JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Procyanidin Fractions from Pine (*Pinus pinaster*) Bark: Radical Scavenging Power in Solution, Antioxidant Activity in Emulsion, and Antiproliferative Effect in Melanoma Cells

SONIA TOURIÑO,† ARIADNA SELGA,† AURORA JIMÉNEZ,† LLUÍS JULIÁ,† CARLES LOZANO,†,‡ DANEIDA LIZÁRRAGA,‡ MARTA CASCANTE,‡ AND JOSEP LLUÍS TORRES*,†

Institute for Chemical and Environmental Research (IIQAB-CSIC), Jordi Girona 18-26, 08034 Barcelona, Spain, and Department of Biochemistry and Molecular Biology, Associated Unit to CSIC, University of Barcelona, Martí i Franquès 1-11, 08028 Barcelona, Spain

Pine (*Pinus pinaster*) bark is a rich source of procyanidin oligomers. From a total polyphenolic extract, we have generated fractions of different procyanidin composition. The mixtures, devoid of gallate esters, were active as free radical scavengers against $ABTS^{\bullet+}$, DPPH, and HNTTM. Pine bark fractions were tested for antioxidant activity in solution (hydrogen donation and electron transfer) and emulsion (inhibition of lipid peroxidation) and compared with their galloylated counterparts from grape origin. While galloylation clearly influenced the free radical scavenging efficiency in solution, it did not seem to play a determinant role in protection against lipid peroxidation in emulsion. The fractions were very mild inhibitors of cell proliferation. Because gallate esters appear to interfere with crucial cell functions, gallate free pine procyanidins may be the innocuous chemopreventative agents of choice for many applications in food and skin protection.

KEYWORDS: ***Pinus pinaster*; polyphenols; procyanidins; catechins; antioxidants; free radical scavenging activity; emulsions; cell proliferation**

INTRODUCTION

Polyphenols from plant origin, particularly flavonoids, are widely 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*−*9*). Moreover, the efficiency of any antioxidant very much depends on its distribution within the different biological and physicochemical environments where oxidation of key molecules (e.g., lipids, proteins, DNA) takes place (*10*, *11*). Because so many factors influence the oxidative degradation of biomolecules, the antioxidant activity should be evaluated by more than one experimental setup including methods in solution and emulsion (*12*). This is particularly true for the protection of colloidal systems such as food products and skin care formulations.

Apart from their direct antioxidant properties, flavonoids exert other activities that may or may not be related to their radical scavenging capacity. These activities, mostly mediated by receptor−ligand interactions, include antiproliferation, cell cycle regulation, and induction of apoptosis (*13*−*17*). It is becoming evident that the activity of plant flavonoids as preventive agents must be evaluated from different angles to cover not only the scavenging power but also the influence of the physicochemical environment on the antioxidant effectiveness and the occurrence of other biological activities.

Proanthocyanidins, which include both procyanidins and prodelphinidins (**Figure 1B**), are a particularly interesting type of flavonoids consisting of oligomers of flavan-3-ols (catechins). They are potent free radical scavengers, efficient antioxidants, and antiproliferative and antimflammatory agents (*18*, *19*). The size and composition of oligomeric proanthocyanidins appear to be related to their antioxidant activity, through differences in both the intrinsic scavenging capacity and the physicochemical properties governing their partition behavior within biological environments (*20*−*22*).

One of the main interests in our laboratories is to assess the suitability of proanthocyanidins (**Figure 1B**) obtained from plant byproducts as food antioxidants and preventative agents against skin aging and cancer. Oligomeric proanthocyanidins of the prodelphinidin type are present in grape skin and stems (*23*−*26*), whereas grape seeds contain only procyanidins (*27*, *28*). The three components (skin, seeds, and stems) of the grape pomace are galloylated (presence of gallate esters in C-3, **Figure 1B**) to some extent (*23*−*28*). Pine bark appears to contain only procyanidins with no measurable galloylation (*29*−*31*). The fact

* Author to whom correspondence should be addressed [telephone 34 93 400 61 12; fax 34 93 204 59 04; e-mail jltqbp@iiqab.csic.es].
† Institute for Chemical and Environmental Research-CSIC.
‡ University of Barcelona.

10.1021/jf050262q CCC: $30.25
Published on Web 04/30/2005

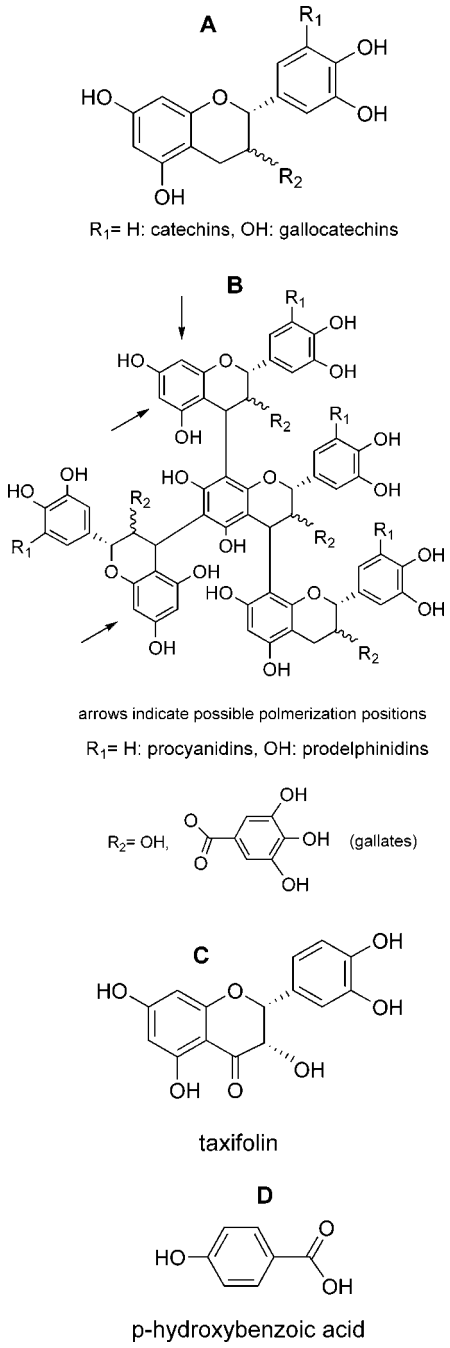


Figure 1.

that pine bark extracts are devoid of gallocatechins and gallates makes them particularly interesting because there have been reports of possible adverse effects of the pyrogallol group due to a putative prooxidant action (*32*, *33*). We have obtained a mixture of pine (*Pinus pinaster*) bark polyphenols and generated fractions with different content in monomeric catechins and oligomeric procyanidins. To evaluate the potential application of the fractions mainly in skin care formulations, and also as functional ingredients of food products, we have measured their efficiency as free radical scavengers, inhibitors of lipid peroxidation in oil-in-water emulsion, and antiproliferative activity. Because galloylation is an important parameter for defining the suitability of procyanidins and for exploring the influence of the galloyl moiety on the activity of the fractions, the results have been compared with those obtained with homologous fractions of grape origin.

MATERIALS AND METHODS

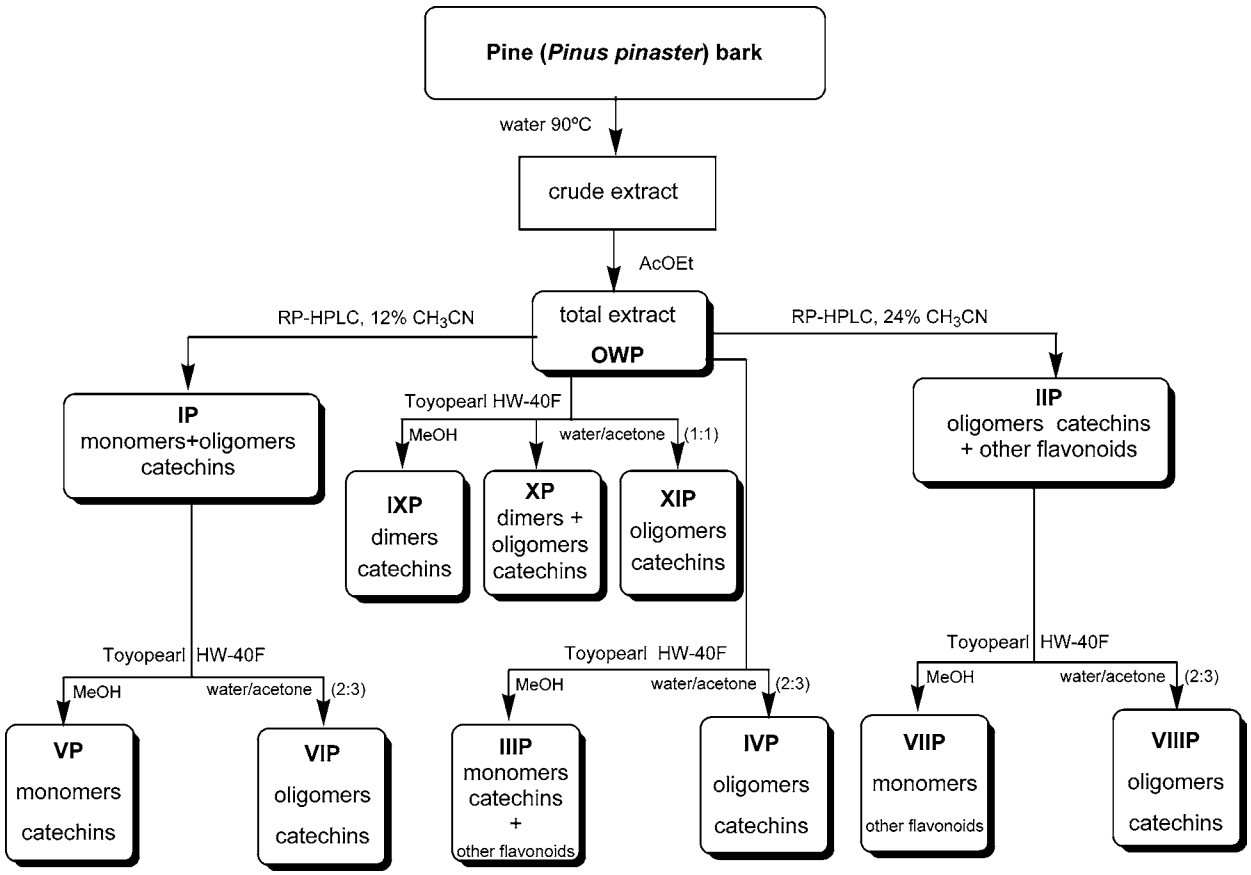
Materials and Chemicals. The starting material was pine (*Pinus pinaster*) bark (byproduct of a saw-mill) provided by Manuel Bouzas SA (Vedra, A Coruña, Spain). After air-drying, the solid was ground (GR-250 mill with 3 mm pore size from Oliver Batlle SA, Badalona, Spain) before extraction.

Water and solvents were deionized water, analytical grade MeOH (Panreac, Montcada i Reixac, Spain), and analytical grade acetone (Carlo Erba, Milano, Italy) for semipreparative chromatography; milli-Q water and HPLC grade CH_3CN (E. Merck, Darmstadt, Germany) for analytical RP-HPLC; analytical grade MeOH (Panreac) for the thioacidolysis and free radical scavenging assays; and analytical grade CH_3Cl (Panreac) for the HNTTM assay. Trifluoroacetic acid (TFA, Fluorochem, Derbyshire, UK) biotech grade was distilled in-house. Cysteamine hydrochloride was from Sigma-Aldrich Chemical (Steinheim, Germany), and 37% HCl was from Merck. 1,1-Diphenyl-2-picrylhydrazyl free radical (DPPH) (95%) was from Aldrich (Gillingham-Dorset, UK), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) (97%) was from Aldrich (Milwaukee, WI), and (−)-epicatechin was from Sigma Chemical (Saint Louis, MO). 2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) crystallized diammonium salt and horseradish peroxidase type IV (RZ $A_{403}/A_{275} < 3$) were obtained from Sigma Chemical. Hydrogen peroxide (3% v/v) was from Sigma Chemical. Tris(2,4,6-trichloro-3,5-dinitrophenyl)methyl (HNTTM) free radical was synthesized as described (*34*). Corn oil stripped of tocopherols was from Ácros Organics (New Jersey, NJ), and soybean lecithin (L-α-phosphatidylcholine) was from Sigma Chemical. Dulbecco 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 extraction and fractionation with solvents were performed at a pilot plant (A+/LGAI, Bellaterra, Spain). The polyphenolic fraction **OWP** was obtained essentially as described before for grape pomace (*35*) except that water at 90 °C was used instead of 70% EtOH in the extraction step, which was performed in an Autoclave Engineers Reactor (Dispermax, De Dietrich Niederbronn, France). Briefly, ground pine bark (10 kg) was suspended in deionized water (35 L) and kept at 90 °C, 1 atm, under stirring (80 rpm) and continuous nitrogen flow (8 L/min) for 2 h. The solid was then filtered off (Centrifugal Filter Riera Nadeu, SA, Montcada i Reixac, Catalonia, Spain), and the filtrates were extracted with ethyl acetate (3 × 20 L) after saturating the water phase with NaCl. The organic phase was then dried under vacuum, the pellet was dissolved in deionized water, and the solution was filtered through a porous plate. The dry fraction **OWP** (8.96 g, fraction from pine bark soluble in both ethyl acetate and water) was obtained by lyophilization.

Chromatography. OWP was fractionated as summarized in **Scheme 1**. Fractions **IP** and **IIP** were obtained by preparative RP-HPLC essentially as described before (*36*). The rest of the fractions were obtained by semipreparative chromatography on Toyopearl TSK HW-40F (TosoHass, Tokyo, Japan) following a protocol previously described by us (*22*). TSK HW-40F 32−63 μm particle size stationary phase was packed into a flash chromatography type 240 × 25 mm i.d. glass column and equilibrated with MeOH. Fractions **OWP** (total of 9 mL of MeOH solution, 900 mg in three runs × 3 mL), **IP**, and **IIP** (total of 6 mL of MeOH solution each fraction, 600 mg, in two runs × 3 mL) were separately fractionated in two steps: MeOH (250 mL) and water/acetone (2:3) (250 mL) following elution protocols described in the literature (*37*, *38*). The flow rate was 10 mL/min (MeOH) and 2.5 mL/min (water/acetone). The solvent was then evaporated under vacuum, and the residue was dissolved in milli-Q water (ca. 100 mL) and lyophilized to give slightly colored fluffy solids (from fraction **OWP**, 501 mg of **IIIP** and 271 mg of **IVP**; from fraction **IP**, 341 mg of **VP** and 173 mg of **VIP**; and from fraction **IIP**, 340 mg of **VIIP** and 153 mg of **VIIIP**). Alternatively, **OWP** (four runs × 300 mg) was more extensively fractionated with MeOH and water/acetone 1:1 to generate a fraction of monomers, essentially equal to **IIIP**, 54 mg

Scheme 1



fraction **IXP**, 82 mg fraction **XP**, and 186 mg fraction **XIP**. The fractions were analyzed by RP-HPLC on a Smart System (Amersham-Pharmacia Biotech, Uppsala, Sweden) equipped with a μPeak Monitor (Amersham-Pharmacia Biotech) and fitted with a μRPC C2/C18 SC 2.1/10 (100 × 2.1 mm i.d.) column (Amersham-Pharmacia Biotech). Elution: [A] 0.10% (v/v) aqueous TFA, [B] 0.08% (v/v) TFA in water/CH_3CN 1:4, gradient 8 to 23% [B] over 38 min. Flow rate 200 μL/min. Detection by triple wavelength 214, 280, 320 nm.

Thiolysis with Cysteamine and RP-HPLC. The size and composition of the procyanidins within the fractions were estimated from the HPLC analysis of the depolymerized fractions as described (*39*). 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 (μRPC column, gradient 8−23% [B] over 45 min), and the molar amount (nanomoles) of all of the released moieties was calculated from the peak areas and calibration curves obtained with pure samples:

mean degree of polymerization (mDP) = total nmol/nmol terminal units;

mean molecular weight (mMW) = total mass/nmol terminal units.

Free Radical Scavenging Activity. *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 (*40*, *41*) with some modifications. To prepare the initial radical solution, H_2O_2 3‰ (45 μL) 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. Polyphenolic solutions (50 μL) at concentrations of 0.3, 0.2, 0.15, 0.10, and 0.05 mg/mL in MeOH were then 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. 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 either in micromolar units or as μg/mL. The molar concentrations were calculated using the mean molecular weight values estimated by thioacidolysis. The Trolox equivalent antioxidant capacity (TEAC) was calculated as the ratio between the slopes of the dose−response curves of the samples and Trolox, and also as mass concentration of fraction equivalent to a 1 μM solution of Trolox. The total antioxidant activity (TAA) of the fractions was expressed in millimoles of Trolox equivalents.

DPPH Assay. The antiradical activity of the fractions was also evaluated by the DPPH stable radical method (*42*, *43*). 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 $Abs_{517nm} = 11\,345 \times C_{DPPH}$ 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 either micrograms or micromoles of product able to consume one-half the amount of free radical divided by micromoles of initial DPPH. The molecular amounts (micromoles) of procyanidin mixtures were calculated with the mean molecular weights (mMW) estimated by thiolysis with cysteamine. The results were expressed as antiradical power (ARP), which is the inverse of ED_{50}.

HNTTM Assay. EPR measurements were performed on a Varian (Palo Alto, CA) E-109 spectrometer equipped with a dual cavity (V-4532 mode). 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)) (*34*), and the mixture

was incubated for 30 min. The exact initial concentration of radical, around 60 μM, was calculated for every experiment from calibration curves made by measuring the intensity (I_0) of the EPR signal (peak to peak line distance) of standard samples of the radical at different concentrations. The equations of the curves were $I = 1980 \times C_{radical}$ or $I = 2262 \times C_{radical}$ depending on the experiment. The results were plotted as the percentage of signal intensity disappearance [$(1 - I/I_0) \times 100$] against the amount of sample divided by the initial micromoles of the radical. 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 one-half the amount of free radical divided by micromoles of initial HNTTM. As for the DPPH assay, the mean molecular weight was estimated by thioacidolysis and RP-HPLC.

Inhibition of Lipid Peroxidation in an Oil-in-Water Emulsion. Lipid peroxidation in corn oil emulsion was monitored by measuring UV absorbance at 234 nm, corresponding to the formation of conjugated dienes upon air oxidation (*10*, *44*−*46*). The emulsion was formed essentially as described (*47*, *48*). A mixture of corn oil stripped of natural antioxidants (10% w/w) and soybean lecithin (1% w/w) in 25 mM potassium phosphate pH 5 buffer was mixed with an Ultra-turrax T25 (Ika-Labortechnik, Staufen, Germany) at 8500 rpm for 5 min until the complete emulsification. The emulsion was then homogenized with a Microfluidics Corp. (Newton, MA) 110 L high-pressure homogenizer in six cycles at 600−630 bar. The average particle size of the fresh emulsions was 0.35−0.40 μm determined with an optical microscopy Reichert Polyvar (Leica Microsystems AG, Wetzlar, Germany) equipped with a video camera and a PC with a Leica IM500 software for image capture. Polyphenolic fractions dissolved in buffer (100 μL) were added to the emulsion (9.9 mL) to obtain initial concentrations ranging from 2 to 50 μg/mL. The samples (2.5 mL) were placed into 10-mL screw-capped test tubes and air oxidized at 60 °C in a shaker bath for 4 days. Every day, aliquots (50 μL) were taken, dissolved in MeOH (10 mL), to obtain absorbance values in the linear range, and the UV absorbance at 234 nm was recorded. The lipid peroxidation was calculated as millimoles of hydroperoxydes per kilogram of oil using an absorptivity of 26 000 for linoleate hydroperoxydes (*49*) and plotted against time. The experiments were done in triplicate. The antioxidant activity was expressed as percentage inhibition of hydroperoxide formation $100 \times [(C - S)/C]$, where C is the amount of hydroperoxides in the control and S is the amount of hydroperoxides in each sample with antioxidants (*48*).

Proliferation Assay on SK-Mel-28 Human Melanoma Cell Line. SK-MEL-28 adherent cells (ATCC #HTB-72) were grown in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), in the presence of 0.1% (v/v) antibiotics (10 000 U/mL penicillin, 10 000 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. 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.

Cell growth was determined using the Mosmann assay (*50*) 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 the addition of the samples dissolved in DMEM. Control wells were treated with equal volume 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−5% CO_2 during 1 h. The supernatant was removed, the blue MTT formazan precipitated was dissolved in DMSO (100 μL), and the optical density (OD) was measured at 550 nm on a multi-well reader (Merck ELISA System MIOS).

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

RESULTS AND DISCUSSION

Characterization of the Fractions and Free Radical Scavenging Activity. The pine bark polyphenolic extract **OWP**, soluble in both ethyl acetate and water, contained catechins, both monomeric and oligomeric (**Figure 1A**,**B**), and other monomeric flavonoids, mainly taxifolin (**Figure 1C**), hydroxycinnamic acids, and *p*-hydroxybenzoic acid (**Figure 1D**). From this mixture, a set of fractions differing in composition and procyanidin structure were generated using a combination of two chromatographic techniques (**Scheme 1**). RP-HPLC discriminates among solutes by hydrophobicity, while Toyopearl HW-40 has been shown to separate monomeric flavonoids and oligomeric catechins in order of increasing size (*37*, *51*) by the combined action of gel permeation and adsorption phenomena. From the total extract **OWP**, we generated fractions homologous to those obtained from grape (*22*). The fractions of pine bark origin contained catechin monomers, mainly (+)-catechin, and other flavonoid monomers, mainly taxifolin (**VP** and **VIIP**, respectively), procyanidin dimers (**IXP**), procyanidin oligomers (**IVP**, **VIP**, **VIIIP**, **XP**, **XIP**), and different combinations of monomers and oligomers (**IP**, **IIP**, **IIIP**). Procyanidin size was estimated by thiolysis with cysteamine. The results are summarized in the second and third columns of **Table 1**. While grape procyanidins were galloylated to some extent, galloylated catechin monomers or oligomers were not detected in any of the fractions from pine bark. This was the main difference between homologous procyanidin containing fractions from grape and pine. Moreover, the pine extract was richer than the grape extract in monomeric species (flavonoids and catechins in pine bark, flavonols and catechins in grape pomace) as shown by comparing the amounts of monomeric fractions obtained: **VP** (341 mg), **IIIP** (501 mg), and particularly **VIIP** (340 mg) versus homologous grape fractions **V** (218 mg), **III** (293 mg), and **VII** (85 mg) in ref *22*. The yield in procyanidins with mDP from 2.0 to 2.9 (**IVP**, **VIP**, **IXP**, **XP** and the homologous **IV**, **VI**, **IX**, **X**) was similar for both origins, while pine bark contained less oligomers with a mean degree of polymerization of 3 and higher: **VIIIP** (153 mg) and **XIP** (186 mg) versus **VIII** (305 mg) and **XI** (243 mg) in ref *22*). The total antioxidant activity of the polyphenolic mixture **OWP** and its fractions was measured by the ABTS cation radical method, which is a widely used assay for the evaluation of natural antioxidant mixtures as well as their pure components (*52*−*55*). The results are arranged and summarized in **Figure 2**, which gives a comparative idea of the activity that can be recovered in each fraction from the total mixture **OWP**. To compare the antioxidant efficiency of the fractions, the results were also expressed in terms of relative potency as compared to Trolox, as summarized in the fourth and fifth columns of **Table 1**, using either mass or molar concentration. The former provides an idea of the weighed amount of fraction needed to exert a given antiradical effect, and the latter carries information on the mean efficiency of the procyanidin components of that fraction, using the mean molecular weight (mMW) estimated by thioacidolysis followed by RP-HPLC. All of the fractions except **VIIP** were more efficient scavengers than Trolox. **VIIP** contained part of the taxifolin from **OWP** and other material not of polyphenolic nature.

The free radical scavenging power was also measured against two stable radicals, DPPH and HNTTM. These two radicals, which can be stored indefinitely and determined accurately before every experiment, can be used to calculate the stoichiometry of the redox reaction and to discriminate between hydrogen donation and electron-transfer mechanisms of radical

Table 1. Mean Size of the Components and Antiradical Power of Polyphenolic Fractions from Pine Bark

	thioacidolysis		ABTS•+ assay		DPPH assay			
fractions	mDP[a]	mMW[a]	TEAC[b]	molar TEAC[c]	ARP[d] $(1/ED_{50}) \times 10^3$	molar ARP[e] $(1/ED_{50})$	stoichiometric value	H atoms per molecule
OWP	2.1	601	0.23	2.3	12.5	7.5	0.27	3.8
IP	1.9	546	0.23	2.2	11.6	6.3	0.31	3.2
IIP	2.9	835	0.26	3.0	9.6	8.0	0.25	4.0
VP	1.0	290[f]	0.43		6.5	1.9	1.05	0.9
IIIP	1.0		0.30		9.5			
VIIP	1.0		1.80		4.5			
IXP	1.9	559	0.18	3.2	16.8	9.4	0.21	4.7
XP	2.2	639	0.14	4.1	22.2	14.2	0.14	7.1
VIP	2.7	777	0.17	4.5	20.9	17.8	0.12	8.6
IVP	2.9	833	0.15	4.5	16.8	14.1	0.14	7.1
VIIIP	3.0	876	0.22	4.2	11.9	10.4	0.19	5.2
XIP	3.4	999	0.13	6.6	21.0	21.0	0.09	10.5
controls								
Trolox	1.0	250	0.53	1.0	15.8	3.95	0.51	2.0
EC	1.0	290			21.0	6.0	0.33	3.0

[a] Mean of three independent thioacidolysis experiments with three RP-HPLC replicate injections, ABTS•+ assay, mean of three experiments. [b] Micrograms/microliter of fraction equivalents to 1 μM (0.53 μg/mL) Trolox (the lower the more efficient). [c] Micromolar concentration of Trolox equivalent to a 1 μM solution of fraction (the higher the more efficient). [d] ED_{50} in micrograms of fraction/micromoles DPPH mean of three experiments. [e] ED_{50} in estimated micromoles of fraction/micromoles DPPH only for procyanidin fractions devoid of other flavonoids. [f] Not calculated mass corresponding to catechin monomer. Standard deviation ($n = 3$): $\leq$0.4 (molar ARP), 0.03 (stoichiometric value), 0.2 (H atoms per molecule).

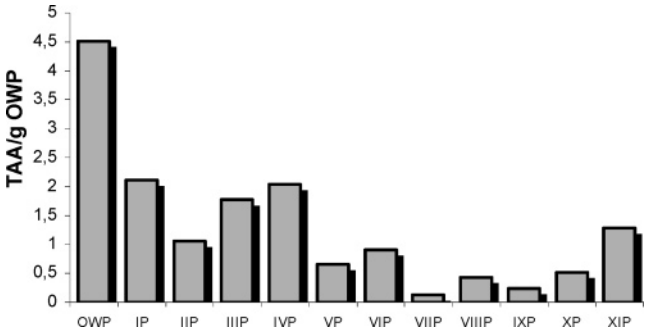


Figure 2. Total antioxidant activity (TAA) of the fractions by the ABTS cation radical method. TAA expressed as millimoles of Trolox equivalents obtained per gram of **OWP**.

scavenging. Whereas the DPPH assay measures the combined hydrogen donation and electron-transfer capacity of the polyphenols (the hydrogenated species are the end products, and no electron-transfer intermediates have been detected) (*43*, *56*, *57*), the HNTTM stable radical (*34*) exerts its action exclusively by electron transfer and the end product is the anion, as detected by UV spectrophotometry (*58*). The differential capacity of electron transfer and hydrogen donation is a relevant parameter to be measured because electron transfer is regarded sometimes as an undesired effect (*59*). Under certain conditions, flavonoids such as the pyrogallol containing (−)-epigallocatechin (EGC) and (−)-epigallocatechin-gallate (EGCG) may participate in redox cycling with the production of active superoxide radical anion ($O_2^{\bullet -}$) (*32*, *33*, *60*). Although this is less likely to occur with (−)-epicatechin or (+)-catechin or their polymers (procyanidins), redox cycling has been described also for catechols (*33*). For both DPPH and HNTTM assays, the antiradical power was expressed as the inverse of ED_{50} (micrograms or micromoles of fraction able to consume one-half the amount of free radical divided by micromoles of initial DPPH or HNTTM). By multiplying the ED_{50} by 2, the stoichiometric value (theoretical concentration of antioxidant able to reduce 100% of the radical) is obtained. The inverse of this value represents the moles of radical reduced by one mole of antioxidant and gives an estimate of the number of hydrogen atoms donated (DPPH assay) or electrons transferred (HNTTM assay) per molecule of antioxidant. **Table 1**, columns 6−9, summarizes the results of the DPPH assay. In general, the results are similar to those obtained with the ABTS cation radical. Pine bark fractions followed the general trend that the higher is the degree of polymerization, the higher is the number of hydroxyls and the higher is the free radical scavenging power per molecule. As compared to the homologous fractions from white Parellada grape pomace (*22*), pine polyphenols were less potent scavengers. This must be due to the absence of galloyl esters, which confer the extra scavenging capacity of their three hydroxyl groups. The electron-transfer capacity (HNTTM assay) of selected fractions from pine bark and grape pomace was measured and compared with the results of the DPPH assay. **Table 2** summarizes the results with the HNTTM stable radical. The fractions selected for the study contained only procyanidins of different mean degrees of polymerization (mDP). For the sake of simplicity, only the number of hydrogen atoms or electrons per molecule is shown. Homologous fractions presented essentially the same mDP. The number of hydrogen atoms per molecule increased with size (i.e., number of hydroxyls) in both cases with a plateau between 2.2 and 2.9 mDP. This was also the case for the electron-transfer capacity of grape procyanidins. Interestingly, the electron-transfer capacity of pine fractions did not completely follow this trend. Fraction **XIP** (mDP, 3.4) showed a high hydrogen donation capacity (10.5 hydrogen atoms per molecule), while keeping the electron-transfer capacity low (5.8 electrons per molecule). Because low electron transfer may imply reducing the odds for pro-oxidant effects, this result may have important implications for the use of procyanidin fractions from pine bark as safe chemopreventive agents. Alternatively, the low number of electrons transferred to HNTTM may be due to other causes such as low accessibility of the reagent in the reaction medium.

Antioxidant Activity in an Oil-in-Water Emulsion. The three methods described above provide information about the intrinsic scavenging power of the fractions in solution with solvents of different polarity going from $CHCl_3$ (HNTTM) and protic organic (MeOH, DPPH) to water (ABTS•+). The results indicate that the order of antiradical power was essentially the same for the three assays, meaning that the experimental conditions (type of radical and polarity of the solvent) did not influence the behavior of the polyphenolic fractions with the

Table 2. Hydrogen Donation versus Electron-Transfer Capacity of Pine Bark and Grape Pomace Procyanidin Fractions

	pine bark fractions				grape pomace fractions[c]		
	mDP	H/mol[a]	e/mol[b]		mDP	H/mol[a]	e/mol[b]
IXP	1.9	4.7	4.3	**IX**	2.0	7.1	4.7
XP	2.2	7.1	5.8	**X**	2.2	12.5	6.8
IVP	2.9	7.1	5.8	**IV**	2.7	12.5	8.1
XIP	3.4	10.5	5.8	**XI**	3.7	16.7	13.7

[a] Hydrogen atoms per molecule or moles of reduced DPPH per mole antioxidant. [b] Electrons per molecule or moles of reduced HNTTM per mole antioxidant. Standard deviation ($n = 3$): ≤ 0.2. [c] Grape pomace fractions and their mDP values were those obtained in ref *22*.

Table 3. Hydroxyperoxide (Hx) Formation[a] in an Oil-in-Water Emulsion after 4 days of Oxidation at 60 °C

		fraction concentration				fraction concentration		
	blank	2 μg/mL	10 μg/mL	50 μg/mL		2 μg/mL	10 μg/mL	50 μg/mL
OWP	63.5 ± 18.5	nd	25.9 ± 4.2	39.7 ± 9.1	**OW**	nd	38.5 ± 5.3	21.4 ± 4.9
IVP	66.1 ± 14.6	50.3 ± 2.8	83.6 ± 14.1	42.6 ± 1.6	**IV**	49.6 ± 2.4	43.2 ± 10.4	22.7 ± 4.1
VIIIP	78.2 ± 8.3	83.8 ± 3.4	71.3 ± 1.5	70.5 ± 12.3	**VIII**	60.2 ± 3.3	54.8 ± 3.9	28.4 ± 0.7
IXP	84.2 ± 10.3	77.4 ± 5.9	54.7 ± 14.6	58.3 ± 8.9	**IX**	76.8 ± 5.9	60.9 ± 10.4	44.6 ± 4.8
XP	64.0 ± 7.2	76.5 ± 6.9	64.9 ± 3.3	46.3 ± 0.3	**X**	61.9 ± 7.6	59.6 ± 3.0	48.3 ± 3.5
XIP	69.6 ± 28.4	70.8 ± 3.0	63.5 ± 5.2	36.4 ± 1.1	**XI**	54.5 ± 2.7	45.9 ± 2.3	31.5 ± 2.3

[a] Results expressed in mmoles Hx/kg oil ±SD, $n = 3$; nd, not determined.

possible exception of fraction **XIP** (mDP = 3.4). Because most food and living systems are colloids rather than solutions in solvents of a given polarity, and because lipids deteriorate fast at interfaces with air or water, assays in models of interfacial (e.g., lipid−water) oxidation must be part of the evaluation of antioxidants (*12*). The antioxidant activity of the fractions and Trolox against lipid peroxidation was tested in an oil-in-water emulsion made up with corn oil stripped of natural antioxidants under the conditions described in Materials and Methods. In this model, protection against lipid peroxidation is believed to depend mainly on the tendency of an antioxidant molecule to be located at water−oil interfaces (*11*, *12*, *45*, *61*). One of the goals of this paper was to establish the influence of galloylation on the antioxidant efficacy of plant procyanidins in emulsion as a model of biologically significant systems. Because the gallate moiety has been shown to be behind the influence of catechins on the cell replicating and survival machinery (cell cycle, apoptosis) (*13*, *62*), it may be preferable, in some cases, to use nongalloylated catechins for applications pertaining to food and skin protection. Our previous study with procyanidin fractions from white grape pomace recorded a relation between activity in emulsion and both size and galloylation indistinguishably (*22*). Because procyanidins from pine origin were not galloylated, they provided an excellent tool to test the influence of galloylation in lipid protection in emulsion by comparing them with grape procyanidins.

Table 3 summarizes the results obtained with pairs of significant homologous fractions of both origins. **Figure 3** depicts the percentage inhibition of lipid peroxidation exerted by the polyphenolic fractions at a concentration of 50 μg/mL after 4 days of air oxidation. The general trend was again that the galloylated fractions were the most effective. Interestingly though, nongalloylated **XP** (mDP = 2.2) and **XIP** (mDP = 3.4) were as effective as the homologous **X** (mDP = 2.2) and **XI** (mDP = 3.7), which were relatively highly galloylated (30% and 31%, respectively). This suggests that galloylation has little influence on the capacity of oligomeric procyanidins with degree of polymerization between two and four to protect lipids from peroxidation in emulsion and corroborates the hypothesis that interfacial phenomena are more important that intrinsic scavenging activity in complex multiphasic systems. This result, together with the result on low electron-transfer capacity of the same

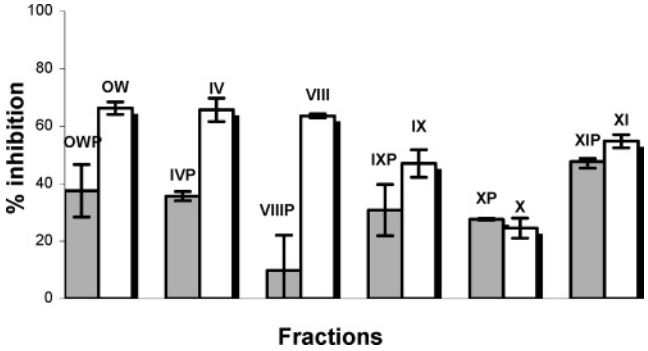


Figure 3. Inhibition of corn oil oxidation in an oil-in-water emulsion. Comparison between fractions from grape pomace and pine bark. Air oxidation for 4 days at 60 °C in the presence of procyanidin fractions at a concentration of 50 μg/mL.

procyanidins (fraction **XIP**), may have important implications in the definition of the possible applications of pine procyanidins. Pine bark procyanidins appear to protect lipids from peroxidation as effectively as other preparations, which include putatively less innocuous gallate containing components. Interestingly, fraction **XIP** contains a relatively high portion (ca. one-third) of the antioxidant activity of **OWP** (**Figure 2**) while being devoid of monomers, which, due to reports of side effects attributed to high doses of catechin (*63*, *64*), are sometimes regarded as undesirable components.

Proliferation of SK-Mel 28 Human Melanoma Cells. Selected fractions (**OWP**, **IIIP**, **IVP**, **VP**, **VIP**, **VIIIP**) showing different mean procyanidin degrees of polymerization were assayed for their influence on the proliferation of melanoma cells. Their homologous counterparts form grape origin had been assayed before on the same assay (*22*). The fractions exerted a weak antiproliferative effect on this tumoral cell line. The IC_{50} values obtained were 122 μg/mL (**OWP**), 137 μg/mL (**IVP**), 213 μg/mL (**VP**), 146 μg/mL (**VIP**), and 134 μg/mL (**VIIIP**). In overall agreement with the results obtained for grape pomace procyanidins, the monomers (**VP**) were the least efficient agents. Our results are also in agreement with those obtained with SK-MEL-1 and/or SK-MEL-28 cells after treatment with flavonoids of the flavonol and flavone type (*65*, *66*).

In conclusion, oligomeric procyanidin fractions from pine bark appear to be efficient and safe antioxidant agents. Because they do not include gallate esters, they are less potent free radical scavengers in solution than the corresponding galloylated fractions of grape origin. Interestingly, the galloyl moiety is not necessary for lipid protection in emulsion. This has important implications for the application of mixtures of this kind in food manufacturing and the formulation of skin care products. Because the galloyl moiety influences crucial biochemical pathways in cell replication and survival, efficient gallate free mixtures such as our fraction **XIP** may be the best option for products designed to offer antioxidant protection by exclusively scavenging an excess of radicals without altering the normal cell functions.

ABBREVIATIONS USED

ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid); EC, (−)-epicatechin; DMEM, Dulbecco 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; MTT, 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide; RP-HPLC, reversed-phase high-performance liquid chromatography; TAA, total antioxidant activity; TEAC, Trolox equivalent antioxidant capacity; TFA, trifluoroacetic acid; Trolox, 2,5,7,8-tetramethyl-chroman-2-carboxylic acid.

ACKNOWLEDGMENT

We are thankful to Drs. C. Solans and J. Esquena for their advice and help in the preparation of the emulsion.

Supporting Information Available: RP-HPLC chromatograms obtained for all of the fractions before and after thioacidolysis, plots of time course oil oxidation in emulsion, and dose−response curves from the proliferation assay. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Rusznyák, S. P.; Szent-Györgyi, A. Vitamin P: flavonols as vitamins. *Nature* **1936**, *138*, 27.

(2) Ross, J. A.; Kasum, C. M. Dietary flavonoids: Bioavailability, metabolic effects, and safety. *Annu. Rev. Nutr.* **2002**, *22*, 19−34.

(3) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med.* **1996**, *20*, 933−956.

(4) Yokozawa, T.; Chen, C. P.; Dong, E.; Tanaka, T.; Nonaka, G.-I.; Nishioka, I. Study on the inhibitory effect of tannins and flavonoids against the 1,1-diphenyl-2-picrylhydrazyl radical. *Biochem. Pharmacol.* **1998**, *56*, 213−222.

(5) Brown, J. E.; Khodr, H.; Hider, R. C.; Rice-Evans, C. A. Structural dependence of flavonoid interactions with Cu^{2+} ions: implications for their antioxidant properties. *Biochem. J.* **1998**, *330*, 1173−1178.

(6) Hider, R. C.; Liu, Z. D.; Khodr, H. H. Metal chelation of polyphenols. In *Flavonoids and Other Polyphenols*; Packer, L., Ed.; Academic Press Inc.: San Diego, CA, 2001; pp 190−203.

(7) Katiyar, S. K.; Afaq, F.; Perez, A.; Mukhtar, H. Green tea polyphenol (−)-epigallocatechin-3-gallate treatment of human skin inhibits ultraviolet radiation-induced oxidative stress. *Carcinogenesis* **2001**, *22*, 287−294.

(8) Ishige, K.; Schubert, D.; Sagara, Y. Flavonoids protect neuronal cells from oxidative stress by three distinct mechanisms. *Free Radical Biol. Med.* **2001**, *30*, 433−446.

(9) Inal, M. E.; Kahraman, A. The protective effect of flavonol quercetin against ultraviolet a induced oxidative stress in rats. *Toxicology* **2000**, *154*, 21−29.

(10) Frankel, E. N.; Huang, S.-W.; Kanner, J.; German, J. B. Interfacial phenomena in the evaluation of antioxidants: Bulk oils vs emulsions. *J. Agric. Food Chem.* **1994**, *42*, 1054−1059.

(11) Frankel, E. N. Interfacial lipid oxidation and antioxidation. *J. Oleo Sci.* **2001**, *50*, 387−391.

(12) Frankel, E. N.; Meyer, A. S. The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. *J. Sci. Food Agric.* **2000**, *80*, 1925−1941.

(13) Liang, Y. C.; Lin-shiau, S. Y.; Chen, C. F.; Lin, J. K. Suppression of extracellular signals and cell proliferation through EGF receptor binding by (−)-epigallocatechin gallate in human A431 epidermoid carcinoma cells. *J. Cell Biochem.* **1997**, *67*, 55−65.

(14) Ahmad, N.; Gali, H.; Javed, S.; Agarwal, R. Skin cancer chemopreventive effects of a flavonoid antioxidant silymarin are mediated via impairment of receptor tyrosine kinase signaling and perturbation in cell cycle progression. *Biochem. Biophys. Res. Commun.* **1998**, *247*, 294−301.

(15) Ahmad, N.; Cheng, P. Y.; Mukhtar, H. Cell cycle dysregulation by green tea polyphenol epigallocatechin-3-gallate. *Biochem. Biophys. Res. Commun.* **2000**, *275*, 328−334.

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

(17) Williams, R. J.; Spencer, J. P. E.; Rice-Evans, C. Flavonoids: Antioxidants or signaling molecules? *Free Radical Biol. Med.* **2004**, *36*, 838−849.

(18) Santos-Buelga, C.; Scalbert, A. Proanthocyanidins and tannin-like compounds − nature, occurrence, dietary intake and effects on nutrition and health. *J. Sci. Food Agric.* **2000**, *80*, 1094−1117.

(19) Cos, P.; De Bruyne, T.; Hermans, N.; Apers, S.; Berghe, D. V.; Vlietinck, A. J. Proanthocyanidins in health care: Current and new trends. *Curr. Med. Chem.* **2004**, *11*, 1345−1359.

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

(21) Saint-Cricq de Gaulejac, N.; Provost, C.; Vivas, N. Comparative study of polyphenol scavenging activities assessed by different methods. *J. Agric. Food Chem.* **1999**, *47*, 425−431.

(22) Torres, J. L.; Varela, B.; García, M. T.; Carilla, J.; Matito, C.; Centelles, J. J.; Cascante, C.; 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*, 7548−7555.

(23) Escribano Bailón, M. T.; Guerra, M. T.; Rivas Gonzalo, J. C.; Santos Buelga, C. Proanthocyanidin composition in skin and seed from grapes. *Polyphenols* **1994**, 225−226.

(24) Souquet, J.-M.; Cheynier, V.; Brossaud, F.; Moutounet, M. Polymeric proanthocyanidins from grape skins. *Phytochemistry* **1996**, *43*, 509−512.

(25) Kennedy, J. A.; Hayasaka, Y.; Vidal, S.; Waters, E. J.; Jones, G. P. Composition of grape skin proanthocyanidins at different stages of berry development. *J. Agric. Food Chem.* **2001**, *49*, 5348−5355.

(26) Souquet, J. M.; Labarbe, B.; LeGuerneve, C.; Cheynier, V.; Moutounet, M. Phenolic composition of grape stems. *J. Agric. Food Chem.* **2000**, *48*, 1076−1080.

(27) Prieur, C.; Rigaud, J.; Cheynier, V.; Moutounet, M. Oligomeric and polymeric procyanidins from grape seeds. *Phytochemistry* **1994**, *36*, 781−784.

(28) Fuleki, T.; Ricardo da Silva, J. M. Catechin and procyanidin composition of seeds from grape cultivars grown in Ontario. *J. Agric. Food Chem.* **1997**, *45*, 1156−1160.

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

(30) Rohdewald, P. A review of the French maritime pine bark extract (Pycnogenol (R)), a herbal medication with a diverse clinical pharmacology. *Int. J. Clin. Pharmacol. Ther.* **2002**, *40*, 158−168.

(31) 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*, 467−473.

(32) 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.

(33) Long, L. H.; Clement, M. V.; Halliwell, B. Artifacts in cell culture: Rapid generation of hydrogen peroxide on addition of (−)-epigallocatechin, (−)-epigallocatechin gallate, (+)-catechin, and quercetin to commonly used cell culture media. *Biochem. Biophys. Res. Commun.* **2000**, *273*, 50−53.

(34) Torres, J. L.; Varela, B.; Brillas, E.; Juliá, L. Tris(2,4,6-Trichloro-3,5-dinitrophenyl)methyl radical: a new stable coloured magnetic species as a chemosensor for natural polyphenols. *Chem. Commun.* **2003**, 74−75.

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

(36) Matito, C.; Mastorakou, F.; Centelles, J. J.; Torres, J. L.; Cascante, M. Antiproliferative effect of antioxidant polyphenols from grape in murine Hepa-1c1c7. *Eur. J. Nutr.* **2003**, *42*, 43−49.

(37) Sun, B. S.; Belchior, G. P.; Ricardo da Silva, J. M.; Spranger, M. I. Isolation and purification of dimeric and trimeric procyanidins from grape seeds. *J. Chromatogr., A* **1999**, *841*, 115−121.

(38) Labarbe, B.; Cheynier, V.; Brossaud, F.; Souquet, J. M.; Moutounet, M. Quantitative fractionation of grape proanthocyanidins according to their degree of polymerization. *J. Agric. Food Chem.* **1999**, *47*, 2719−2723.

(39) Torres, J. L.; Selga, A. Procyanidin size and composition by thiolysis with cysteamine hydrochloride and chromatography. *Chromatographia* **2003**, *57*, 441−445.

(40) Cano, A.; Hernández-Ruiz, J.; García-Cánovas, F.; Acosta, M.; Arnao, M. An end-point method for estimation of the total antioxidant capacity in plant material. *Phytochem. Anal.* **1998**, *9*, 196−202.

(41) Alcolea, J. F.; Cano, A.; Acosta, M.; Arnao, M. B. Hydrophilic and lipofilic antioxidant activities of grapes. *Nahrung* **2002**, *46*, 353−356.

(42) Blois, M. S. Antioxidant determinations by the use of stable free radical. *Nature* **1958**, *181*, 1199−1200.

(43) Brand-Williams, W.; Cuvelier, M. E.; Berset, C. Use of a free radical method to evaluate antioxidant activity. *Lebensm.-Wiss. Technol.* **1995**, *28*, 25−30.

(44) Buege, J. A.; Aust, S. D. Microsomal lipid peroxidation. *Methods Enzymol.* **1978**, *52*, 302−310.

(45) Huang, S.-W.; Hopia, A.; Schwarz, K.; Frankel, E. N.; German, J. B. Antioxidant activity of α-tocopherol and trolox in different lipid substrates: Bulk oils vs oil-in-water emulsions. *J. Agric. Food Chem.* **1996**, *44*, 444−452.

(46) Sánchez-Moreno, C.; Satué-Gracia, M. T.; Frankel, E. N. Antioxidant activity of selected spanish wines in corn oil emulsions. *J. Agric. Food Chem.* **2000**, *48*, 5581−5587.

(47) Huang, S.-W.; Satué-Gracia, M. T.; Frankel, E. N.; German, J. B. Effect of lactoferrin on oxidative stability of corn oil emulsions and liposomes. *J. Agric. Food Chem.* **1999**, *47*, 1356−1361.

(48) Schwarz, K.; Huang, S. W.; German, J. B.; Tiersch, B.; Hartmann, J. Activities of antioxidants are affected by colloidal properties of oil-in-water and water-in-oil emulsions and bulk oils. *J. Agric. Food Chem.* **2000**, *48*, 4874−4882.

(49) Chan, H. W.-S.; Lewet, G. Autoxidation of methyl linoleate. Separation and analysis of isomeric mixtures of methyl linoleate hydroperoxides and methyl hydroxylinoleates. *Lipids* **1977**, *12*, 99−104.

(50) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, 55−63.

(51) Derdelinckx, G.; Jerumanis, J. Separation of malt and hop proanthocyanidins on Fractogel TSK HW-40S. *J. Chromatogr.* **1984**, *285*, 231−234.

(52) Miller, N. J.; Rice Evans, C. The relative contributions of ascorbic acid and phenolic antioxidants to the total antioxidant activity of orange and apple fruit juices and blackcurrant drink. *Food Chem.* **1997**, *60*, 331−337.

(53) Pietta, P.; Simonetti, P.; Mauri, P. Antioxidant activity of selected medicinal plants. *J. Agric. Food Chem.* **1998**, *46*, 4487−4490.

(54) Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biol. Med.* **1999**, *26*, 1231−1237.

(55) Luximon-Ramma, A.; Bahorun, T.; Soobrattee, M. A.; Aruoma, O. I. Antioxidant activities of phenolic, proanthocyanidin, and flavonoid components in extracts of Cassia fistula. *J. Agric. Food Chem.* **2002**, *50*, 5042−5047.

(56) Dangles, O.; Fargeix, G.; Dufour, C. Antioxidant properties of anthocyanins and tannins: a mechanistic investigation with catechin and the 3′,4′,7-trihydroxyflavylium ion. *J. Chem. Soc., Perkin Trans. 2* **2000**, 1653−1666.

(57) Goupy, P.; Dufour, C.; Loonis, M.; Dangles, O. Quantitative kinetic analysis of hydrogen transfer reactions from dietary polyphenols to the DPPH radical. *J. Agric. Food Chem.* **2003**, *51*, 615−622.

(58) Jiménez, A.; Selga, A.; Torres, J. L.; Juliá, L. Reducing activity of polyphenols with stable radicals of the TTM series. Electron transfer versus H-abstraction reactions in flavan-3-ols. *Org. Lett.* **2004**, *6*, 4583−4586.

(59) Kovacic, P.; Sacman, A.; Wu-Weis, M. Nephrotoxins: widespread role of oxidative stress and electron transfer. *Curr. Med. Chem.* **2002**, *9*, 823−847.

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

(61) Huang, S.-W.; Frankel, E. N.; Aeschbach, R.; German, J. B. Partition of selected antioxidants in corn-water model systems. *J. Agric. Food Chem.* **1997**, *45*, 1991−1994.

(62) Liberto, M.; Cobrinik, D. Growth factor-dependent induction of p21(CIP1) by the green tea by polyphenol, epigallocatechin gallate. *Cancer Lett.* **2000**, *154*, 151−161.

(63) Jaeger, A.; Wälti, M.; Neftel, K. Side effects of flavonoids in medical practice. In *Plant Flavonoids in Biology and Medicine II: Biochemical, Cellular, and Medicinal Properties*; Cody, V., Ed.; Alan R. Liss Inc.: New York, 1988; pp 379−394.

(64) Stuhlinger, W.; Berek, K.; Grosswang, F.; Schauer, N. Several years observation of a Catergen (cyanidanol-3) induced immunohemolysis. *Schweiz. Med. Wochenschr.* **1990**, *120*, 345−348.

(65) Rodríguez, J.; Yáñez, J.; Vicente, V.; Alcaraz, M.; Benavente-García, O.; Castillo, J.; Lorente, J.; Lozano, J. A. Effects of several flavonoids on the growth of B16F10 and SK-MEL-1 melanoma cell lines: relationship between structure and activity. *Melanoma Res.* **2002**, *12*, 99−107.

(66) Lama, G.; Angelucci, C.; Bruzzese, N.; Iacopino, F.; Nori, S. L.; D'Atri, S.; Turriziani, M.; Bonmassar, E.; Sica, G. Sensitivity of human melanoma cells to oestrogens, tamoxifen and quercetin: is there any relationship with type I and II oestrogen binding site expression. *Melanoma Res.* **1998**, *8*, 313−322.

Received for review February 4, 2005. Accepted March 22, 2005. Financial support from the Spanish Ministry of Education and Science (research grants PPQ2003-06602-C04-01 and -04, and doctoral fellowships to C.L. and D.L.) is acknowledged. A private donation by Esperança and Rosa Soler Módena is also gratefully acknowledged.

JF050262Q