

Cytotoxicity and Antiproliferative Activities of Several Phenolic Compounds Against Three Melanocytes Cell Lines: Relationship Between Structure and Activity

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Abstract: Polyphenolic compounds are widely distributed in the vegetable kingdom and are therefore consumed regularly in the human diet. Epidemiological studies suggest that foods rich in polyphenolic compounds contribute to reducing the risk of cancer. The purpose of our work is to: 1) study the possible cytotoxicity and antiproliferative effects of 13 polyphenolic compounds on 3 cell lines of melanocytes, 2 of melanoma (B16F10 and SK-MEL-1), and 1 of nontransformed melanocytes (Melan-a); and 2) identify the possible relationship between the chemical structure of the tested compounds and their effect on cellular viability. The said polyphenolic compounds corresponded to 8 flavonoids with varying hydroxyl and methoxyl substituents, related structurally through the oxidation state of their flavonoid skeleton, a catechin polymer and 4 phenolic acids. The cytotoxic activity of all the studied compounds was modest or not apparent. The flavonoids luteolin, tangeretin, baicalein, quercetin, and myricetin, and gallic acid showed antiproliferative effects on the tested lines. Our results suggest that a correlation exists between the structural oxidation state and the position, number, and nature of substituents of the polyphenolic compounds studied and their antiproliferative effects.

Introduction

Phenolic compounds constitute a group of substances that are widely present in the plant kingdom, where more than 8,000 are known, with different chemical structures and activities. They are found in fruits, vegetables, nuts, and seeds, as well as in tea, red wine, citrus fruits, and other food sources. They are consumed regularly as part of the human diet (1,2). They intervene in the organoleptic properties of foods, contributing to their color, bitter taste, and astringency; they also contribute to the browning of vegetables (3).

The main classes of phenolic compounds in the diet are phenolic acids, flavonoids, lignans, stilbenes, coumarins, and tannins (4). Many epidemiological studies have suggested that there is a link between the consumption of some foods and drinks with a high phenolic content and the prevention of some diseases (5–7), whereas the revision carried out by Block and coworkers (8) showed that, of 156 epidemiological studies, 128 stated that the consumption of fruit and vegetables was inversely related to the risk of acquiring cancer. Furthermore, the studies of Steinmetz and Potter (9–11) strongly suggest that vegetable-based diets contain, besides all the traditional nutrients, other substances that reduce the risk of cancer. Among the properties of phenolic compounds, they have been found to protect plants against oxidative damage and may have the same role in humans (12,13). They have a wide range of action, which includes antitumoral, antiviral, antibacterial, cardioprotective, and antimutagenic activities (1,14,15). They may act in different stages of the development of malignant tumors by protecting the DNA from oxidative damage. They inactivate carcinogens by inhibiting the expression of mutagenic genes; they also inactivate the enzymes charged with activating procarcinogens and activate the systems responsible for the detoxification of xenobiotics (16).

Melanoma is a tumor of great significance because it has increased alarmingly among the white population in the last 50 years (17) and is now the 4th most common cancer in Australia and New Zealand and the 10th most common in the United States (18). Although it represents less than 10% of skin cancers, it is responsible for more than 75% of skin cancer-related deaths (19). Surgical intervention is the most effective treatment in its initial phases but is of little use in advanced stages, when a great variety of therapeutic measures have been tried (dacarbazine, alkylating agents, nitrosoureas, etc). However, melanoma presents one of the worst response

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rates to chemotherapy (20) because of resistance phenomena (21), besides the important known side effects. Recent years have seen a substantial increase in studies into the effects of polyphenols on different cancers. The studies related to their possible antiproliferative and antimetastatic capacity in regard to melanoma are of note (22–27).

In this study, we examined the possible cytotoxic action and antiproliferative effect of several groups of polyphenolic compounds (flavanones, flavones, flavonols, procyanidin, and phenolic acids) that are present in multiple foods and the relation between their structure and biological activity shown against melanoma cell lines.

Material and Methods

Cell Cultures

We used 3 cell lines: B16F10 established from a mouse primary melanoma C57BL/6JB57 (from the European Collection of Cell Cultures, Salisbury, UK), SK-MEL-1 from the lymphatic metastasis of a human cutaneous melanoma (ATCC, MD, USA), and the line of nontransformed murine melanocytes Melan-a (kindly provided by Dr. Bennet of St. George's Hospital Medical School, London, UK). The B16F10 and SK-MEL-1 cells were cultured in EMEM and Melan-a in RPMI-160 medium. All media (Gibco, Langley, VA, USA) were supplemented at 10% with fetal bovine serum (Gibco) and streptomycin plus penicillin (100 ug/ml and 100 u/ml, respectively; Sigma Co., Madrid, Spain). Nonesential amino acids were added when culturing SK-MEL-1. In the case of Melan-a, 2 μM TPA (Sigma), a potent tumor promoter, was added to the medium on the day of use. All the processes were carried out in a Cultair ABS type II vertical laminar flow chamber. The melanoma cell lines and Melan-a cultures were kept at 37°C, 98% relative humidity with 5% and 7–10% CO₂ atmosphere, respectively, in a Cytoperm heater.

Phenolic Compounds

Of the 13 phenolic compounds assayed, 9 corresponded to different types of flavonoids with varying hydroxyl and methoxyl substituents and 4 to different phenolic acids (Fig. 1). Eriodictyol, hesperetin, 7,3'-dimethylhesperetin, luteolin, quercetin, and procyanidin were obtained from Furfural Español S.A. (Murcia, Spain). Tangeretin was obtained from Extrasynthèse (Genay, France), and baicalein, myricetin, and gallic, trimethoxybenzoic, sinapinic, and isofeluric acids were obtained from Sigma Co. All phenolic compounds were dissolved in dimethylsulphoxide (DMSO; Sigma Co.) at 10 mM. To determine the maximal stable concentration of flavonoids that can be achieved in vitro, DMSO stock were diluted in phosphate buffered saline (PBS; Sigma), PBS with bovine serum albumin (BSA; Sigma; 20 mg/ml), and ultrapure water (18.2 MΩ, MilliQ-UF-Plus, Millipore, Bed-

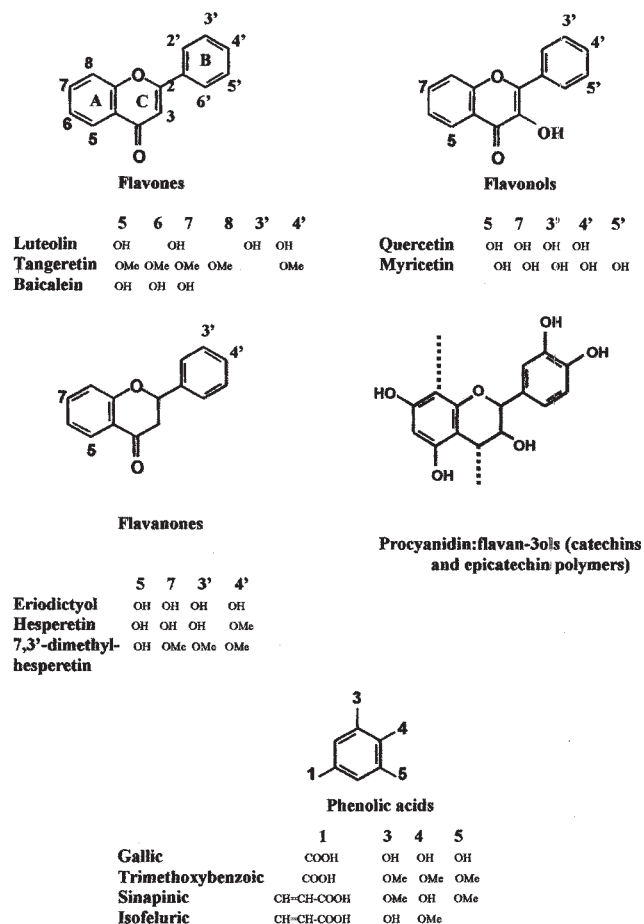


Figure 1. Structure of the different chemical classes of phenolic compounds used in this study: flavones (luteolin, tangeretin and baicalein), flavonols (quercetin and myricetin), flavanones (eriodictyol, hesperetin, and 7,3'-dimethylhesperetin), procyanidin (catechins and epicatechin polymers), and phenolic acids (gallic, trimethoxybenzoic, sinapinic, and isofeluric).

ford, MA, USA). Different dilutions of each compound ranging from 1/5 to 1/500, were centrifuged at 13,000 g (Microfuge-K, Beckman, Fullerton, CA, USA) for at least 20 seconds. The concentrations at which a stable solution was obtained were ascertained from observing the vials where no pellet was formed. The DMSO final concentration was not greater than 1% (v/v) in all experiments.

In Vitro Assay

Two types of assay were carried out: treatment lasting 24 h to evaluate the cytotoxic effect and lasting 72 h to evaluate the antiproliferative effect. For this, a sufficient number of exponentially growing cells to avoid confluence of the culture during the treatments were seeded at 2,500 cells/well (B16F10 and SK-MEL-1) or 5,000 cells/well (Melan-a) to evaluate cytotoxicity; and 100 cells/well (B16F10) or 2,500 (SK-MEL-1 and Melan-a) to evaluate possible antiproliferative effects in Nunc 96-well plates. The treatment started 24 h after seeding (to improve environment ad-

aptation), alter of treatments (24 or 72 h), the medium was replaced, and the cultures were maintained for another 24 h before the cell viability quantification. Control was always treated with the same amount of DMSO as used in the corresponding experiments.

Cell Viability Quantification: MTT Test

The tetrazolium salt MTT works as an indicator of mammalian cell survival and proliferation (28). The assay detects living cells only and is used to measure cytotoxicity and proliferation (29–31). The methods of Carmichael and coworkers (32,33) and Alley and coworkers (34) were adapted to our culture conditions. Briefly, the cultures in 96-well plates were incubated with 200 μ l of fresh supplemented medium and 50 μ l of MTT (8 mg/ml) in the incubator (4 h, 37°C and 5% CO₂). After centrifugation (240 g, 10 min) to carefully remove the medium and nonmetabolized MTT, 100 μ l of DMSO were added to each well to solubilize the MTT formazan produced by the cultured cells. After shaking for 30 min at room temperature, the plates were read with a Multiskan MCC/340P spectrophotometer using 570 nm for the reading and 690 nm for the reference wavelengths.

Data Analysis

In the graphs (Figs. 2–4), each point corresponds to a given flavonoid concentration and is the mean of 4 to 6 measurements. All the experiments were performed in triplicate at least. The bars that appear in some graphs are calculated from the standard error of the mean. A variance analysis of repeated means was carried out to compare the percentages of surviving cells in the cultures with different concentrations of the various compounds complemented by least significant difference to contrast pairs and means. The analyses were carried out by logarithmically transforming the data to comply with analysis of variance conditions.

The IC₅₀ values were obtained from the curve fitted to the means of the absorbance quotients with respect to the control.

Results

Depending on the results obtained in the solubility assays, the solutions of the different phenolic compounds were prepared in: PBS (baicalein, myricetin, sinapinic, and gallic and trimethoxybenzoic acids), PBS with BSA (hesperetin, 7,3'-dimethylhesperetin, eriodictyol, tangeretin, luteolin, and quercetin), or water (procyanidins and isofeluric acid) according to the maximal stable concentrations obtained. The phenolic compounds were tested in concentrations ranging from 3.12 to 50 μ M. Concentrations above 50 μ M could not be tested because of the poor solubility of some compounds. Cell viability is expressed as the percentage of survival compared with the control.

Cytotoxic Effects

To evaluate the cytotoxic effect on the melanoma cells, we carried out a series of 24-h assays with the different compounds, the results pointing to moderate or no effect (Table 1). All the 13 compounds did not show significant cytotoxic effects at lower concentrations. In the case of B16F10, only the flavones, tangeretin, and luteolin had a significant effect ($P < 0.05$) on cell growth between the minimum (3.12 μ M) and the maximum (25 and 50 μ M) concentrations used. These same flavones (tangeretin and, to a lesser extent, luteolin) had a similar effect on the SK-MEL-1 cell line. The results obtained with Melan-a were more variable. The trihydroxylated flavonoids, baicalein and myricetin, affected cell growth at concentrations above 12.5 μ M. Myricetin had a greater cytotoxic effect; it was significantly different from that of the other phenolic compounds assayed.

Antiproliferative Effects

To evaluate the antiproliferative activity, the assays lasted 72 h because, besides any inhibition of growth caused by cytotoxicity, other situations may arise at longer times, such as the quiescence, metabolic stopping, or induction of apoptosis. The melanoma cells were more sensitive to the methoxylated compounds and, as occurred in the 24-h treatments, B16F10 was more sensitive than SK-MEL-1. In general, the Melan-a cells were the most sensitive to treatment with the phenolic compounds, especially in the case of the trihydroxylated compounds.

Our results (Table 1) showed that the flavones tangeretin and baicalein and the flavonols myricetin and gallic acid had the greatest effect on cell growth, except in the SK-MEL-1 cells, in which none of the compounds assayed had a significant inhibitory effect. The flavanone 7,3'-dimethylhesperetin, the flavone luteolin, and the flavonol quercetin had a moderate effect; the flavanones eriodictyol and hesperitin, procyanidin and trimethoxybenzoic, and sinapinic and isofeluric acids had no significant effect on cell growth in any of the 3 cell lines treated.

With regard to the effect of the flavones in the 72-h assay, there was a clear dose-response curve in the case of the B16F10 cell (Fig. 2), with tangeretin having the greatest effect. Treatment with baicalein showed significant differences ($P < 0.05$) between the different concentrations assayed. Of the flavonols assayed, myricetin had the best response, which was significantly different ($P < 0.05$) from the response obtained with quercetin. The inhibitory response of gallic acid is represented by a sigmoid curve and was significantly different ($P < 0.05$) from the response obtained with the other compounds assayed, except with tangeretin. Of the 3 flavanones assayed, only 7,3'-dimethylhesperetin at 50 mM had a significant inhibitory effect on cell growth.

None of the compounds had a marked effect on the cell growth of SK-MEL-1 (Fig. 3), although, as in the case of the other melanomas studied, the flavones tangeretin and

Table 1. Cytotoxicity and Antiproliferative Effects of Selected Polyphenolic Compounds on B16F10, SK-MEL-1 and Melan-a Cell Cultures^a

Polyphenolic Compounds	B16F10				SK-MEL-1				Melan-a			
	24 h		72 h		24 h		72 h		24 h		72 h	
	(1)	(2)	(1)	(3)	(1)	(2)	(1)	(3)	(1)	(2)	(1)	(3)
Eriodictyol	109.21	127.45	124.99	>50	90.35	104.85	97.99	>50	99.32	83.11	86.61	>50
Hesperetin	108.41	111.36	142.07	>50	96.99	94.88	159.21	>50	100.85	92.16	93.79	>50
7,3'-Dimethylhesperetin	101.22	127.26	47.64	50	86.38	98.53	69.73	>50	119.46	97.08	80.25	>50
Luteolin	70.23	128.73	78.54	>50	76.66	105.39	79.37	>50	69.37	87.54	35.43	23.4
Tangeretin	57.18	87.55	23.07	11.2	67.64	106.92	67.68	>50	101.13	97.99	78.68	>50
Baicalein	84.43	95.20	29.31	33.13	94.59	88.72	64.75	>50	51.93	93.87	9.87	5.84
Quercetin	82.57	104.11	66.64	>50	87.09	98.89	90.2	>50	110.65	98.57	51.03	63.9
Myricetin	81.33	85.81	37.18	38.34	126.03	122.24	79.46	>50	34.42	112.64	7.6	21.9
Procyanidin	97.00	97.60	95.98	>50	101.49	88.49	105.8	>50	98.11	98.62	73.03	>50
Gallic acid	74.61	76.25	21.01	13.45	106.2	98.39	106.73	>50	63.64	105.07	0.54	30
Isofeluric acid	92.74	100.31	80.14	>50	76.2	71.50	82.48	>50	96.16	90.14	82.27	>50
Sinapinic acid	108.04	108.43	84.42	>50	119.73	111.81	98.16	>50	88.3	83.99	66.17	>50
Trimethoxybenzoic acid	88.67	79.58	90.6	>50	87.97	85.80	105.68	>50	98.53	97.29	103.15	>50

a: (1), percent surviving at 50 μ M with respect to the control. Values are the mean of 4 to 6 measures. (2), percent surviving at 3.12 μ M with respect to the control. Values are the mean of 4 to 6 measures. (3), IC₅₀ value (μ M). They were obtained from the curve fitted to the means of the absorbance quotients with respect to the control.

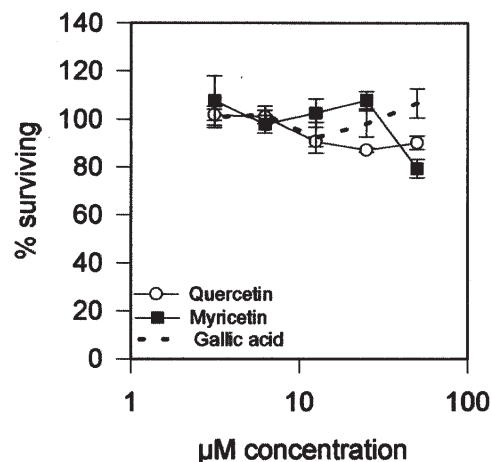
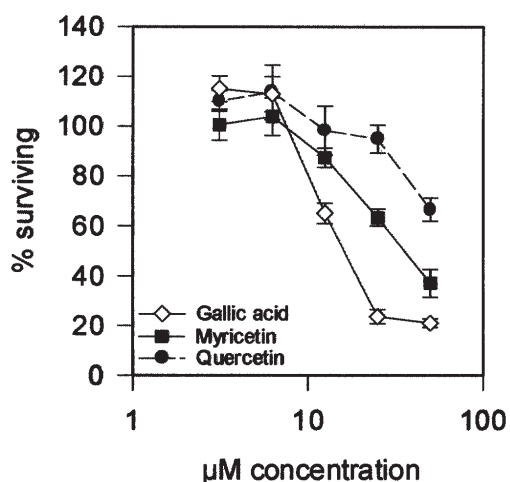
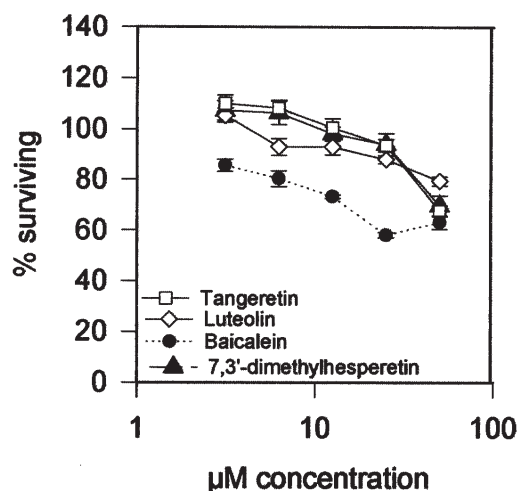
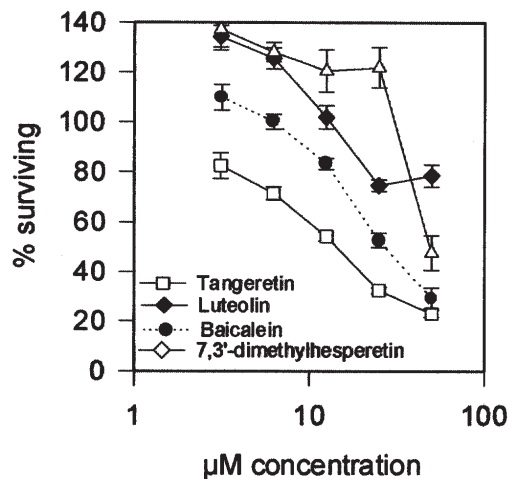


Figure 2. Cell viability of B16F10 cells after 72 h of treatment with the flavones tangeretin, luteolin, and baicalein; the flavonols quercetin and myricetin; the flavanone 7,3'-dimethylhesperetin; and gallic acid. The survival was determined with respect to the control by MTT assay. Each point corresponds to a given flavonoid concentration (from 3.12 to 50 μM) and is the mean (\pm SEM where shown) of 4 to 6 measurements.

Figure 3. Cell viability of SK-MEL-1 cells after 72 h of treatment with the flavones tangeretin, luteolin, and baicalein; the flavonols quercetin and myricetin; the flavanone 7,3'-dimethylhesperetin; and gallic acid. Survival was determined with respect to the control by MTT assay. Each point corresponds to a given flavonoid concentration (from 3.12 to 50 μM) and is the mean (\pm SEM where shown) of 4 to 6 measurements.

baicalein showed the greatest antiproliferative activity. Tangeretin showed a significant difference ($P < 0.01$) among the concentrations of 50, 25, and 12.5 μM. As occurred with the B16F10 cells, 7,3'-dimethylhesperetin inhibited growth at the highest concentration, with significant differences ($P < 0.01$) among the results obtained with the different concentrations.

In the case of Melan-a, the polyhydroxylated compounds (gallic acid, baicalein, myricetin, and luteolin) had the greatest effect, all showing a dose-response curve (Fig. 4). Of the 3 flavones assayed, baicalein and luteolin showed a high response at 72 h of treatment and significant differences ($P < 0.05$) among different concentrations. With regard to treatment with flavonols, myricetin had the best effect, with significantly different ($P < 0.05$) results from those obtained with quercetin. Of the phenolic acids assayed, gallic acid significantly inhibited cell growth.

Discussion

We conducted an *in vitro* study of the cytotoxic and antiproliferative activity of 13 phenolic compounds (9 flavonoids with different hydroxyl and methoxyl substituents and 4 phenolic acids), whose structures are depicted in Fig. 1, used on 3 types of melanocyte cells: 2 melanomas (the murine B16F10 and the human SK-MEL-1) and 1 nonneoplastic mouse melanocyte cell line.

The 3 cell lines showed differing degrees of sensitivity to the compounds assayed. The B16F10 was the most sensitive, confirming previous observations made by our group (35). The lower resistance of these cells and the greater resistance of SK-MEL-1 seem to be related to the characteristics of each line and perhaps their origin because the former corresponds to a primary tumor culture and the latter to a metastatic cell.

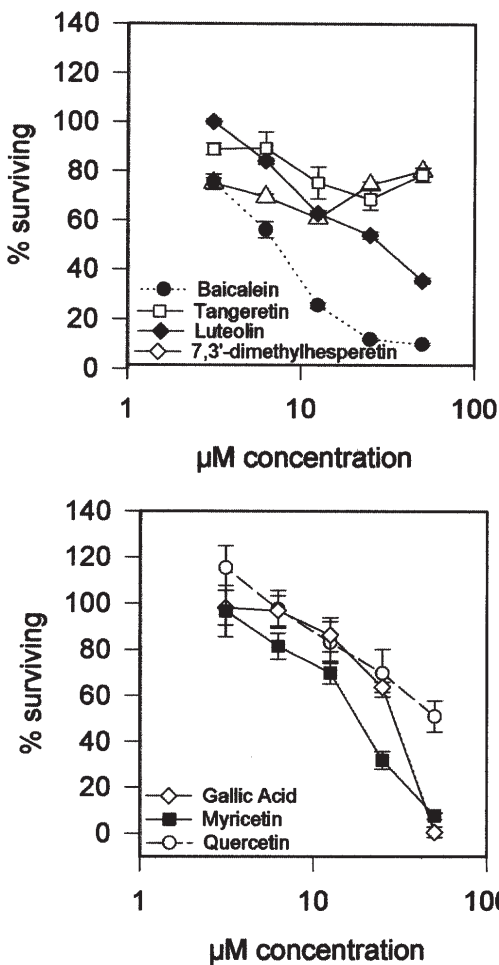


Figure 4. Cell viability of Melan-a cells after 72 h of treatment with the flavones tangeretin, luteolin, and baicalein; the flavonols quercetin and myricetin; the flavanone 7,3'-dimethylhesperetin; and gallic acid. Survival was determined with regard to control by MTT assay. Each point corresponds to a given flavonoid concentration (from 3.12 to 50 μM) and is the mean (\pm SEM where shown) of 4 to 6 measurements.

The cytotoxicity of the polyphenols assayed was moderate or null except in the case of baicalein and myricetin on Melan-a. These findings agree with other studies that evaluate the cytotoxic effect of certain flavonoids, such as those of Kuntz and coworkers (36), who studied the effect of 36 flavonoids on 2 colon cancer cell lines (HT-29 and Caco-2), concluding that almost all had an antiproliferative effect and showed no cytotoxicity. In another study, Sánchez and coworkers (37) demonstrated the low cytotoxicity of 4 polymethoxylated flavones that had an antiproliferative effect on 2 animal tumor cells (LLC-MK2 and C6).

With regard to the activity of the phenolic compounds assayed in this experiment on the inhibition of cell growth, tangeretin was the most effective flavonoid on the B16F10 cells, followed by gallic acid, baicalein, myricetin, 7,3'-dimethylhesperetin, quercetin, and luteolin. On Melan-a, on the other hand, gallic acid was the most effective flavonoid, followed by baicalein, myricetin, luteolin, quercetin, and tangeretin. In the bibliography consulted on

the antineoplastic activity of flavonoids, the most used cell lines have been carcinoma of the thyroid (38), breast (39–42), and cervix (43), and non-small-cell lung carcinoma (44,45); however, few studies have been carried out with melanoma cultures (25,46–49) and most of them are exclusively related to metastatic processes (22–24,50).

A previous study of ours on treating B16F10 and SK-MEL-1 melanoma cells (48) showed the flavone tangeretin (tetramethoxylated in the A ring) to be the most effective flavonoid of those assayed in inhibiting cell growth in vitro in both lines. We have found no literature on the effect of tangeretin on melanoma cell lines, although some studies do exist on the activity of this flavonoid (36,51,52). With regard to gallic acid, which in our study had a highly antiproliferative effect on the B16 and Melan-a lines, Ohno and coworkers (53) had already indicated that this compound inhibited the proliferation of tumor cells, among which parental B16 cells were included.

As for any structure-activity relationship, previous studies suggest that the position, number, and substitution of the hydroxyl of the A and B rings may be important factors affecting cytotoxic and/or antiproliferative activities of these polyphenols (37,54–58). The results obtained in our experiments with melanoma lines (B16F10 and SK-MEL-1) and nonneoplastic melanocytes (Melan-a) add weight to the idea of there being a structure-antiproliferative activity relationship.

Figure 5 shows a scheme of oxidation sequential that permits ordering of the structures of the different flavonoids tested and establishment of a relationship between each specific structural modification with their effect on cytotoxic and/or antiproliferative activities. Several concepts are combined in this figure: a comparison of the flavanone, flavone, and flavonol skeletons, and the number of substituents on the A and B rings and their nature (free hydroxyls or methylated).

The first mentioned comparison confirms that the presence of a double C2-C3 bond in polyhydroxylated flavonoids increases the antiproliferative activity in the 3 cell lines used, as can be seen from the results obtained with luteolin compared with those obtained with eriodyctiol. This greater activity of the flavones compared with the flavanones was also observed in studies by Agullo and coworkers (54) and Fotsis and coworkers (55) in other cell lines. Flavonols, like flavones, are flat structures with a characteristic 3-hydroxyl substituent. However, the activity shown by luteolin was similar (in B16F10) and even more pronounced (in Melan-a) than that of quercetin, its corresponding flavonol, demonstrating that in these cell lines, at least, the 3-hydroxylation on the flavone nucleus does not confer a greater inhibitory effect.

Another structural element that may influence antiproliferative activity is the number and position of the substituents in the flavonoid base skeleton. Eriodyctiol (flavanone), luteolin (flavone), and quercetin (flavonol) have a catechol structure (O-hydroxy) in its B ring. Taking quercetin as an example, the presence of a new hydroxyl in this ring leads to the myricetin structure (flavonol). The greater activity of this flavonol compared with that of

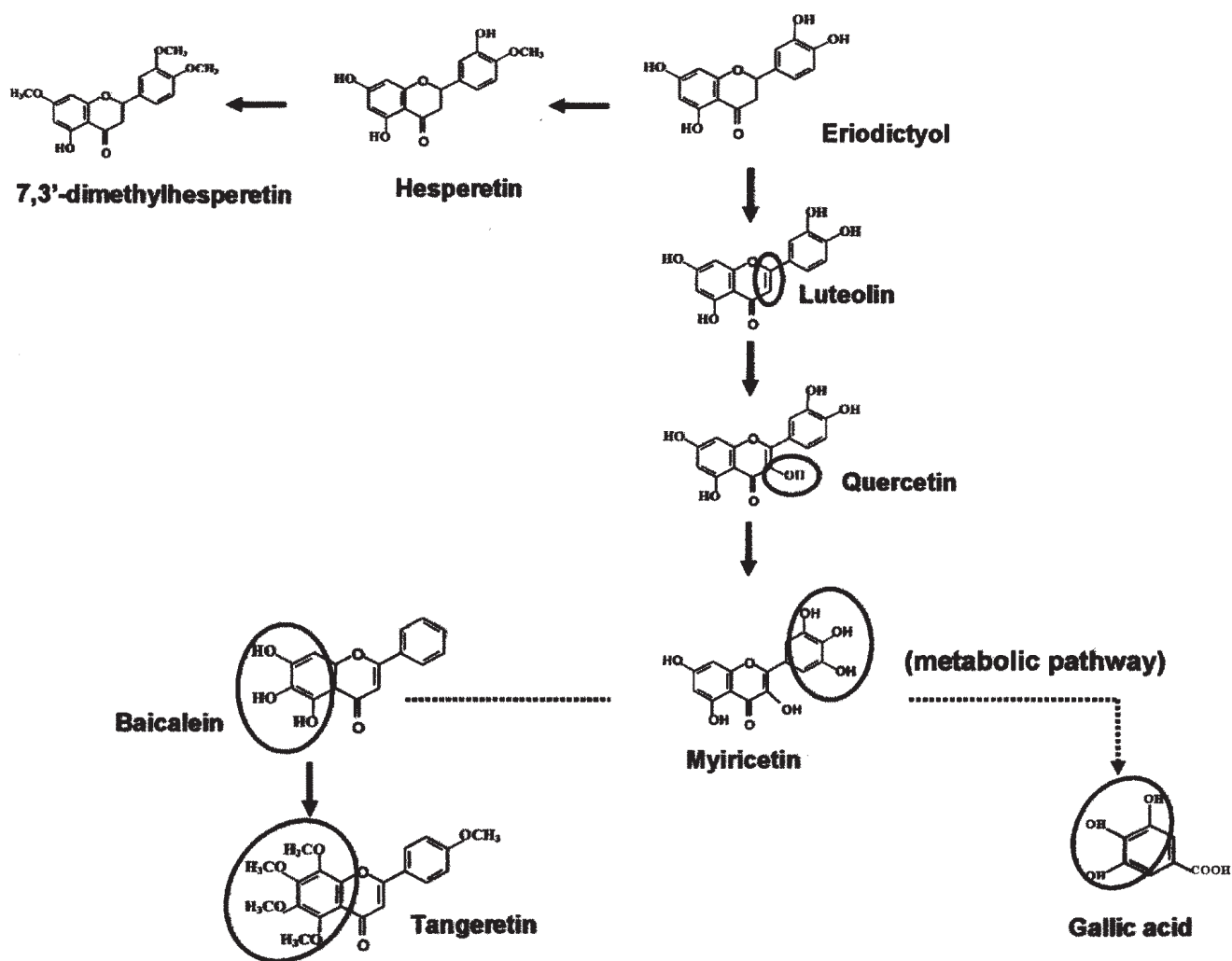


Figure 5. Schematic representation of the sequential oxidation and chemical structures of gallic acid and the 8 flavonoids discussed in this article: 7,3'-dimethylhesperetin, hesperetin, eriodictyol, luteolin, quercetin, myricetin, baicalein, and tangeretin.

quercetin and luteolin on the B16F10 and Melan-a cell lines suggests that the presence of at least 3 adjacent hydroxyl groups confers much greater antiproliferative power, an observation also made by Agullo and coworkers (59). In this sense, the results obtained with the flavone baicalein (trihydroxylated in the A ring) confirm the previously mentioned statements, namely that 3 adjacent hydroxyl groups in an aromatic nucleus confer a strong antiproliferative effect, whether situated in the A ring (as in baicalein) or in the B ring (myricetin) of the flavonoid skeleton.

With regard to the nature of the substituents, methylation of the hydroxyls does not reduce the antiproliferative capacity, and even appears to increase it, because the activity shown by 7,3'-dimethylhesperetin was higher than that shown by the other 2 flavanones, hesperetin and eriodictyol (37), suggesting that the presence of a methoxy group in position C-4 may be related to greater cytostatic activity. Furthermore, in both the melanoma lines, the flavone tangeretin (tetrahydroxylated in the A ring) had a greater antipro-

liferative effect than baicalein (trihydroxylated in the A ring). With regard to gallic acid, which displayed a strong antiproliferative effect on B16 and Melan-a, our results confirm those of Ohno and coworkers (60) and Qiu and coworkers (61), who suggested that such effects are due to its hydroxylated aromatic structure and not to the presence of the carboxyl group because trimethoxybenzoic acid showed no such effect on the inhibition of cell growth.

Nowadays, flavonoids are considered potentially important constituents of the human diet for the chemoprevention of cancer because of their biological activities at the cell level, and several hypotheses have been proposed to explain their antitumoral activities. These include their potential cytotoxic activity, the inhibition of cell proliferation, and their effect on cell differentiation and angiogenesis processes (62). Various mechanisms have been proposed to explain these hypotheses in different murine and human melanoma lines, such as their anti-invasive and antimetastatic potential (50), the induction of apoptosis by negative retroregulation

of the protein Bcl-2 or inhibition of the transmembrane transport of glucose and the promotion of Bax expression in both cases (26), the inhibition of the kinases CDK1 and CDK2 by the positive retroregulation of inhibitors of p27 and p21, or phosphorylation of the residues Tyr 15 of kinase (46).

Obviously, further studies are necessary to elucidate the cellular mechanism underlying the antiproliferative effects of these polyphenolic compounds on the growth and invasive potential of melanoma cell lines. However, our results using 2 melanoma and 1 nonneoplastic melanocyte cell lines clearly show that there is a direct correlation between the structural oxidation state and antiproliferative effects of the polyphenolic compounds used.

Acknowledgments and Notes

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