figure 2b) for pentagalloylglucose and IC_{50} in DMEM = 20 μg/mL ± 4.5 (Figure 2a)/IC_{50} in DMEM with catalase =13 μg/mL ± 4.6 (Figure 2b) for hamamelitannin), whereas F800H4 cytotoxicity was shown to be partially attributable to H_2O_2-mediated modulation (IC_{50} in DMEM = 38 μg/mL ± 4.4 (Figure 2a)/IC_{50} in DMEM with catalase = 95 μg/mL ± 8.7 (Figure 2b)). This effect is probably triggered by the highly reactive pyrogallol moieties in the condensed tannins. Interestingly, the results obtained for the positive control, EGCG, a flavan-3-ol with a pyrogallol B-ring, are in accordance with this hypothesis. Consequently, the difference between the IC_{50} value of F800H4 determined in HT29 cells incubated with catalase (Figure 2b) and the value established in NCM460 cells (Figure 2c) is not as high as when we compared the results obtained for HT29 without catalase (Figure 2a), which were artifactual, with NCM460 (Figure 2c). This demonstrates that, as with pentagalloylglucose, F800H4 is not completely specific against cancer cells. Interestingly, the cytotoxic activity of hamamelitannin was not modified by the addition of catalase to the medium.

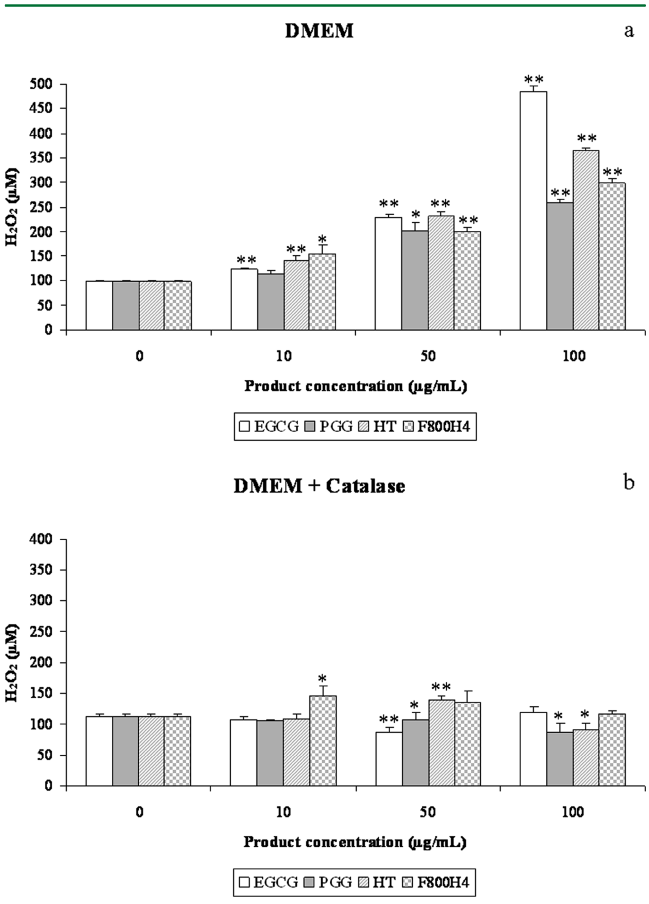

Figure 5. (a) H_2O_2 concentration in cell culture medium (DMEM + 10% FCS + 0.1% streptomycin/penicillin) with pentagalloyl glucose, hamamelitannin, and the proanthocyanidin-rich fraction F800H4 in medium. (b) H_2O_2 concentration produced in DMEM culture medium with catalase (100 U/mL) after incubation with witch hazel compounds. Epigallocatechin gallate is used as a positive control. Mean ± standard deviation of two independent experiments. $^{**}p < 0.001$ and $^{*}p < 0.05$, significant difference with respect to the corresponding value in untreated cells (Ct).

In summary, we conclude that pentagalloylglucose and the proanthocyanidin-rich fraction F800H4 do not show specificity for cancerous cells, whereas hamamelitannin is a promising chemotherapeutic agent, which might be used for the treatment of colon cancer without compromising the viability of normal colon cells. Hamamelitannin appears to contain a highly reactive phenolic position that can be detected by the stable radical TNPTM, which may explain its efficacy at inhibiting colon cancer cell growth. These findings may lead to a better understanding of the structure–bioactivity relationship of tannins, which should be of assistance for formulations of chemopreventive and chemotherapeutic agents.

EXPERIMENTAL SECTION

General Experimental Procedures. UV measurements were made on a Cary 50-Bio UV spectrophotometer (Varian, Palo Alto, CA, USA). Semipreparative chromatography was conducted on a Waters system (Milford, MA, USA) using an X-Terra C_{18} (19 × 250 mm, 10 μm) column. HPLC was carried out on a Hitachi (San Jose, CA, USA) system equipped with a quaternary pump, autosampler, and diode array detector and an analytical Kromasil C_{18} (Teknokroma, Barcelona, Spain) column. All chemicals were purchased from Sigma-Aldrich Co. (St Louis, MO, USA), unless otherwise specified. For extraction, we used deionized water, bulk EtOH (Momplet y Esteban, Barcelona, Spain), bulk acetone (Quimivita, Sant Adrià del Besòs, Spain), and bulk hexane (alkanes mixture) (Quimivita). For purification, deionized water, analytical grade MeOH (Panreac, Montcada i Reixac, Spain), 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 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. TFA (Fluorochem, Derbyshire, UK) biotech grade was distilled in-house. HCl (37%) and HOAc were from E. Merck. Et_3N (E. Merck) was of buffer grade. Deuterated solvents for NMR were from SDS (Peypin, France). DPPH (95%) was from Aldrich (Gillingham-Dorset, UK), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (97%) was from Aldrich (Milwaukee, WI, USA). HNTTM and TNPTM radicals were synthesized as described elsewhere.[30,47] Antibiotics (10 000 U/mL penicillin, 10 000 μg/mL streptomycin) were obtained from Gibco-BRL (Eggenstein, Germany), fetal calf serum (FCS) was from Invitrogen (Paisley, UK), and trypsin EDTA solution C (0.05% trypsin–0.02% EDTA) was from Biological Industries (Kibbutz Beit Haemet, Israel). The annexin V/FITC kit was obtained from Bender System (Vienna, Austria). M3Base medium was purchased from INCELL (San Antonio, TX, USA).

Extraction, Fractionation, and Characterization of F800H4. Polyphenols were obtained from witch hazel bark by extraction with acetone–water (7:3) and fractionation with EtOAc,[22] which produced fraction OWH (polyphenols soluble in EtOAc and H_2O) and fraction AH (polyphenols only soluble in H_2O). To generate fraction F800H4, AH (800 mg) was dissolved in 50% MeOH and fractionated on a Sephadex LH-20 column (50 × 2.5 cm i.d.) using a gradient of MeOH in H_2O and a final step of washing with acetone, as previously reported.[48] Five subfractions (800H1 to 800H5) were collected, and their absorbance was measured at 280 and 400 nm; yield, 8% from

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fraction AH; 0.05% from witch hazel bark. Table 1 shows the chemical composition of fraction F800H4, which was estimated as previously described.[22] The content of condensed tannins was estimated by thioacidolytic depolymerization in the presence of cysteamine and HPLC analysis of the cleaved units. The hydrolyzable tannins were determined directly from the fraction by HPLC and standards.

Purification of Pentagalloylglucose. Pentagalloylglucose was purified from fraction OWH by semipreparative chromatography on a Waters system (Milford, MA, USA) using an X-Terra C_{18} (19 × 250 mm, 10 μm) column. A total amount of 2 g of OWH was processed in successive chromatographic runs with loads of 200 mg, 4 mL each, and elution by a binary system [solvent A, 0.1% aqueous TFA; solvent B, 0.08% TFA in H_2O−CH_3CN (1:4)] under the following conditions: 10 min at 16% B and two gradients, 16−36% B over 40 min, and 36−55% B over 5 min, at a flow rate of 10 mL/min with detection at 235 nm. The purity of the pentagalloylglucose was ascertained by HPLC on a Hitachi (San Jose, CA, USA) system equipped with a quaternary pump, autosampler, and diode array detector and an analytical Kromasil C_{18} (Teknokroma, Barcelona, Spain) column under the same elution conditions at a flow rate of 1 mL/min. Pentagalloylglucose was lyophilized, and its identity was confirmed by chromatography coupled to high-resolution mass spectrometry and NMR; purity, 95% by HPLC; yield, 3.8% from fraction OWH, 0.03% from witch hazel bark.

DPPH Assay. The antiradical capacity of the polyphenols was evaluated by the DPPH stable radical method.[49] Fresh MeOH solutions (2 mL) at concentrations ranging from 2 to 30 μM were added to a freshly prepared radical solution (2 mL, 120 μM) in deoxygenated MeOH. The mixture was incubated for 30 min at room temperature in the dark, and the UV absorbance at 517 nm was measured. 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 concentration of DPPH. Each data point was the result of three independent determinations. A dose−response curve was obtained for every sample. The results are expressed as the efficient concentration, EC_{50}, given as the amount of polyphenols that consumes half the amount of free radical divided by the initial amount of DPPH in micromoles. The results are also expressed as antiradical power (ARP), which is the inverse of EC_{50}. UV measurements were made on a Cary 50-Bio UV spectrophotometer (Varian, Palo Alto, CA, USA).

Electron Transfer Capacity against the Stable Free Radicals HNTTM and TNPTM. Fresh solutions of the polyphenols (2 mL) at concentrations ranging from 2 to 62 μM were added to a freshly prepared solution of HNTTM (2 mL, 120 μM) in deoxygenated $CHCl_3$−MeOH (2:1). The mixture was incubated for 7 h at room temperature in the dark, and the UV absorbance was measured at 384 nm. The results are plotted as the percentage of absorbance disappearance $[(1 - A/A_0) \times 100]$ against the amount of sample divided by the initial amount of the radical in micromoles, as described for DPPH. Each data point was the result of three independent determinations. A dose−response curve was obtained for every sample. The results are expressed as the efficient concentration, EC_{50}, and as ARP. The working conditions with TNPTM were essentially those described for HNTTM[30] with some differences. The concentration range was 10−120 μM, the incubation time was 48 h, and the absorbance was measured at 378 nm. The results are plotted as described for HNTTM.

Cell Culture. Human colorectal adenocarcinoma HT29 cells (obtained from the American Type Culture Collection, HTB-38) were grown as a monolayer culture in Dulbecco's modified Eagle's medium (DMEM) in the presence of 10% heat-inactivated fetal calf serum and 0.1% streptomycin/penicillin in standard culture conditions. NCM460 cells, obtained by a Material Transfer Agreement with INCELL, are from an epithelial cell line derived from the normal colon mucosa of a 68-year-old Hispanic male.[43] They were grown as a monolayer culture in M3Base medium (which contains growth supplements and antibiotics) supplemented with 10% heat-inactivated fetal calf serum and 2.5 mM D-glucose (final concentration 5 mM glucose). The cells were cultured at 37 °C in a 95% air, 5% CO_2 humidified environment.

Determination of Cell Viability. The assay was performed using a variation of the MTT assay described by Mosmann.[50] The assay is based upon the principle of reduction of MTT into blue formazan pigments by viable mitochondria in healthy cells. The cells were seeded at densities of 3×10^3 cells/well (HT29 cells) and 1×10^4 cells/well (NCM460 cells) in 96-well flat-bottom plates. After 24 h of incubation at 37 °C, the polyphenolic samples were added to the cells at different concentrations in fresh medium. Some experiments were performed in the presence of catalase (100 U/mL, from bovine liver) to examine the potential influence on extracellular H_2O_2. The use of an antioxidant enzyme in the cell medium allows us to rule out the effects of exogenous H_2O_2 generated during the incubation with polyphenols. The addition of this enzyme does not affect the cellular markers, since it does not enter the cells and is removed after incubation. In all cases the antitumor agent EGCG was used as standard. The culture was incubated for 72 h. Next the medium was removed, and 50 μL of MTT (1 mg/mL in PBS) with 50 μL of fresh medium was added to each well and incubated for 1 h. The MTT reduced to blue formazan, and the precipitate was dissolved in 100 μL of DMSO; absorbance values were measured on an ELISA plate reader (550 nM) (Tecan Sunrise MR20-301, Tecan, Salzburg, Austria). Absorbance was taken as proportional to the number of living cells. The concentrations that caused 50% cell growth inhibition (IC_{50}) were estimated from the dose−viability curves.

Cell Cycle Analysis by FACS. The cell cycle was analyzed by measuring the cellular DNA content using the fluorescent nucleic acid dye propidium iodide (PI) to identify the proportion of cells in each stage of the cell cycle. The assay was carried out using flow cytometry with a fluorescence-activated cell sorter (FACS). HT29 cells were plated in six-well flat-bottom plates at a density of 87×10^3 cells/well. After 24 h of incubation at 37 °C, the polyphenolic fractions were added to the cells at their respective IC_{50} values. We used the G1/S cell cycle inhibitor HU at 1 mM as standard. The cultures were incubated for 72 h in the absence or presence of the polyphenolic fractions. The cells were trypsinized, pelleted by centrifugation (1500 rpm for 5 min), and stained in Tris-buffered saline containing 50 μg/mL PI, 10 μg/mL RNase free of DNase, and 0.1% Igepal CA-630. They were incubated in the dark for 1 h at 4 °C. Cell cycle analysis was performed by FACS (Epics XL flow cytometer, Coulter Corp., Hialeah, FL, USA) at 488 nm.[51]

Apoptosis Analysis by FACS. Double staining with annexin V-FITC and PI measured by FACS was used to determine the percentage of apoptotic cells. Annexin+/PI− cells were considered early apoptotic cells. Annexin+/PI+ and annexin−/PI+ cells were classed together as late apoptotic/necrotic cells, since this method does not differentiate necrotic cells from cells in late stages of apoptosis, which are also permeable to PI. The cells were seeded, treated, and collected as described in the previous section. ST (1 μM) was utilized as a control of apoptosis induction. After centrifugation (1500 rpm for 5 min), they were washed in binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and resuspended in the same buffer. Annexin V-FITC was added using the annexin V-FITC kit. Afterward, the cells were incubated for 30 min at room temperature in the dark. Next, PI was added 1 min before the FACS analysis at 20 μg/mL. Fluorescence was measured at 495 nm (annexin V-FITC) and 488 nm (PI).

Determination of H_2O_2 (FOX Assay). H_2O_2 in the cell culture medium was determined using the ferrous oxidation xylenol orange (FOX) assay.[52] After oxidation of Fe(II) to Fe(III) by H_2O_2, the resulting xylenol orange−Fe(III) complex was quantified spectrophotometrically (560 nm). The cells were incubated for 72 h with a range of concentrations of witch hazel compounds in culture medium (DMEM or M3Base) either alone or in the presence of catalase (100 U/mL, from bovine liver) under cell culture conditions (96-well flat-bottom plate, in the absence of cells). EGCG was used as a positive control in this assay given that it has already been reported that this product generates high levels of ROS in cell culture media. Next, 100 μL of medium was transferred to a new 96-well flat-bottom plate. FOX reagent (900 μL) was added to each aliquot: 100 μM xylenol orange, 250 μM ferrous ammonium sulfate, 25 mM H_2SO_4 and 4 mM BHT in

90% (v/v) MeOH. After 30 min, absorbance at 560 nm was measured in a microplate reader (Tecan Sunrise MR20-301, Tecan). Peroxides were quantified by comparing the absorbance to a standard curve (H_2O_2 concentrations: 0−150 μM).

Data Presentation and Statistical Analysis. Data are given as the means ± SD (standard deviation). For each assay, the parametric unpaired two-tailed independent sample *t* test was used for statistical comparison with the untreated control cells, and differences were considered to be significant when $p < 0.05$ and $p < 0.001$.

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REFERENCES

(1) Theodoratou, E.; Kyle, J.; Cetnarskyj, R.; Farrington, S. M.; Tenesa, A.; Barnetson, R.; Porteous, M.; Dunlop, M.; Campbell, H. *Cancer Epidemiol. Biomarkers Prev.* **2007**, *16*, 684−693.

(2) Cutler, G. J.; Nettleton, J. A.; Ross, J. A.; Harnack, L. J.; Jacobs, D. R. Jr.; Scrafford, C. G.; Barraj, L. M.; Mink, P. J.; Robien, K. *Int. J. Cancer.* **2008**, *123*, 664−671.

(3) Vennat, B.; Pourrat, H.; Pouget, M. P.; Gross, D.; Pourrat, A. *Planta Med.* **1988**, *54*, 454−457.

(4) Hu, H.; Chai, Y.; Wang, L.; Zhang, J.; Lee, H. J.; Kim, S. H.; Lu, J. *Mol. Cancer Ther.* **2009**, *8*, 2833−2843.

(5) Mutanen, M.; Pajari, A. M.; Paivarinta, E.; Misikangas, M.; Rajakangas, J.; Marttinen, M.; Oikarinen, S. *Asia Pac. J. Clin. Nutr.* **2008**, *17* (Suppl 1), 123−125.

(6) Engelbrecht, A. M.; Mattheyse, M.; Ellis, B.; Loos, B.; Thomas, M.; Smith, R.; Peters, S.; Smith, C.; Myburgh, K. *Cancer Lett.* **2007**, *258*, 144−153.

(7) Chung, W. G.; Miranda, C. L.; Stevens, J. F.; Maier, C. S. *Food Chem. Toxicol.* **2009**, *47*, 827−836.

(8) Gosse, F.; Guyot, S.; Roussi, S.; Lobstein, A.; Fischer, B.; Seiler, N.; Raul, F. *Carcinogenesis* **2005**, *26*, 1291−1295.

(9) Kolodziel, H.; Heberland, C.; Woerdenbag, H. J.; Konings, A. W. T. *Phytother. Res.* **1995**, *9*, 410−415.

(10) Hu, H.; Lee, H. J.; Jiang, C.; Zhang, J.; Wang, L.; Zhao, Y.; Xiang, Q.; Lee, E. O.; Kim, S. H.; Lu, J. *Mol. Cancer Ther.* **2008**, *7*, 2681−2691.

(11) Kuo, P. T.; Lin, T. P.; Liu, L. C.; Huang, C. H.; Lin, J. K.; Kao, J. Y.; Way, T. D. *J. Agric. Food Chem.* **2009**, *57*, 3331−3339.

(12) Huh, J. E.; Lee, E. O.; Kim, M. S.; Kang, K. S.; Kim, C. H.; Cha, B. C.; Surh, Y. J.; Kim, S. H. *Carcinogenesis* **2005**, *26*, 1436−1445.

(13) Miyamoto, K.; Kishi, N.; Koshiura, R.; Yoshida, T.; Hatano, T.; Okuda, T. *Chem. Pharm. Bull. (Tokyo)* **1987**, *35*, 814−822.

(14) Chen, W. J.; Chang, C. Y.; Lin, J. K. *Biochem. Pharmacol.* **2003**, *65*, 1777−1785.

(15) Chen, W. J.; Lin, J. K. *J. Biol. Chem.* **2004**, *279*, 13496−13505.

(16) Oh, G. S.; Pae, H. O.; Oh, H.; Hong, S. G.; Kim, I. K.; Chai, K. Y.; Yun, Y. G.; Kwon, T. O.; Chung, H. T. *Cancer Lett.* **2001**, *174*, 17−24.

(17) Ho, L. L.; Chen, W. J.; Lin-Shiau, S. Y.; Lin, J. K. *Eur. J. Pharmacol.* **2002**, *453*, 149−158.

(18) Habtemariam, S. *Toxicon* **2002**, *40*, 83−88.

(19) Sakai, H.; Yamada, Y.; Shimizu, M.; Saito, K.; Moriwaki, H.; Hara, A. *Chem. Biol. Interact.* **2010**, *184*, 423−430.

(20) Hartisch, C.; Kolodziej, H.; von Bruchhausen, F. *Planta Med.* **1997**, *63*, 106−110.

(21) Wasilewicz, M. P.; Kolodziej, B.; Bojulko, T.; Kaczmarczyk, M.; Sulzyc-Bielicka, V.; Bielicki, D.; Ciepiela, K. *Int. J. Colorectal Dis.* **2010**, *25*, 1079−1085.

(22) Touriño, S.; Lizarraga, D.; Carreras, A.; Lorenzo, S.; Ugartondo, V.; Mitjans, M.; Vinardell, M. P.; Julia, L.; Cascante, M.; Torres, J. L. *Chem. Res. Toxicol.* **2008**, *21*, 696−704.

(23) Lizarraga, D.; Tourino, S.; Reyes-Zurita, F. J.; de Kok, T. M.; van Delft, J. H.; Maas, L. M.; Briede, J. J.; Centelles, J. J.; Torres, J. L.; Cascante, M. *J. Agric. Food Chem.* **2008**, *56*, 11675−11682.

(24) Masaki, H.; Atsumi, T.; Sakurai, H. *Free Radical Res. Commun.* **1993**, *19*, 333−340.

(25) Masaki, H.; Atsumi, T.; Sakurai, H. *Biol. Pharm. Bull.* **1995**, *18*, 59−63.

(26) Halliwell, B. *FEBS Lett.* **2003**, *540*, 3−6.

(27) Bellion, P.; Olk, M.; Will, F.; Dietrich, H.; Baum, M.; Eisenbrand, G.; Janzowski, C. *Mol. Nutr. Food Res.* **2009**, *53*, 1226−1236.

(28) Sang, S.; Hou, Z.; Lambert, J. D.; Yang, C. S. *Antioxid. Redox Signal.* **2005**, *7*, 1704−1714.

(29) Foti, M. C.; Daquino, C.; Geraci, C. *J. Org. Chem.* **2004**, *69*, 2309−2314.

(30) Torres, J. L.; Carreras, A.; Jimenez, A.; Brillas, E.; Torrelles, X.; Rius, J.; Julia, L. *J. Org. Chem.* **2007**, *72*, 3750−3756.

(31) Sato, M.; Toyazaki, H.; Yoshioka, Y.; Yokoi, N.; Yamasaki, T. *Chem. Pharm. Bull. (Tokyo)* **2010**, *58*, 98−102.

(32) Singh, B. N.; Shankar, S.; Srivastava, R. K. *Biochem. Pharmacol.* **2011**, *82*, 1807−1821.

(33) Yang, C. S.; Wang, H.; Li, G. X.; Yang, Z.; Guan, F.; Jin, H. *Pharmacol. Res.* **2011**, *64*, 113−122.

(34) Iacomino, G.; Medici, M. C.; Napoli, D.; Russo, G. L. *J. Cell. Biochem.* **2006**, *99*, 1122−1131.

(35) Elsaba, T. M.; Martinez-Pomares, L.; Robins, A. R.; Crook, S.; Seth, R.; Jackson, D.; McCart, A.; Silver, A. R.; TomLinson, I. P.; Ilyas, M. *PLoS One.* **2010**, *5*, e10714.

(36) Hu, H.; Zhang, J.; Lee, H. J.; Kim, S. H.; Lu, J. *Carcinogenesis* **2009**, *30*, 818−823.

(37) Dauer, A.; Hensel, A.; Lhoste, E.; Knasmuller, S.; Mersch-Sundermann, V. *Phytochemistry* **2003**, *63*, 199−207.

(38) McDougall, G. J.; Ross, H. A.; Ikeji, M.; Stewart, D. *J. Agric. Food Chem.* **2008**, *56*, 3016−3023.

(39) Maldonado-Celisa, M. E.; Roussia, S.; Foltzer-Jourdainne, C.; Gosse, F.; Lobstein, A.; Habold, C.; Roessner, A.; Schneider-Stock, R.; Raul, F. *Cell. Mol. Life Sci.* **2008**, *65*, 1425−1434.

(40) Ascensao, A. A.; Magalhaes, J. F.; Soares, J. M.; Ferreira, R. M.; Neuparth, M. J.; Appell, H. J.; Duarte, J. A. *Int. J. Sports Med.* **2005**, *26*, 258−267.

(41) Dhakshinamoorthy, S.; Long, D. J. 2nd; Jaiswal, A. K. *Curr. Top Cell Regul.* **2000**, *36*, 201−216.

(42) Havens, C. G.; Ho, A.; Yoshioka, N.; Dowdy, S. F. *Mol. Cell. Biol.* **2006**, *26*, 4701−4711.

(43) Moyer, M. P.; Manzano, L. A.; Merriman, R. L.; Stauffer, J. S.; Tanzer, L. R. *In Vitro Cell Dev. Biol. Anim.* **1996**, *32*, 315−317.

(44) Chai, P. C.; Long, L. H.; Halliwell, B. *Biochem. Biophys. Res. Commun.* **2003**, *304*, 650−654.

(45) Elbling, L.; Weiss, R. M.; Teufelhofer, O.; Uhl, M.; Knasmueller, S.; Schulte-Hermann, R.; Berger, W.; Micksche, M. *FASEB J.* **2005**, *19*, 807−809.
(46) Long, L. H.; Clement, M. V.; Halliwell, B. *Biochem. Biophys. Res. Commun.* **2000**, *273*, 50−53.
(47) Torres, J. L.; Varela, B.; Brillas, E.; Julia, L. *Chem. Commun. (Cambridge, U.K.).* **2003**, 74−75.
(48) Jerez, M.; Touriño, S.; Sineiro, J.; Torres, J. L.; Núñez, M. J. *Food Chem.* **2007**, *104*, 518−527.
(49) Brand-Williams, W.; Cuvelier, M. E.; Berset, C. *LWT−Food Sci. Technol.* **1995**, *28*, 25−30.
(50) Mosmann, T. *J. Immunol. Methods* **1983**, *65*, 55−63.
(51) Lozano, C.; Torres, J. L.; Julia, L.; Jimenez, A.; Centelles, J. J.; Cascante, M. *FEBS Lett.* **2005**, *579*, 4219−4225.
(52) Jiang, Z.-Y.; Hunt, J. V.; Wolff, S. P. *Anal. Biochem.* **1992**, *202*, 384−389.