Effects of Wine Phenolics and Sorghum Tannins on Tyrosinase Activity and Growth of Melanoma Cells

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In this study, three different phenolic (anthocyanin, other flavonoid, and phenolic acid) fractions from wine and a condensed tannin preparation from sorghum were tested for their effects on melanogenesis of normal cells and growth of human melanoma cells. The wine phenolic fractions decreased melanogenic activity (tyrosinase activity) at concentrations that resulted in a slight variation in melanocyte viability. Sorghum tannins, however, increased melanogenic activity, although no increase was found in total melanin at the concentrations that least affect melanocyte viability. Incubation of human melanoma cells with the wine fractions and sorghum tannins resulted in a decrease in colony formation, although the effect was not dose dependent in all cases. These results suggest that all of these phenolic fractions have potential as therapeutic agents in the treatments of human melanoma, although the mechanisms by which cellular toxicity is effected seem to be different among the fractions.

Keywords: *Wine phenolics; sorghum tannins; melanogenesis; melanocyte; melanoma*

INTRODUCTION

The enzyme tyrosinase (EC 1.14.18.1) catalyzes the oxidation of tyrosine and certain diphenolic intermediate products to quinones, which polymerize to give rise to melanin. In vertebrates, tyrosinase is located in specialized cells (melanocytes). Several pure compounds are reported to act as inhibitors/activators of tyrosinase activity (*1*−*4*). Tyrosinase inhibitors are used in depigmentation drugs and in cosmetics (*5*−*7*), whereas compounds that increase melanogenesis may protect human skin from ultraviolet irradiation damage (*8*). The tyrosinase metabolic pathway also seems to be involved in the antimelanoma effects of some tyrosine analogues (*6*). These substances are oxidized by tyrosinase-generating reactive *o*-quinones with cytotoxic potential (*6*, *9*).

Some of the beneficial effects of wine in human health are attributed to its phenolic components. In vitro antioxidant activity of wine phenolics against free radicals and human low-density lipoproteins (LDL) is widely reported (*10*, *11*). Ingestion of alcohol-free red wine by healthy human subjects increased plasma antioxidant capacity parallel to increases of circulating levels of polyphenols (*12*). In addition, supplementation of caffeic acid, one of the hydroxycinnamic acids present in wines, has been shown to increase the α-tocopherol concentration in both plasma and lipoprotein in rats (*13*). Some of the main phenolics found in wine have been shown to arrest tumor growth as well as to inhibit carcinogenesis in different experimental models (*14*).

The aim of this study was to evaluate the potential of some plant phenolic preparations as therapeutic agents in the treatment of malignant melanoma. Three different wine phenolic fractions and a sorghum tannin preparation were obtained, characterized, and assayed for their in vitro antioxidant activity. They were tested for their effects in melanogenesis (tyrosinase activity) and cell viability of normal melanocytes in culture. An in vitro assay for tumorigenesis was carried out with human melanoma cells growing in soft agar containing the wine and the sorghum phenolics. The effects of these compounds on colony formation were assessed by direct colony counts.

MATERIALS AND METHODS

Isolation and Identification of Wine Phenolic Fractions. A Spanish "crianza" red wine (D.O. Rioja) was used. Wine phenolics were extracted and fractionated using the method described by Ghiselli et al. (*11*) slightly modified. In brief, wine (400 mL) was adjusted to pH 2.0 and was extracted with diethyl ether (100 mL) three times and then with ethyl acetate (100 mL) three times. The aqueous residue was dried with a nitrogen stream (wine anthocyanin fraction), and the organic phases were combined, concentrated to dryness, and dissolved in water (100 mL) at pH 7.0. The solution was then extracted with diethyl ether (100 mL) three times and then with ethyl acetate (100 mL) three times. The organic phases were combined, dried with anhydrous sodium sulfate, filtered, concentrated to dryness, redissolved in a small volume of distilled water/methanol (1:1), and dried with a nitrogen stream (wine flavonoid fraction). The aqueous residue from this extraction was adjusted to pH 2.0 and extracted again with diethyl ether (100 mL) three times and then with ethyl acetate (100 mL) three times. The organic phases were combined, dried with anhydrous sodium sulfate, filtered, concentrated to dryness, redissolved in a small volume of distilled water/methanol (1:1), and dried with a nitrogen stream (wine phenolic acid fraction).

Phenolics in wine fractions were analyzed by HPLC-DAD. A chromatographic system equipped with a model 600E pump system, a U6K injector, and a model 991 photodiode array detector (Waters, Milford, MA) was used. Previously published

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methods were followed for the analysis of benzoic and cinnamic acids, aldehydes, flavan-3-ols, procyanidins, and flavonols (*15*) and for anthocyanins (*16*). Chromatographic peaks were identified by comparing retention times and data of spectral parameters with those of standards (*15*, *17*). Relative amounts of each phenolic compound were estimated as percentage of peak area (at 280 nm for the flavonoid and phenolic acid fractions and at 546 nm for anthocyanins) to total area of all the peaks detected.

Sorghum Condensed Tannins. Sorghum condensed tannins were obtained as reported by Jiménez-Ramsey et al. (*18*). In brief, sorghum phenolics were extracted with methanol/hydrochloric acid (99:1, v/v) and fractionated through Sephadex LH-20. Sorghum condensed tannins were eluted with 80% acetone and concentrated to dryness, redissolved in a small volume of distilled water, and freeze-dried (sorghum tannins). Sorghum condensed tannins were analyzed by HPLC as described above.

In Vitro Antioxidant Activity. The antioxidant activities of the wine fractions, sorghum condensed tannins, and some phenolic standards [malvidin 3-*O*-glucoside, (+)-catechin, (−)-epicatechin, resveratrol, tyrosol, tryptophol, gallic acid, caffeic acid, and *p*-coumaric acid] were determined as the measure of radical scavenging using $DPPH^•$ (*19*). The disappearance of $DPPH^•$ was measured at the steady state. The radical stabilization curves (disappearance of $DPPH^•$ versus phenolic content) were calculated by preparing dilutions of the fractions that yielded percentages of remaining $DPPH^•$ between 100 and 10%. From these curves, the radical-scavenging capacity (C_{50}) was defined as the amount (micrograms) necessary to decrease the initial $DPPH^•$ concentration by 50%.

Activity on Melonogenesis and Cell Viability. Melan-a cells (*20*) were a kind gift of Dr. D. C. Bennett (St. George's Hospital, London, U.K.) and were routinely grown in a humidified atmosphere with 10% CO_2 at 37 °C in complete RPMI 1640 medium containing 5% heat-inactivated fetal calf serum, 50 units/mL penicillin, 50 mg/L streptomycin, 100 μM β-mercaptoethanol, 2 mM L-glutamine, and 200 nM phorbol 12-myristate 13-acetate (PMA).

Upon exposure of melan-a melanocytes to the different phenolic fractions, a standardized protocol was used for the analysis of cell viability, melanogenesis (tyrosinase activity), and total melanin. In brief, melan-a cells were seeded in six well plates at 25000 cells/well. Upon attachment, test fractions were added at various concentrations. Cells were exposed to test compounds for a total of 4 days with one change of medium and compounds. Cells were harvested by brief trypsin/EDTA treatment, and an aliquot of each was seeded for the cell viability (MTT) assay. The remaining cells were centrifuged for 5 min at 1500 *g*, washed with Dulbecco's phosphate buffer saline, and then solubilized in extraction buffer [1% Nonidet P40, 0.01% SDS, 0.1 M Tris-HCl, pH 7.2, containing protease inhibitor cocktail (Boehringer-Mannheim, Indianapolis, IN)]. Aliquots of those extracts were used to determine melanin content by absorbance at 650 nm and for radiometric assays utilizing L-[^{14}C]tyrosine (*4*). Experiments were carried out in duplicate.

Colony Formation in Agar. All media and supplements were from Gibco BRL except where indicated. MNT-1 cells (human melanoma) (*21*) were routinely grown in a 10% CO_2 humidified atmosphere. High-glucose DMEM medium contained the following supplements: 2 mM L-glutamine, 100 units/mL penicillin, 100 mg/L streptomycin, 0.25 mg/L fungizone, 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, 0.16% $NaHCO_3$, 10% Aim V medium, and 20% FBS (Atlanta Biologic, Atlanta, GA). For colony formation in agar, a previously published method was used (*22*) with some modifications (*23*). Feeder layers composed of 0.5% molten agar in 2× DMEM medium (with supplements) were allowed to solidify in six well plates. Cells were harvested by brief trypsinization, and 25000 cells/well were mixed with 0.6% molten agar/2× DMEM medium (containing the test phenolic fractions) for the top layer. Plates were initially covered with Parafilm to preclude desiccation. Three weeks later, the Parafilm was removed, and 100 μL aliquots of fresh medium were added. Growth of colonies was monitored under the microscope every 3 days. Colonies were visible by eye at 4 weeks, and then they were counted and photographed. Colonies >5 μm in diameter were counted. Colony counting was carried out with an AlphaImager 2000 (Alpa Innotech Corp., San Leandro, CA) with their proprietary software AlphaEase. The thresholds required for colony counting were established by counting five random fields in a dissecting microscope and comparing the results.

Table 1. Characterization and Antioxidant Activity of the Phenolic Fractions

phenolic fraction	main phenolic compounds (% of total phenolic composition)	radical scavenging capacity (C_{50})[a]
wine anthocyanin fraction	delphinidin 3-*O*-glucoside (11.4%) cyanidin 3-*O*-glucoside (0.3%), petunidin 3-*O*-glucoside (11.9%) peonidin 3-*O*-glucoside (2.4%) malvidin 3-*O*-glucoside (51.3%) malvidin 3-(6-*O*-*p*-coumaroyl)-glucoside (1.7%)	29.4
wine flavonoid fraction	hydroxytyrosol (4.8%) tyrosol (7.4%) tryptophol (11.7%) (+)-catechin (11.2%) (−)-epicatechin (5.1%) procyanidin B2 (0.6%) procyanidin B5 (1.7%) others procyanidins (1.2%) quercetin glycosides (4.5%) resveratrol (8.1%)	8.0
wine phenolic acid fraction	gallic acid (15.6%) protocatechuic acid (2.8%) *p*-hydroxybenzoic acid (1.4%) vanillic acid (2.9%) syringic acid (9.9%) caffeic acid (22.3%) *p*-coumaric acid (23.7%) ferulic acid (0.6%) 3,4-dihydroxyphenylacetic acid (5.1%) *p*-coumaric esters (5.3%)	36.5
sorghum tannins	polymers greater than procyanidin pentamers	5.2

[a] Expressed as the amount (micrograms) necessary to decrease the initial $DPPH^•$ concentration by 50%.

RESULTS AND DISCUSSION

The wine phenolic fractions were found to contain mainly anthocyanins (anthocyanin fraction), alcohols, flavan-3-ols, procyanidins, and flavonols (flavonoid fraction), and benzoic and cinnamic acids (phenolic acid fraction). The main individual phenolic compounds identified in each fraction are reported in Table 1. In the anthocyanin fraction, 79% of the anthocyanins correspond to single glucosylated forms such as delphinidin 3-glucoside (D-3-gl), cyanidin 3-glucoside (Cy-3-gl), petunidin 3-glucoside (Pt-3-gl), peonidin 3-glucoside (Pn-3-gl), and malvidin 3-glucoside (M-3-gl) and acyl derivatives such as malvidin 3-(6-*O*-*p*-coumaroyl)-glucoside. Alcohols such as hydroxytyrosol, tyrosol, and tryptophol, flavan-3-ols such as (+)-catechin and (−)-epicatechin, procyanidins such as B2 and B5, flavonol glycosides such as quercetin glycosides, and stilbenes such as resveratrol were present in the wine flavonoid fraction. The main compounds detected in the wine phenolic acid fraction were gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, syringic acid, caffeic acid, *p*-coumaric acid, ferulic acid, 3,4-dihydroxy-

phenylacetic acid, and *p*-coumaric esters. Ghiselli et al. (*11*) reported similar phenolic compositions for the anthocyanin, flavonoid, and phenolic acid fractions obtained from a young Italian red wine. These authors identified myricetin 3-glucoside, kaempferol, and kaempferol 3-glucoside in the flavonoid fraction and *p*-coumaroyl- and feruloyltartaric acids in the phenolic acid fraction. These compounds were not detected in our study; however, we identified hydroxytyrosol, tyrosol, and tryptophol in the flavonoid fraction and syringic acid in the phenolic acid fraction, which were not reported by Ghiselli et al. (*11*). The composition in procyanidins of the wine flavonoid fraction found in our study was slightly different from the one reported by Ghiselli et al. (*11*). The sorghum tannin preparation obtained in this study was found to contain polymers greater than procyanidin pentamers because no peaks were detected for procyanidins.

The in vitro antioxidant capacity (radical scavenging capacity, C_{50}) was higher for sorghum tannins and the wine flavonoid fraction than for the wine anthocyanin and phenolic acid fractions (Table 1). The radical scavenging capacity against DPPH$^•$ has been shown to depend on phenolic structure (*19*, *24*). The antioxidant capacity of the wine fractions should be the result of the contributions of the main phenolic compounds present and their possible synergistic effects. The C_{50} value for the anthocyanin fraction (29.4 μg) was similar to the value obtained for the main component, malvidin 3-*O*-glucoside (22.5 μg). (+)-Catechin and (−)-epicatechin with C_{50} values of 6 and 2.5 μg, respectively, seemed to be the major compounds contributing to the antioxidant capacity of the flavonoid fraction. Other components such as resveratrol, tyrosol, and tryptophol showed lower values for radical scavenging capacity (17, 105, and >1600 μg, respectively), and it might be other nonidentified components that also contributed to the total antioxidant capacity of this fraction. The antioxidant capacity of the wine phenolic acid fraction can be attributed to acids such as gallic and caffeic, with C_{50} values of 1.5 and 2.5 μg, respectively, rather than to *p*-coumaric acids, with a C_{50} value of 985 μg.

Wine anthocyanin fractions and sorghum tannins were tested for their melanogenic effects in cultured cells at a concentration range between 500 and 4 mg/L, whereas wine flavonoid and acid fractions were tested at a range between 1000 and 8 mg/L. In all cases, the highest concentration tested resulted in a profound decrease in cell viability. Only concentrations that resulted in a slight decrease in viability are reported here (Figure 1). Standard error in all results shown was ±<5%. The three wine fractions decreased melanogenic activity (tyrosinase activity) at some concentrations while minimally affecting cell viability (Figure 1). Melanogenic activity of wine fractions at concentrations that least affected cell viability (100 mg/L for the anthocyanin fraction, 40 mg/L for the flavonoid fraction, and 8 mg/L for the acid fraction) seemed to be inversely related to their antioxidant activity (see Table 1). This result may be explained by direct competitive inhibition of melanocyte tyrosinase by some of the phenolic compounds contained in these fractions and/or by antioxidant mechanisms affecting tyrosinase expression or other cell signaling mechanisms. Surprisingly, sorghum tannins at 20 mg/L increased melanogenic activity, although no increase was found in total melanin. Compared to the single phenolic structures present in

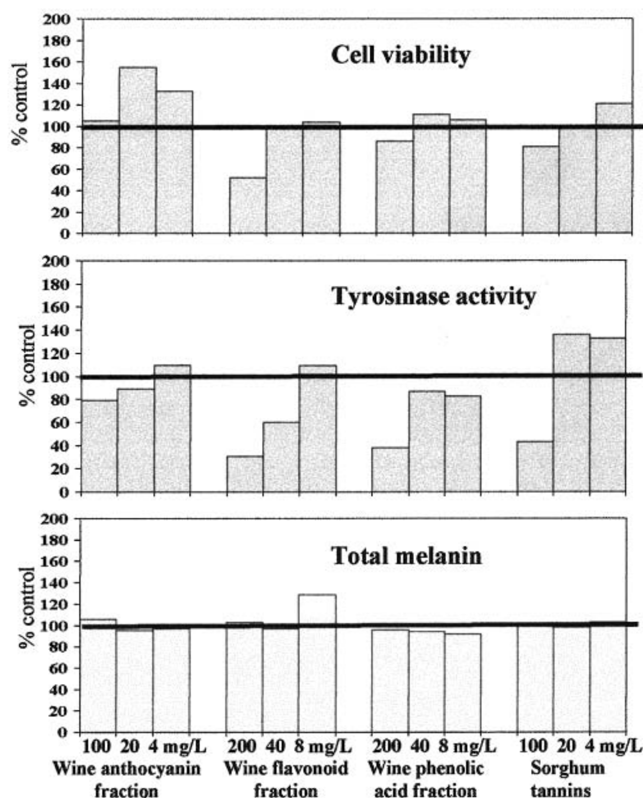

Figure 1. Quantitative analysis of the effects of the phenolic fractions on melanogenic activity (tyrosinase activity) and melanocyte viability in culture: (A) cell viability measured by the MTT assay; (B) tyrosinase activity measured as radioactive melanin formation quantitated by phosphorimager; (C) total intracellular melanin measured as absorbance at 650 nm of the soluble cell extracts. Melan-a mouse melanocytes were treated in culture for 4 days with the phenolic fractions at the concentrations indicated. Each value represents a mean of the duplicate determinations from duplicate samples. This experiment was repeated three times with similar results.

the wine fractions, sorghum tannins have a greater ability to interact with proteins with the consequent decrease on enzymatic activities. Because the tannin preparation showed the highest antioxidant activity (Table 1), melanin production may be inhibited inside the cells, but radioactive melanin can be enzymatically produced from solubilized tyrosinase in the melanogenic assay.

Some phenolic compounds have been shown to inhibit mammalian melanin production in vivo or in vitro. Hydroquinone (*3*, *4*), arbutin (a glucopyranoside of hydroquinone) (*25*−*27*), kojic acid (*28*), and glabridin (*29*) have been reported as potential inhibitors of tyrosinase activity, and their phenolic groups are not in the ortho position, which is required for the dopa oxidase activity of tyrosinase (*30*).

The effects of the wine phenolic fractions and sorghum tannins on the growth of human melanoma cells were also studied. The ability of cells to undergo unlimited proliferation is tested by their ability to form a colony, and the colony formation assay determines the proportion of cells surviving a particular treatment under the conditions used, thus assessing their sensitivity to cytotoxic treatments. In a modification of the colony formation assay using growth on agar, the ability of cancerous cells to grow and migrate unattached to a substrate is tested (anchorage-independent growth). Figure 2 shows the colony formation of MNT-1 human

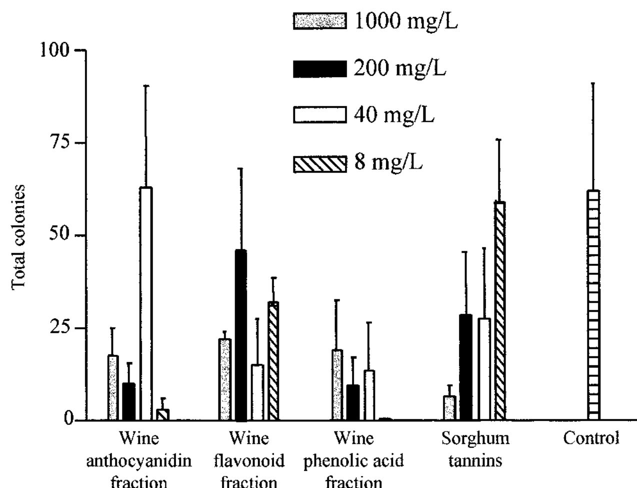

Figure 2. Colony formation assay for the phenolic fractions. Feeder layers composed of 0.5% molten agar in 2× DMEM medium were allowed to solidify in six well plates. MNT-1 human melanoma cells were suspended in 0.6% agar with 2× DMEM medium containing the phenolic fractions. Colonies, visible by eye at 4 weeks, were counted in an AlphaImager and photographed. Three separate experiments were carried, each at four concentrations plus replicate negative controls, covering the range of concentrations shown in the figure.

melanoma cells following exposure to different concentrations of the phenolic fractions. All fractions decreased colony formation, but the effect seemed not to be progressively dose dependent. Concentrations having the least effect on colony formation (i.e., lowest toxicity) varied among the fractions: 40 mg/L for wine anthocyanidins, 200 mg/L for other wine flavonoids, 1000 mg/L for wine phenolic acids, and 8 mg/L for sorghum tannins. The wine phenolic acid fraction and the tannins did have a dose-dependent pattern. Toxicity to melanoma cells may occur mostly by free radical species, and therefore antioxidants present in the cellular milieu should confer protection. Indeed, the wine phenolic acid fraction, with the lowest in vitro antioxidant activity (Table 1), had the lowest colony formation overall, whereas the sorghum tannins, which had the highest in vitro antioxidant activity (Table 1), showed the highest colony formation overall. Other aspects of the action of phenolics should also be considered for the interpretation of these results. First, phenolic antioxidants act as inhibitors of melanin formation, either by inhibition of tyrosinase activity or by competitive inhibition of their oxidation products in the formation of dopa *o*-quinones that are the main precursors of melanin (*31*). Inhibition of melanin formation appears to be inversely correlated with the proliferation of melanized cells (*32*, *33*). Second, oxidation of phenolics could lead to the generation of compounds with different cytotoxic potential against melanoma cells. At the lowest concentration tested (8 mg/L), the survival of melanoma cells in this assay seemed to be inversely related to the antioxidant potential of the phenolic fractions (Table 1). At higher concentrations, the toxicological effect of the oxidized phenolic compounds would be relatively more important and would lead to differences among fractions and to a dose-independent trend, perhaps reflecting the diverse actions of individual phenolics in those fractions (*29*, *34*).

Growth inhibition of malignant cells has been studied mostly on monolayer cultures. Kamei et al. (*35*) reported the growth inhibitory effect of bioflavonoids on cells in culture. Others have reported similar inhibition effected by purified tea polyphenols (*34*, *36*, *37*), resveratrol (*38*), and glabridin (*29*). There are fewer reports of the effects on clonal growth in soft agar, which reflects the anchorage-independent growth associated with metastatic potential. Dong et al. (*39*) reported that (−)-epigallocatechin gallate (EGCG) and theaflavins inhibited epidermal growth factor- or 12-*O*-tetradecanoylphorbol-13-acetate-induced cell transformation in a dose-dependent manner. Quercetin (1 μM) inhibited the clonogenic capacity of human melanoma cells by ~60% (and so did tamoxifen), whereas rutin, a glucoside of quercetin, did not inhibit such growth (*40*). Green tea has been shown to inhibit in vivo metastasis and in vitro invasion of mouse Lewis lung carcinoma LL2-Lu3 cells, which are highly metastatic (*41*). The invasion inhibition may be associated with inhibition of type IV collagenase activity. This activity would be ascribed to the galloyl moiety because gallic acid is a well-known protein cross-linking agent (used of old for tanning).

CONCLUSIONS

Polyphenols from plant sources have been extensively studied, and a plethora of activities has been attributed to them. Most important are antioxidant and detoxifying effects as well as effects on lowering the rate of cell replication, thus controlling neoplasms. Our data show that these phenolic fractions inhibit melanogenic activity in melanocytes and decrease colony forming of melanoma cells, which support their potential as therapeutic agents in the treatments of human melanoma. The mechanisms by which cellular toxicity is effected seem to be different among the fractions but need to be further investigated. We are currently studying these effects in a murine melanoma cell line as well as in vivo by treatment of melanoma tumors in mice.

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LITERATURE CITED

(1) Shoji, T.; Kobori, M.; Shinmoto, H.; Tanabe, M.; Tsushida, T. Progressive effects of phloridzin on melanogenesis in B16 mouse melanoma cells. *Biosci., Biotechnol., Biochem.* **1997**, *61*, 1963−1967.

(2) Watters, D.; Garrone, B.; Coomer, J.; Johnson, W. E.; Brown, G.; Parsons, P. Stimulation of melanogenesis in a human melanoma cell line by bistratene A. *Biochem. Pharmacol.* **1998**, *55*, 1691−1699.

(3) Curto, E. V.; Kwong, C.; Hermersdorfer, H.; Glatt, H.; Santis, C.; Virador, V.; Hearing, V. J.; Dooley, T. P. Inhibitors of mammalian melanocyte tyrosinase: in vitro comparisons of alkyl esters of gentisic acid with other putative inhibitors. *Biochem. Pharmacol.* **1999**, *57*, 663−672.

(4) Virador, V. M.; Kobayashi, N.; Matsunaga, J.; Hearing, V. J. A standardized protocol for assessing regulators of pigmentation. *Anal. Biochem.* **1999**, *270*, 207−219.

(5) Kligman, A. M.; Willis, I. A new formula for depigmenting human skin. *Arch. Dermatol.* **1975**, *111*, 40−48.

(6) Alena, F.; Jimbow, K.; Ito, S. Melanocytotoxicity and antimelanoma effects of phenolic amine compounds in mice *in vivo*. *Cancer Res.* **1990**, *50*, 3743−3747.

(7) Tasaka, K.; Kamei, C.; Nakano, S.; Takeuchi, Y.; Yamato, M. Effects of certain resorcinol derivatives on the tyrosinase activity and the growth of melanoma cells. *Methods Find. Exp. Clin. Pharmacol.* **1998**, *20*, 99−109.

(8) Eller, M. S.; Ostrom, K.; Gilchrest, B. A. DNA damage enhances melanogenesis. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 1087−1092.
(9) Riley, P. A. Radicals and melanomas. *Philos. Trans. R. Soc. London B, Biol. Sci.* **1985**, *311*, 679−689.
(10) Frankel, E. N.; Kanner, J.; German, J. B.; Parks, E.; Kinsella, J. E. Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. *Lancet* **1993**, *341*, 454−457.
(11) Ghiselli, A.; Nardini, M.; Baldi, A.; Scaccini, C. Antioxidant activity of different phenolic fractions separated from an italian red wine. *J. Agric. Food Chem.* **1998**, *46*, 361−367.
(12) Serafini, M.; Maiani, G.; Ferro-Luzzi, A. Alcohol-free red wine enhances plasma antioxidant capacity in humans. *J. Nutr.* **1998**, *128*, 1003−1007.
(13) Nardini, M.; Natella, F.; Gentili, V.; Di Felice, M.; Scaccini, C. Effect of caffeic acid dietary supplementation on the antioxidant defense system in rat: an *in vivo* study. *Arch. Biochem. Biophys.* **1997**, *342*, 157−160.
(14) Soleas, G. J.; Diamandis, E. P.; Goldberg, D. M. Wine as biological fluid: history, production, and role in disease prevention. *J. Clin. Lab. Anal.* **1997**, *11*, 287−313.
(15) Bartolomé, B.; Bengoechea, M. L.; Gálvez, M. C.; Pérez-Ilzarbe, F. J.; Hernández, T.; Estrella, I.; Gómez-Cordovés, C. Photodiode array detection for elucidation of the structure of phenolic compounds. *J. Chromatogr. A* **1993**, *655*, 119−125.
(16) Gonzalez-San Jose, M. L.; Diez, C.; Santa-Maria, G. Analysis by high performance liquid chromatography of the anthocyanins pigments of *Vitis vinifera* grapes. *An. Quim.* **1988**, *84*, 290−294.
(17) Bartolomé, B.; Hernández, T.; Bengoechea, M. L.; Quesada, C.; Gómez-Cordovés, C.; Estrella, I. Determination of some structural features of procyanidins and related compounds by photodiode-array detection. *J. Chromatogr. A* **1996**, *723*, 19−26.
(18) Jiménez-Ramsey, L. M.; Rogler, J. C.; Housley, T. L.; Butler, L. G.; Elkin, R. G. Absorption and distribution of ^{14}C-labeled condensed tannins and related sorghum phenolics in chickens. *J. Agric. Food Chem.* **1994**, *42*, 963−967.
(19) 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.
(20) Bennett, D. C.; Cooper, P. J.; Hart, I. R. A line of non-tumorigenic mouse melanocytes, syngeneic with the B16 melanoma and requiring a tumour promoter for growth. *Int. J. Cancer* **1987**, *39*, 414−418.
(21) Cuomo, M.; Nicotra, M. R.; Apollonj, C.; Fraioli, R.; Giacomini, P.; Natali, P. G. Production and characterization of the murine monoclonal antibody 2G10 to a human T4-tyrosinase epitope. *J. Invest. Dermatol.* **1991**, *96*, 446−451.
(22) MacPherson, I. Soft agar techniques. In *Tissue Culture Methods and Applications*; Kruse, P. F., Patterson, M. K., Eds.; Academic Press: New York, 1973; p 276.
(23) Fletcher, J. M.; Dowdle, E. B. Proteolytic mechanisms involved in the metastasis of human melanoma cells. Ph.D. Thesis, Department of Clinical Science and Immunology, University of Cape Town, 1994.
(24) Sánchez-Moreno, C.; Larrauri, J. A.; Saura-Calixto, F. A procedure to measure the antiradical efficiency of polyphenols. *J. Sci. Food Agric.* **1998**, *76*, 270−276.
(25) Chakraborty, A. K.; Funasaka, Y.; Komoto, M.; Ichihashi, M. Effect of arbutin on melanogenic proteins in human melanocytes. *Pigment Cell Res.* **1998**, *11*, 206−212.
(26) Nakajima, M.; Shinoda, I.; Fukuwatari, Y.; Hayasawa, H. Arbutin increases the pigmentation of cultured human melanocytes through mechanisms other than the induction of tyrosinase activity. *Pigment Cell Res.* **1998**, *11*, 12−17.
(27) Maeda, K.; Fukuda, M. Arbutin: mechanism of its depigmenting action in human melanocyte culture. *J. Pharmacol. Exp. Ther.* **1996**, *276*, 765−769.
(28) Lim, J. T. Treatment of melasma using kojic acid in a gel containing hydroquinone and glycolic acid. *Dermatol. Surg.* **1999**, *25*, 282−284.
(29) Yokota, T.; Nishio, H.; Kubota, Y.; Mizoguchi, M. The inhibitory effect of glabridin from licorice extracts on melanogenesis and inflammation. *Pigment Cell Res.* **1998**, *11*, 355−361.
(30) Hearing, V. J. Mammalian monophenol monooxygenase (tyrosinase): purification, properties, and reactions catalyzed. *Methods Enzymol.* **1987**, *142*, 154−165.
(31) Rorsman, H.; Agrup, G.; Hansson, C.; Rosengren, E. Biochemical recorders of malignant melanoma. In *Malignant Melanoma, Advances of a Decade*; Mekie, R. M., Eds.; Karger: Basel, 1983; pp 93−115.
(32) Kameyama, K.; Vieira, W. D.; Tsukamoto, K.; Law, L. W.; Hearing, V. J. Differentiation and the tumorigenic and metastatic phenotype of murine melanoma cells. *Int. J. Cancer* **1990**, *45*, 1151−1158.
(33) Nakazawa, K.; Damour, O.; Collombel, C. Modulation of normal human melanocyte dendricity by growth-promoting agents. *Pigment Cell. Res.* **1993**, *6*, 406−416.
(34) Suganuma, M.; Okabe, S.; Kai, Y.; Sueoka, N.; Sueoka, E.; Fujiki, H. Synergistic effects of (−)-epigallocatechin gallate with (−)-epicatechin, sulindac, or tamoxifen on cancer-preventive activity in the human lung cancer cell line PC-9. *Cancer Res.* **1999**, *59*, 44−47.
(35) Kamei, H.; Kojima, T.; Hasegawa, M.; Koide, T.; Umeda, T.; Yukawa, T.; Terabe, K. Suppression of tumor cell growth by anthocyanins in vitro. *Cancer Invest.* **1995**, *13*, 590−594.
(36) Ahmad, N.; Feyes, D. K.; Nieminen, A. L.; Agarwal, R.; Mukhtar, H. Green tea constituent epigallocatechin-3-gallate and induction of apoptosis and cell cycle arrest in human carcinoma cells. *J. Natl. Cancer Inst.* **1997**, *89*, 1881−1886.
(37) Chung, J. Y.; Huang, C.; Meng, X.; Dong, Z.; Yang, C. S. Inhibition of activator protein 1 activity and cell growth by purified green tea and black tea polyphenols in H-ras-transformed cells: structure−activity relationship and mechanisms involved. *Cancer Res.* **1999**, *59*, 4610−4617.
(38) Ciolino, H. P.; Daschner, P. J.; Yeh, G. C. Resveratrol inhibits transcription of CYP1A1 in vitro by preventing activation of the aryl hydrocarbon receptor. *Cancer Res.* **1998**, *58*, 5707−5712.
(39) Dong, Z.; Ma, W.; Huang, C.; Yang, C. S. Inhibition of tumor promoter-induced activator protein 1 activation and cell transformation by tea polyphenols, (−)-epigallocatechin gallate, and theaflavins. *Cancer Res.* **1997**, *57*, 4414−4419.
(40) Piantelli, M.; Maggiano, N.; Ricci, R.; Larocca, L. M.; Capelli, A.; Scambia, G.; Isola, G.; Natali, P. G.; Ranelletti, F. O. Tamoxifen and quercetin interact with type II estrogen binding sites and inhibit the growth of human melanoma cells. *J. Invest. Dermatol.* **1995**, *105*, 248−253.
(41) Sazuka, M.; Imazawa, H.; Shoji, Y.; Mita, T.; Hara, Y.; Isemura, M. Inhibition of collagenases from mouse lung carcinoma cells by green tea catechins and black tea theaflavins. *Biosci., Biotechnol., Biochem.* **1997**, *61*, 1504−1506.

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