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-tetramethyl-chroman-2-carboxylic acid; TNPTM, tris(2,3,5,6-tetrachloro-4-nitrophenyl)-methyl.

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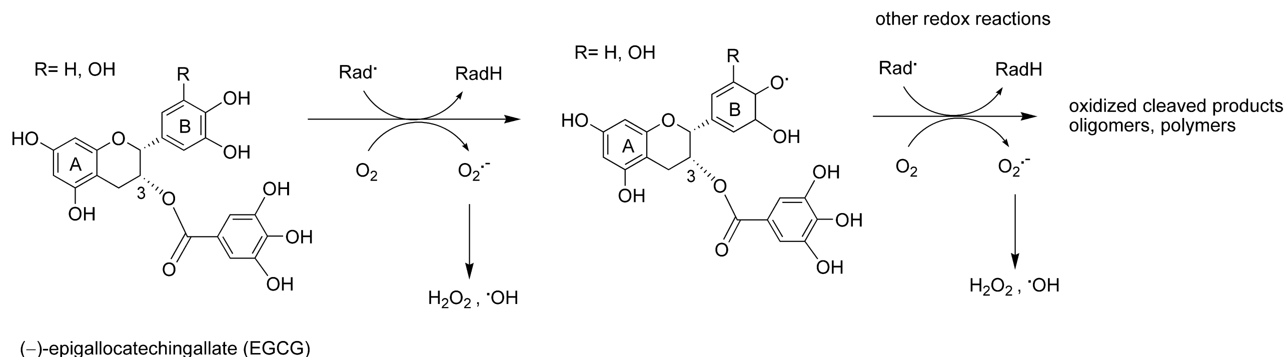


Figure 1. Scavenging of ROS and superoxide formation by catechins.

(*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

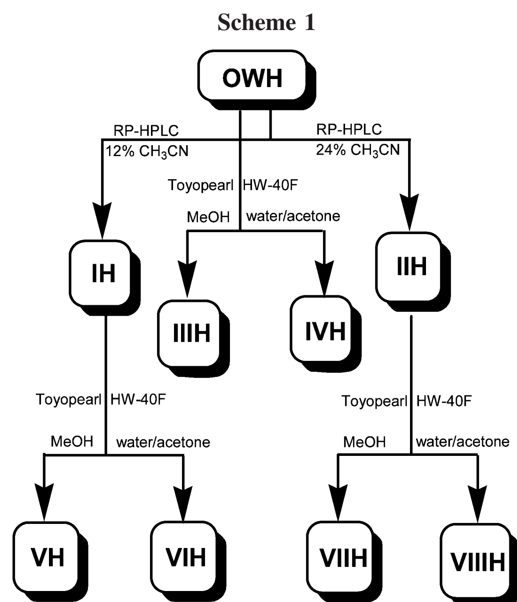
The starting material was witch hazel (*H. virginiana*) chopped stems provided by Martin Bauer GMBH (Alveslohe, Germany). The sample was stored in the dark at room temperature.

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 $\pm$ 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) $\times$ 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,

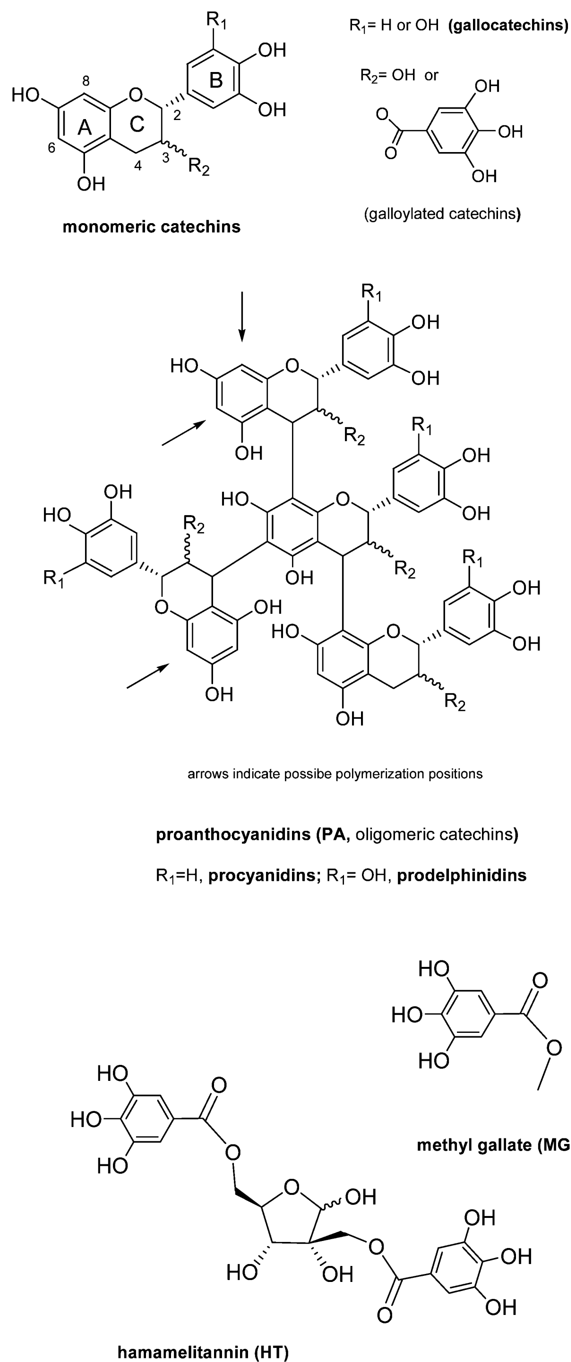


Figure 2. Structures of polyphenolics in *H. virginiana* bark extract.

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

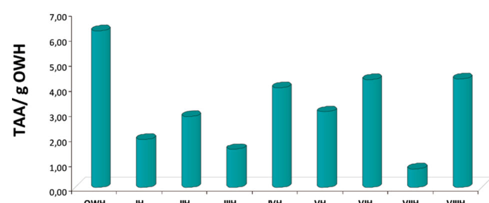


Figure 3. Total antioxidant activity (TAA) of the fractions by the ABTS cation radical method. TAA expressed as mmol Trolox equiv obtained per g of OWH.

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

fractions	DPPH ED_{50}[a]	DPPH ARC[b]	HNTTM ED_{50}	HNTTM ARC[b]	TNPTM ED_{50}[a]	TNPTM 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.

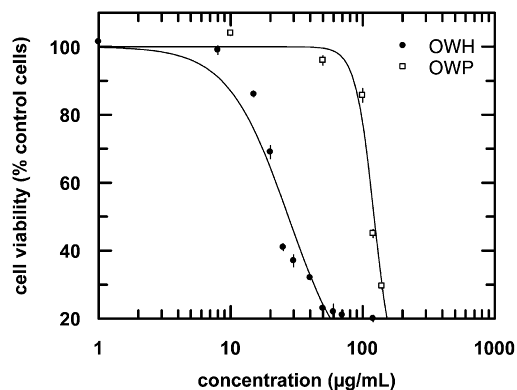


Figure 4. Percentage of proliferation of SK-Mel 28 human melanoma cells as a function of polyphenolic mixture concentration. Samples shown are the crude mixtures soluble in both ethyl acetate and water OWH (□) and OWP (●). Cells were incubated for 72 h with medium alone (control) or containing the polyphenols.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

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