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REVIEWS

Update on Uses and Properties of *Citrus* Flavonoids: New Findings in Anticancer, Cardiovascular, and Anti-inflammatory Activity

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Significantly, much of the activity of *Citrus* flavonoids appears to impact blood and microvascular endothelial cells, and it is not surprising that the two main areas of research on the biological actions of *Citrus* flavonoids have been inflammation and cancer. Epidemiological and animal studies point to a possible protective effect of flavonoids against cardiovascular diseases and some types of cancer. Although flavonoids have been studied for about 50 years, the cellular mechanisms involved in their biological action are still not completely known. Many of the pharmacological properties of *Citrus* flavonoids can be linked to the abilities of these compounds to inhibit enzymes involved in cell activation. Attempts to control cancer involve a variety of means, including the use of suppressing, blocking, and transforming agents. Suppressing agents prevent the formation of new cancers from procarcinogens, and blocking agents prevent carcinogenic compounds from reaching critical initiation sites, while transformation agents act to facilitate the metabolism of carcinogenic components into less toxic materials or prevent their biological actions. Flavonoids can act as all three types of agent. Many epidemiological studies have shown that regular flavonoid intake is associated with a reduced risk of cardiovascular diseases. In coronary heart disease, the protective effects of flavonoids include mainly antithrombotic, anti-ischemic, anti-oxidant, and vasorelaxant. It is suggested that flavonoids decrease the risk of coronary heart disease by three major actions: improving coronary vasodilatation, decreasing the ability of platelets in the blood to clot, and preventing low-density lipoproteins (LDLs) from oxidizing. The anti-inflammatory properties of the *Citrus* flavonoids have also been studied. Several key studies have shown that the anti-inflammatory properties of *Citrus* flavonoids are due to its inhibition of the synthesis and biological activities of different pro-inflammatory mediators, mainly the arachidonic acid derivatives, prostaglandins E_2, F_2, and thromboxane A_2. The anti-oxidant and anti-inflammatory properties of *Citrus* flavonoids can play a key role in their activity against several degenerative diseases and particularly brain diseases. The most abundant *Citrus* flavonoids are flavanones, such as hesperidin, naringin, or neohesperidin. However, generally, the flavones, such as diosmin, apigenin, or luteolin, exhibit higher biological activity, even though they occur in much lower concentrations. Diosmin and rutin have a demonstrated activity as a venotonic agent and are present in several pharmaceutical products. Apigenin and their glucosides have been shown a good anti-inflammatory activity without the side effects of other anti-inflammatory products. In this paper, we discuss the relation between each structural factor of *Citrus* flavonoids and the anticancer, anti-inflammatory, and cardiovascular protection activity of *Citrus* flavonoids and their role in degenerative diseases.

KEYWORDS: Flavonoid; *Citrus*; anti-oxidant; anticarcinogenic; anti-inflammatory; anti-aging; platelet aggregation; cardiovascular diseases; brain diseases

INTRODUCTION

Flavonoids are part of a family of naturally occurring polyphenolic compounds characterized by a common benzo-γ-pyrone structure. They are one of the most important compounds present in vegetables, especially in the genus *Citrus* (family Rutaceae) (*1*). More than 8000 compounds with a flavonoid structure have been identified. This large number arises from the various combinations of multiple hydroxyl, methoxyl, and *O*-glycoside group substituents on the basic benzo-γ-pyrone ($C_6-C_3-C_6$) (*2*).

Four types of flavonoids (flavanones, flavones, flavonols, and anthocyanins, the last only in blood oranges) occur in *Citrus*. In this genus, flavanones are accumulated in greater quantity

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than flavones. The concentration of these compounds depends upon the age of the plant, and the highest levels are detected in tissues showing pronounced cell divisions (*3–7*). These compounds not only play an important physiological and ecological role but are also of commercial interest because of their multitude of applications in the food and pharmaceutical industries (*1, 8–11*).

Significantly, much of the activity of *Citrus* flavonoids appears to impact blood and microvascular endothelial cells, and it is not surprising that the two main areas of research on the biological actions of *Citrus* flavonoids have been inflammation and cancer (*12*).

Epidemiological and animal studies point to a possible protective effect of flavonoids against cardiovascular diseases and some types of cancer. Although flavonoids have been studied for about 50 years, the cellular mechanisms involved in their biological action are still not completely known (*13*). Many of the pharmacological properties of *Citrus* flavonoids can be linked to the abilities of these compounds to inhibit enzymes involved in cell activation. *In vitro*, flavonoids have demonstrated their capacity to modify the activity of enzymatic systems in mammals (kinases, phospholipases, ATPase, lipooxygenases, cyclooxygenases, phosphodiesterases, etc.), a correlation having been observed in some cases between the flavonoid structure and its enzymatic activity (*1, 14–18*). Much of these effects can be attributed to the abilities of flavonoids to interact with the nucleotide-binding sites of regulatory enzymes (*12*). Research has also shown that *Citrus* flavonoids are potent radical scavengers and, thus, able to help in many aging and degenerative events involving reactive oxygen species (*1*).

In this paper, the discussion focuses on the main health-related properties of flavonoids, which are based in their anti-oxidant activity and their ability to modulate some key regulatory enzymes. These properties have been found to include anticancer, anti-inflammatory, cardiovascular protection, effects on platelet aggregation, and effects on brain diseases.

ANTICARCINOGENIC PROPERTIES

Cancer may be controlled by a variety of means, including suppression, blockage, and transformation. Suppressing agents prevent the formation of new cancers from procarcinogens; blocking agents prevent carcinogenic compounds from reaching critical initiation sites; and transformation agents act to facilitate the metabolism of carcinogenic components into less toxic materials or prevent their biological actions. Flavonoids can act in all three ways (*12*).

Flavonoids may act in the different development stages of malignant tumors by protecting DNA against oxidative damage, inactivating carcinogens, inhibiting the expression of the mutagenic genes and enzymes responsible for activating procarcinogenic substances, and activating the systems responsible for xenobiotic detoxification (*16*). Within the past decade, reports on flavonoid activities have been largely associated with enzyme inhibition and antiproliferative activity. Many of these studies have shown a structural−functional relationship, demonstrating that anti-oxidant, enzyme-inhibition, or antiproliferative activities of flavonoids are dependent upon particular structural motifs (*19–23*). Other studies have shown that structural factors would explain the anti-oxidant, antiproliferative, and antimetastasic properties of some *Citrus* flavonoids (*17, 18, 24, 25*).

Important structural factors that may condition flavonoid activity are structure oxidation stage (flavanone, flavone, etc.), substituents (position, number, and nature of groups in both the A and B ring of the flavonoid structure), and the presence of glycosylation (*1, 21, 26*).

A. Antiproliferative Effects. Although most flavonoids appear to be nontoxic to humans and animals, they have been demonstrated to inhibit proliferation in many kinds of cancerous cell lines. It has been reported that *Citrus* flavonoids (tangeretin, nobiletin, quercetin, and taxifolin) (*27*) have antiproliferative effects on squamous cell carcinoma HTB43. Quercetin at 10 μM has demonstrated its antiproliferative activity against meningioma cells (*28*) and against colon cancer cells, Caco-2 and HT-29, with a dose-dependent effect (*29*). Diosmin, another important *Citrus* flavonoids, which is in pharmacy as a venotonic, has shown antiproliferative activity in Caco-2 and HT-29 colon cancer cell lines ($IC_{50} = 203$ μM), although with less efficacy than quercetin (*30*).

Flavonoids also have shown inhibitory effects on the growth of leukemia HL-60 cell, with an IC_{50} value ranging from 10 to 940 ng/mL in a nontoxic mechanism (*31*), which are almost equivalent to the effects of currently used anticancer agents. In more recent trials, *Citrus* flavones have shown their ability to inhibit the proliferation of MDA-MB-435 and MCF-7 human breast cancer cells at low IC_{50} (*21*). In another study, 27 *Citrus* flavonoids were examined for their antiproliferative activities against several tumor and normal cell lines. As a result, seven flavonoids were judged to be active against the tumor cell lines, including lung carcinoma A549 and gastric TGBC11TKB cancer cells, while they did not significantly affect the proliferation of normal cell lines (*19*). Most studies have found that the IC_{50} for the inhibition of cell proliferation by active flavonoids was in the low micromolar range, physiologically available concentrations (*32*).

Results on different melanoma cell lines have demonstrated the antiproliferative effects of flavonoids against these cancerous cells and no cytotoxic effects (*17, 18*). These results point to a correlation between antiproliferative activity and flavonoid structure. More specifically, preliminary studies on melanoma lines B16F10 and SK-MEL-1, using several flavonoids of a *Citrus* origin, showed that the presence of the C_2-C_3 double bond on the C ring, conjugated with the 4-oxo function, was critical for this biological activity (*17*). Subsequent studies have shown that the presence of three or more hydroxyls in any of the rings of the flavonoid skeleton significantly increased the antiproliferative activity observed in B16F10 cell cultures (*18*). More recently, an *in vitro* study on the cytotoxic and antiproliferative activity of 13 structurally different phenolics compounds and phenolic acids (**Figure 1**), three types of melanocyte cell, two melanomas (the murine B16F10 and human SK-MEL-1), and one non-neoplasic mouse melanocyte cell line, Melan-a, was performed (*26*).

The three cell lines used showed differing degrees of sensitivity to the compounds (**Table 1**), with the B16F10 being the most sensitive, confirming previous observations made by our group (*17*). The lower resistance of these cells and the greater resistance of SK-MEL-1 seem to be related to the particular characteristics of each line and the origin and invasive behavior of cell lines, with SK-MEL-1 being the most invasive cell line.

The cytotoxicity of the polyphenols assayed was moderate or null, except for baicalein and myricetin on Melan-a. These findings agree with other studies that evaluated the cytotoxic effect of certain flavonoids, such as those of Kuntz et al. (*30*) on HT-29 and Caco-2

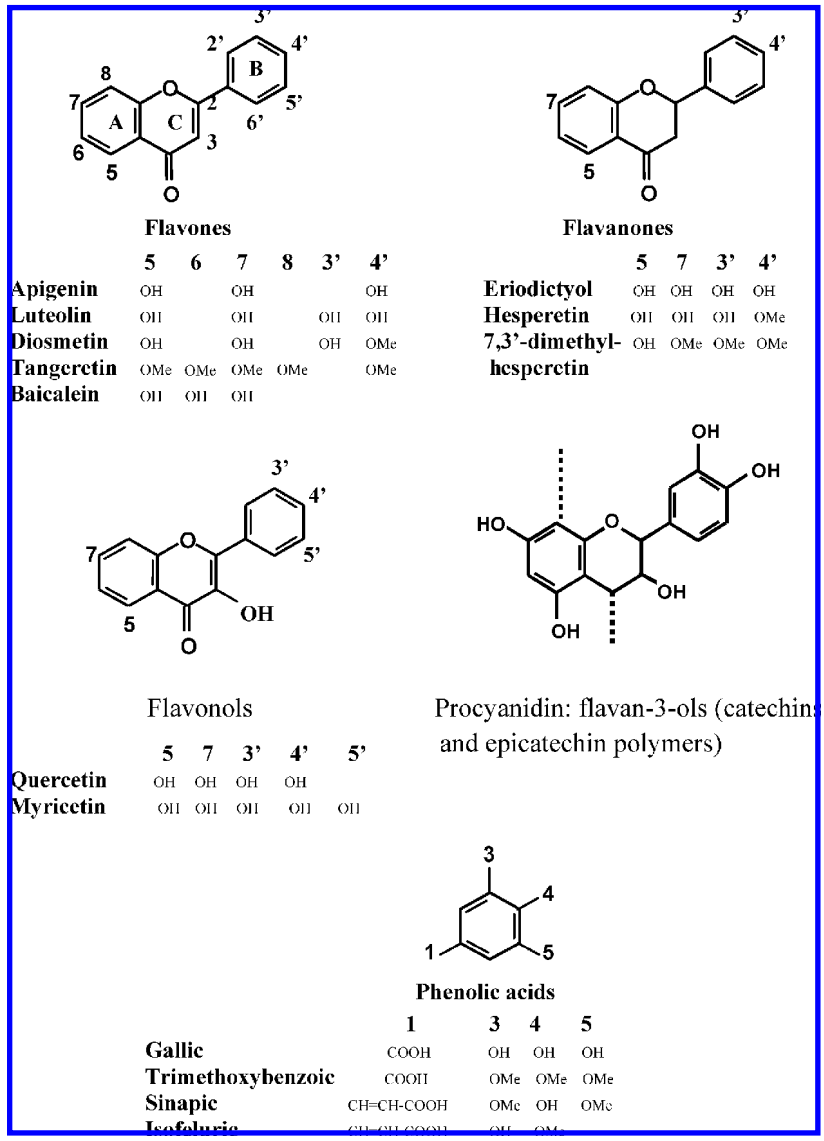

Figure 1. Structure of the different chemical classes of phenolic compounds used in the antiproliferative assay.

Table 1. Cytotoxicity and Antiproliferative Effects of Selected Phenolic Compounds in B16F10, SK-MEL-1, and Melan-a Cell Cultures

	B16F10				SK-MEL-1				Melan-a			
	24 h		72 h		24 h		72 h		24 h		72 h	
	a	*b*	*a*	*c*	*a*	*b*	*a*	*c*	*a*	*b*	*a*	*c*
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
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
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
sinapic 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] Percent surviving at 50 μM. [b] Percent surviving at 3.12 μM. [c] IC_{50} value (in micromolars).

colon cancer cell lines. Sanchez et al. (*33*) also demonstrated the low cytotoxicity of four polymethoxyflavones that had an antiproliferative effect on two animal tumor cells (LLC-MK2 and C6).

On the effect of phenolic compounds assayed on the inhibition of cell growth, tangeretin (**Figure 1**) was the most effective flavonoid on the B16F10 cells, followed by gallic acid, baicalein, myricetin, 7,3′-dimethylhesperetin, quercetin, and luteolin. On Melan-a, gallic acid was the most effective compound, followed by baicalein, myricetin, luteolin, quercetin, and tangeretin (**Table 1**).

A previous study on treating B16F10 and SK-MEL-1 melanoma cells (*17*) showed the flavone tangeretin (tetramethoxylated in the A ring) to be the most effective flavonoid of those assayed for inhibiting *in vitro* cell growth in both lines. Recent studies on the effect of tangeretin on the SK-MEL-5 melanoma cell line (*21*) confirm these results. The effects of tangeretin on other cancer cell lines, such as HL-60 leukemia cells (*34, 35*), human hepatoma Hep G2, Hep 3B, and PLC/PRF/5 cells (*36*), and human lung DMS-14, colon HT-29, breast MCF-7 and MDA-MB-435, and prostate DU-145, agree with those reported for melanoma cell lines (*21*).

As for any structure−activity relationship, previous studies suggest that the position, number, and substitution of the hydroxyl in the flavonoid A and B rings may be important factors affecting cytotoxic and/or antiproliferative activities of these polyphenols (*19, 32, 33, 35, 37, 38*). Our results on melanoma cell lines add weight to the idea of there being a structure−antiproliferation relationship.

Several flavonoids were studied to establish a relationship between each specific structural modification and its effect on cytotoxic and/or antiproliferative activities. Several concepts are combined in this study: a comparison of the flavanone, flavone, and flavonol structures and the number of substituents on the A and B rings and their nature (free hydroxyls or methylated) (**Figure 1**).

The first comparison confirmed that the presence of a double C_2-C_3 bond in polyhydroxylated flavonoids increased the antiproliferative activity in the three cell lines used, as can be seen from the results obtained for luteolin compared to those obtained with eriodictyol. This greater activity of flavones compared to flavanones is a general observation in all of the most important lung, colon, breast, prostate, and melanoma cancerous cell lines (*17, 21, 35, 37*). Flavonols, such as flavones, are flat structures with characteristic 3-hydroxyl substituents. However, the activity shown by luteolin was similar (in B16F10 and SK-MEL-1) and even more pronounced (in Melan-a) than that of quercetin, its corresponding flavonol, demonstrating that, in these cell lines, the 3-hydroxylation on the flavone nucleus does not confer a greater inhibitory effect. This result was confirmed again by Manthey and Guthrie in lung, colon, breast, prostate, and melanoma cell lines (*21*) and Casagrande and Darbon (*20*) in the melanoma OCM-1 cell line. However, Agullo et al. (*37*), using the same colonic adenocarcinoma HT-29 cell line as Manthey, found a stronger inhibitory effect for quercetin than for luteolin.

Another structural element that may influence antiproliferative activity is the number and position of the substituents in the flavonoid base structure. Eriodictyol (flavanone), luteolin (flavone), and quercetin (flavonol) have a catechol structure (*O*-hydroxyl) in their B ring. Taking quercetin as an example, the presence of a new hydroxyl in this ring leads to another flavonol structure, myricetin. The greater activity of this flavonol compared to that of quercetin and luteolin on the B16F10 and Melan-a cell lines suggest that the presence of at least three adjacent hydroxyl groups confers much greater antiproliferative power, an observation also made by Agullo et al. (*39*). In this sense, the results obtained with the flavone baicalein (trihydroxylated in the A ring) confirm the previously mentioned statement, namely, that three adjacent hydroxyl groups in an aromatic nucleus confer a strong antiproliferative effect (*40*), whether situated in the A (as baicalein) or B (as myricetin) ring 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 two flavanones, hesperetin and eriodictyol, suggesting that the presence of a methoxyl 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 antiproliferative effect than baicalein (trihydroxylated in the A ring). The highest antiproliferative effects of tangeretin are again in agreement with the results obtained by Manthey and Guthrie (*21*) in six human cancer cell lines, Kandaswami et al. (*27*) in squamous cell carcinoma HTB43, and Sugiyama et al. (*41*) and Hirano et al. (*34*) in human HL-60 leukemia cell lines. The less polar and planar structure of tangeretin may play a critical role in its biological activity, enhancing its permeability to biological membranes and its binding properties (*21*).

With regard to gallic acid (a myricetin metabolite), which displayed a strong antiproliferative effect on B16F10 and Melan-a, results in this study confirm those of Ohno et al. (*42*) and Qiu et al. (*43*), who suggested that such effects are due to its hydroxylated aromatic structure and not to the presence of a carboxyl group, because trimethoxybenzoic acid showed no such effect on the inhibition of cell growth.

Various mechanisms have been proposed to explain the antiproliferative activity of flavonoids. Flavonoids have been shown to inhibit several kinases involved in signal transduction, such as protein kinases C, tyrosine kinases, PI 3-kinases, or S6 kinase (*20*). They can also interact with estrogen type-II binding sites (*44, 45*). Flavonoids have been found to arrest cell-cycle progression at either G1/S or G2/M boundaries. However, the molecular mechanisms involved in cell-cycle arrest by flavonoids remain largely unclear, although the modulation of multiple cell-cycle regulatory proteins seems to be involved. The eukaryotic cell cycle is regulated through the sequential activation and inactivation of cyclin-dependent kinases (Cdks) that drive cell-cycle progression through the phosphorylation and dephosphorylation of several regulatory proteins (*46*). Progression from the G1 to S phase in mammalian cells is regulated by the accumulation of cyclins D, E, and A, which bind to and activate different Cdk catalytic subunits. The transition from the early to mid G1 phase is regulated by the Cdk4−cyclin D and/or Cdk6−cyclin D complex. Transition from the mid G1 to S phase is regulated by activation of the Cdk2−cyclin E complex. Progression from the late G1 to S phase also required the presence of the Cdk2−cyclin A complex (*47*). G2/M transition is regulated by the Cdk1−cyclin A/B complex.

Recent studies have established a structure−activity relationship for the inhibition of Cdks by flavonoids. Casagrande and Darbon (*20*) demonstrated that a C ring with an oxo function at position 4, a C_2-C_3 double bond, and a catechol group at the 3′ and 4′ positions was required for maximal inhibition of Cdk1 and Cdk2. Interestingly, a similar structure requirement was demonstrated with regard to the inhibition of protein kinase C (PKC) and PI-3-kinase (*39*). Previous studies showed that many flavonoids, such as apigenin, kaempferol, or genistein, induce G2/M arrest in several cell lines (*48, 49*). On the other hand, flavonoids, such as tangeretin, induce G1 arrest in human colorectal carcinoma COLO 205 cells (*46*). These results suggest that the arrest of the cell cycle by flavonoids or tangeretin is an effect that is dependent upon the structural groups of these agents. The induction of G1 arrest in colorectal carcinoma COLO 205 cells is explained by tangeretin inhibition of Cdk2 and Cdk4 in a dose-dependent manner (*46*). Tangeretin also

increased the content of the Cdk inhibitors p21 and p27 protein, and this effect correlated with an increase in p53 levels.

The same effect on G1 arrest has been found for quercetin and luteolin in human melanoma OCM-1 cell lines (*21*). This study showed that flavonoids with 3′- 4′-catechol groups in the B ring inhibit Cdk2 activity and correlated to a G1 cell-cycle arrest. The upregulation of Cdk inhibitors p21 and p27 is probably responsible for the inhibition of Cdk2. In contrast, flavonoids that lack 3′-OH, do not alter the activity of Cdk2, but inhibit Cdk1 are correlated with a G2/M arrest (*21*).

A similar structure-related inhibition by flavonoids was found for inositol-1,4,5-triphosphate-3-kinases (PI3K) isoenzymes A, B, and C. This enzyme complex is also involved in cell proliferation, among other important functions in mammalian cells. Here again, the same structural elements among the 3-OH group were required for maximum inhibition of PI3K-A (*23*).

It can be concluded that the antiproliferative effects of flavonoids are mediated by the inhibition on several kinases and kinase inhibitors and involved in cell-cycle arrest and apoptosis and that this inhibition depends upon the particular structure of each flavonoid, although a planar structure and at least two small substituents (hydroxyl or methoxyl) in the A or B rings of the flavonoid skeleton are essential.

B. Anti-invasive Effects. Metastasis occurs when cancer cells invade beyond the boundaries of the primary site and establish new tumors in distant organs. Because metastases are responsible for most cancer deaths, attention has focused on the mechanisms by which cancer cells acquire metastatic properties (*50*). The invasion of surrounding tissues by cancer cells involves several steps, including matrix metalloproteinase (MMP) secretion, migration, invasion, and adhesion. *Citrus* flavonoids have shown effects on all of these steps.

The *Citrus* flavonoids, quercetin and apigenin, have been reported to possess the ability to inhibit lung colonization *in vivo* by the melanoma B16-BL6 cell line in a dose-dependent manner (*51*). Polymethoxylated flavones, tangeretin and nobiletin, showed a downregulation of secretion of MMPs in several cancerous lines both *in vitro* and *in vivo* (*52, 53*). Quercetin also inhibits the expression of MMP-2 and MMP-9 in prostate cancer PC-3 cells (*54*). Apigenin has inhibitory effects on the *in vitro* motility and invasiveness of MO_4 mouse cells into embryonic chick heart fragments (*55*) and Hela Cx43 carcinoma cells (*56*). Luteolin, too, has shown a strong antimigration and invasion effect in hepatoma HepG2 cells (*57*).

The effects of several *Citrus* flavonoids on an experimental *in vivo* model of pulmonary metastasis using the melanoma B16F10 cell subline were shown to be highly metastatic in the lung (*58*). Despite representing only about 4% of skin cancers, melanoma is responsible for 80% of skin cancer-related deaths and is considered one of the most frequently metastasizing malignant neoplasias (*59*).

Of the many methods that have been proposed for evaluating the effects of treatment (*60–65*), Lentini's model was chosen (*66*), which complements the macroscopic evaluation of nodule numbers by using a stereoscopic microscope and image analysis at the microscopic level, to calculate three indices in histological sections: the percentage of implantation (percentage of lung tissue area occupied by the metastasis), growth index (size of metastasis), and invasion index (frequency of metastasis foci). These gave a more accurate approximation of the real extent of metastasis in the lung.

We considered as quantifiable metastatic nodules those structures of a blackish color clearly identifiable on the lung surface and sufficiently separated to be counted individually, as was published previously (*25*).

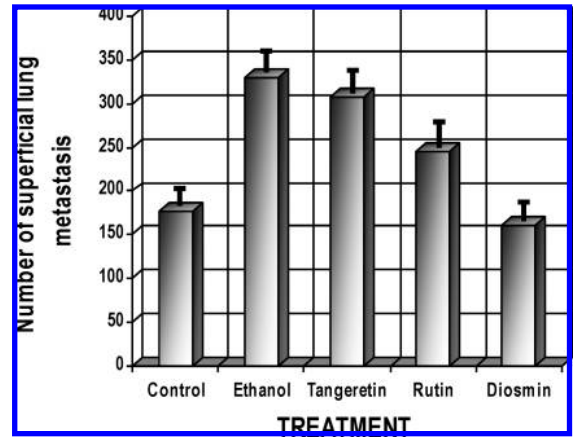

Figure 2. Frequency of the pulmonary metastasic nodules in the control group and groups treated with ethanol, tangeretin, rutin, and diosmin (mean ± error of the mean).

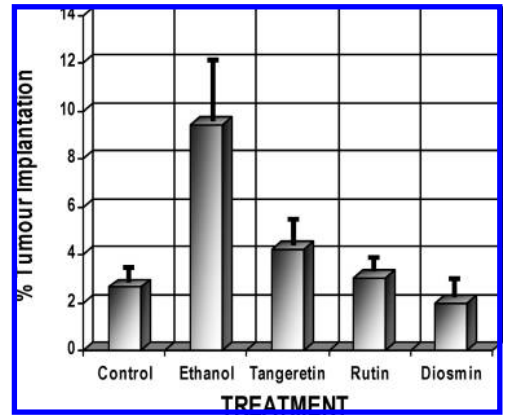

Figure 3. Tumor implantation percentage of control groups and groups treated with ethanol, tangeretin, rutin, and diosmin (mean ± error of the mean).

Figure 2 depicts the pulmonary metastatic nodules counted in the different groups. The control (group I) had a mean of 176.30 ± 19.26 metastatic nodules randomly distributed over the lung surface, while group II (exclusively administered ethanol solution) showed an increase of 87% metastatic nodules over the control. Indeed, group II was effectively the control group because ethanol was used as vehicle to administer the flavonoids.

In decreasing number of metastatic nodules shown were groups III (tangeretin, nonsignificant decrease compared to the ethanol group), group IV (rutin, significant decrease compared to the ethanol group, $p < 0.05$), and group V (diosmin, highly significant decrease with respect to the ethanol group, $p < 0.000\,05$). This last group was the only group to show a smaller number of metastatic nodules than the group I control (with a nonsignificant decrease of 9.09%).

With regard to percentage of implantation (**Figure 3**), group I showed an invasion percentage of the lung parenchyma between 0.61 and 5.75%, with a mean of 2.64 ± 0.54%. Group II, ethanol, the real group for comparison purposes, showed a 255.09% increase over group I levels of invasion ($p < 0.05$). In comparison to group II, the flavonoid-treated groups showed reductions for tangeretin (III) and rutin (IV). The diosmin-treated group (V), showed the greatest reduction in invasion compared to the ethanol group, with the reduction being statistically significant ($p < 0.05$). Furthermore, it was the only group that

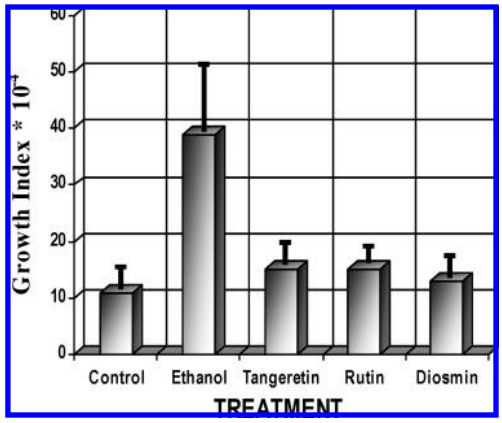

Figure 4. Growth index of control groups and groups treated with ethanol, tangeretin, rutin, and diosmin (mean ± error of the mean).

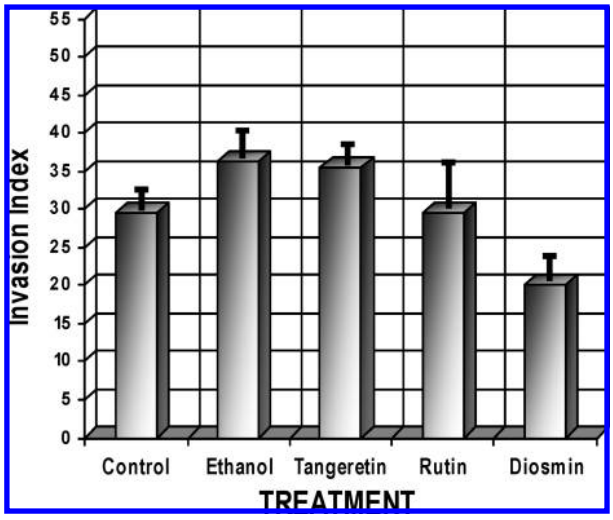

Figure 5. Invasion index of control groups and groups treated with ethanol, tangeretin, rutin, and diosmin (mean ± error of the mean).

showed a reduction with respect to the control (I) (26.8%), although not to a statistically significant degree.

Control group I showed a growth index (**Figure 4**) of 0.0011 ± 0.0004, while ethanol group II showed a 266.57% increase over this group. Rutin (group IV) and tangeretin (group III) showed similar indices, while diosmin (group V) caused the greatest reduction.

Control group I showed an invasion index (**Figure 5**) of 29.46 ± 2.6, with the ethanol group (II) showing a 23.31% increase which respect to the control (I), which was not statistically significant. Among the flavonoids, tangeretin (III) showed the highest index (the reduction over ethanol was not statistically significant), followed by rutin (IV). The greatest decrease in the invasion index corresponded to diosmin (V), which showed a significant reduction with respect to ethanol ($p < 0.005$) and the control (I) (32.28%).

Studies using subcutaneously injected B16F10 cells have shown that ethanol diminishes the number of pulmonary nodules compared to the control (*67*), while injection into the lateral vein of the tail significantly increases the metastatic effect of the ethanol (*68*), which was also observed in this study. These apparently contradictory effects of ethanol have been discussed in several studies: for example, *in vitro* studies have described the stimulatory effects of ethanol on cell migration both in the case of B16F10 melanoma (*69*) and other cell lines, such as breast cancer T47D (*70*); other studies have described its action on endothelial fibrinolysis (*71*) and the inhibitory effect on platelet aggregation (*72*), which may reduce pulmonary metastasis. However, the reasons for which ethanol acts in this way are not fully understood.

The results obtained in the group treated with the ethanol solution (98.8:1.2), namely, a macroscopic (86%) and microscopic (invasion index of 23.31%) increase over the control, suggest tumoral cell transport and invasion of the lung parenchyma. There was also an increase in the index related to the area invaded, a 255.09% increase in the implantation percentage, and a 266.57% increase in the growth index. These data suggest an increase in the invasion of the lung, especially of tumoral cell proliferation.

Another type of study related to the antimetastatic capacity of flavonoids describes how tangeretin inhibits the mobility of sarcoma cells (*55*) and platelet aggregation (*73*) through inhibiting the 12-lipoxygenase activity of the platelets. Similarly, it has been observed that tangeretin diminishes the expression of metaloproteases (MMP-2 and MMP-9) in several cell lines (*52*), which would also be related to its possible antimetastatic activity. Despite these *in vitro* references, our results pointed to no significant antimetastatic activity, because the reductions observed with both controls (groups I and II) were not statistically significant, either macroscopically or microscopically (invasion index).

Following tangeretin, the next flavonoid in order of increasing strength was rutin, a powerful free-radical scavenger (*74*) and basis of some vasoprotective and venotonic pharmaceuticals. Several *in vitro* studies have shown rutin to have antiproliferative effects over the OCM-1 melanoma cell line (*20*) and some colon cancer lines (caco-2 and HT-29) (*30*), carcinoma of human squamous cells (A431) (*75*), and leukemia HL-60 (*35*). Recent *in vivo* studies have shown that orally administered rutin at 4% feed intake has a chemo-protective effect on the colon, where it significantly reduces the number of neoplasia foci (*76*). In this study, rutin reduced the area of invasion, both with regard to the percentage of implantation and growth index, reductions which seem to confirm its antiproliferative effect.

In certain *in vivo* studies (*77*), rutin seemed to show an anti-invasive capacity (71.2% reduction in surface nodules after oral administration). Results in this study pointed to a lower degree of reduction, both macroscopically and microscopically (invasion index); however, a degree of anti-invasive action is clear.

The greatest reduction in the number of metastatic nodules was obtained with diosmin; the same occurred with respect to the percentage of implantation, growth index, and invasion index, all compared to the ethanol group. There was also a reduction in the number of metastatic nodules (9.09%), implantation percentage (26.8%), and invasion index (32.28%) compared to the control (group I). Although several *in vitro* studies have suggested that the antiproliferative effects of diosmin are scant (*30, 78*), other *in vivo* studies have pointed to a degree of antiproliferative action in several tumoral cell lines, including esophagus (*74*), colon (*79*), oral (*80*), and bladder (*81*). Results agree with the latter findings because there was a decrease in the implantation percentage and growth index.

It has been described that diosmin diminishes vein distensibility at the microcirculation level, reinforcing capillary resistance, thus perhaps inhibiting the invasion of tumoral cells (*82*). This anti-invasion effect would be complemented by a capacity to inhibit the release of inflammation mediators, such as prostaglandins (PGE2) (*83*), or act on key enzymes in their biosynthesis (*84*), modulating the adhesion of leukocytes, thereby preventing endothelial damage (*85*). All or any of these mechanisms could explain the significant antimetastasic activity

Table 2. Percentage of Reduction on the Macro- and Microscopic Evaluations of Tumor Development of Each Flavonoid-Treatment Group versus Control-Ethanol Group (II)

parameter	tangeretin (III)	rutin (IV)	diosmin (V)
Antimetastasic Activity			
percent superficial lung metastasis	7.0	25.5	52.0
percent invasion index	3.0	18.8	45.2
Antiproliferative Activity			
percent tumor implantation	59.2	68.1	79.4
percent growth index	60.3	61.1	67.5

that diosmin showed in our study both with regard to the reduced number of nodules at the macroscopic level and the invasion index at the microscopic level. **Table 2** summarizes the data described in **Figures 2–5** expressed as a percentage of reduction in the antiproliferative and antimetastasic index of the different flavonoid treatments versus the control-ethanol group (II).

Efforts were made to relate the results obtained in this study with the plasmatic concentration and structure of each flavonoid, although this antimetastatic activity would be related not only to the flavonoid ingested but also to the metabolites of each flavonoid. Identification and measurement of the physiological flavonoid conjugates are key prerequisites toward understanding the role of dietary polyphenols in human health. Most flavonoid glycosides are deglycosylated by β-glucosidases in the small intestine; subsequently, the polyphenols are found in the blood as conjugates, glucuronate or sulfate, with or without methylation of the B-ring catechol functional group (*86*). According to several authors, the diosmin is metabolized by bacterial flora to form an aglycone, diosmetin, which is then metabolized to form glucuronides and sulfate, the major metabolite (*87, 88*). As in the case of diosmin, the first metabolite formed with rutin is the aglycone quercetin. This subsequently forms conjugates in the form of glucuronides or sulfates, including, in this case, methylation in the 3′ position of the B ring, thus generating as the major metabolite, a conjugate of isorhamnetin.

Finally, with regard to tangeretin, a highly methoxylated compound to which few *in vivo* and *in vitro* references exist, the biotransformation of this flavonoid by rat liver microsomes leads to demethylation of the position 4′ of the B ring, with the possible formation of the corresponding glucuronide (*89, 90*).

The plasmatic levels or concentrations of flavonoids studied should be considered. The literature describes very similar levels for all three at around 15−60 μM (*88–91*). To quantitatively justify the effects observed in the results in this study by reference to these concentrations would seem difficult, and there would, at first sight, seem to be little relation between the two. It is more likely that structural factors would better explain the results.

However, structural factors influence the two activities studied in the present work (antiproliferative and antimetastasic activity) differently and, obviously, as a function of the action mechanisms of each activity. In the first case, the differences in the antiproliferative capacity of the three flavonoids are slight and there is no evidence to support the direct influence of some structural elements on these differences, whether we consider the flavonoids or their major metabolites. However, this is not the case when the antimetastasic activity of the flavonoids is compared, because the group [diosmin−diosmetin glucuronide] showed significantly better results than the groups [rutin−quercetin−isorhamnetin glucuronides] and [tangeretin−tangeretin glucuronide].

This suggests the existence of certain structural factors that would regulate the level of antimetastasic activity and that would basically be related with the greater inhibition capacity of certain enzymatic activities or the ability to block the receptors responsible for the release of mediators of inflammation (*83, 84*) or platelet aggregation (*92*). In no case would these activities be related with the well-referenced and widely (sometimes inaccurately) acclaimed anti-oxidant activity, which is lacking in practically all of the mentioned metabolites. Whatever the case, the findings of future quantitative and qualitative studies of these metabolites will be used to carry out *in vitro* studies with the same cell line and *in vivo* studies controlling the dose of metabolites. Identification of the compounds that contribute to the inhibition of cancerous cell lines and determination of their inhibitory concentration will throw light on the mechanisms behind such inhibition and the basis of the anticarcinogenic properties of these polyphenols.

The different results obtained with this *in vivo* model compared to those obtained *in vitro* with regard to both the antiproliferative and anti-invasive activity of these flavonoids should be noted. Results of this study and many other studies on several cancer cell lines have shown that tangeretin is one of the most active antiproliferative flavonoids *in vitro* and that glycosyl flavonoids have no significant effect on this activity. However, in our *in vivo* model, the differences in the antiproliferative capacity of the three flavonoids, tangeretin, rutin, and diosmin, were slight and both glycosides showed higher antimetastatic capacity than tangeretin (aglycone). As suggested above, the mechanisms governing the antiproliferative and anti-invasive activity of flavonoids may be very different, with the latter being more related to inflammation.

C. Anti-angiogenic Effects. Tumor growth is strongly dependent upon the formation of new blood vessels, which infiltrate the growing mass of tumor cells, providing the oxygen and nutrients and removing metabolites. Without a supply of new blood vessels, a tumor will only reach a volume of about 2 mm^3 (*93*) because the diffusion of oxygen only occurs within a distance of 100−200 mm. Decreasing the oxygen pressure in the growing tumor leads to hypoxia, one of the strongest stimuli for the expression of mediators of neovascularization (*94*). Blood vessels are formed in three different ways, namely, vasculogenesis, angiogenesis, and arteriogenesis (*95*).

In cancer pathologies, angiogenesis, the formation of new capillaries from pre-existing blood vessels, is the main way in which blood vessels are created. The process is initiated by the dissolution of the endothelial basement membrane by proteinases, whose action weakens the tight contact of endothelial cells with the basement membrane and underlying mural cells, thus changing the phenotype of the endothelial cells, which become permissive to the activity of growth factors (*94*). Several hypotheses have been articulated regarding the critical importance of tumor angiogenesis in the development and metastatic spread of tumors and how preventive/therapeutic inhibition of angiogenesis might be exploited as a novel means of controlling cancer growth. Anti-angiogenic therapy is suggested as one of the most promising approaches to control cancer, because endothelial cells are generally nontransformed cells and are less prone to acquire drug resistance. Tumor vasculature could be an important prognostic marker and an independent predictor of the pathologic stages and malignant potential of cancer (*96*).

The critical role of tumor angiogenesis in cancer progression was postulated in the 1970s in pioneering studies by Folkman et al. (*97*). The feasibility of this initially criticized approach was partially confirmed by demonstration of the efficacy of anti-angiogenic strategies in several experimental models, although, only in recent years has the knowledge of endothelial cell

physiology and tumor angiogenesis provided the necessary background to develop effective anti-angiogenic strategies (*98, 99*). Matrix metalloproteinases (MMPs), angiogenic growth factors, and their receptors are the main targets for testing the therapeutic efficacy of anti-angiogenic agents (*100*).

The endothelial cell is a preferential target for therapy, because it is a cell type common to all solid tumors. Cancer cells are able to produce several angiogenic factors, including vascular endothelial growth factor (VEGF), basic-fibroblast-like growth factor (bFGF), interleukin 8 (IL-8), transforming growth factor-β (TGF-β), and others that cause endothelial cell recruitment and proliferation (*101*). There are then a variety of mechanisms by which anti-angiogenic activity may act. Anti-angiogenic agents can prevent the further growth of micrometastases in a tertiary prevention setting, although there is increasing evidence that the "angiogenic switch", defined as the point at which a tumor induces angiogenesis, occurs very early in tumorigenesis and that early intervention can curtail tumor growth (*102, 103*). Thus, angiogenesis applies to primary prevention, limiting the expansion of hyperplasic foci and subsequent tumor development (*100*).

As a general mechanism, oxidative stress is a common hallmark of inflammation and the tumoral phenotype. Tumor growth produces large amounts of reactive oxygen species (ROS) (*104*), which can activate tumor-infiltrating leukocytes to induce the angiogenic response (*105*). One effect of anti-oxidant chemoprotective agents is to alter the redox equilibrium in target cells, although their influence on the production of extracellular matrix molecules and angiogenic growth factors by tumor cells or surrounding stromal cells may also contribute to their antitumoral activity. The mitogen/cytokine-inducible cyclooxigenase-2 (COX-2), involved in prostaglandin (PGE) generation from arachidonic acid, has been shown to regulate angiogenesis in several cancer lines (*106, 107*). COX-2 overexpression results in angiogenic growth factor release and also provides a superoxide-radical-generating pathway, which in turn can stimulate proliferation of tumor cells (*108–110*). Also, the overexpression of 12-lipoxygenase in cancer cells resulted in an increase in VEGF protein levels when compared to vector control cells (*111*).

Epidemiological studies have indicated that regular consumption of *Citrus* fruits is associated with a reduced risk of coronary heart disease, inflammatory pathologies, and tumor progression. *In vitro* and *in vivo* investigations have indicated that some *Citrus* flavonoids are able to inhibit several key events of the angiogenic process, such as the proliferation and migration of endothelial cells and vascular smooth muscle cells and the expression of two major proangiogenic factors, vascular endothelial growth factor (VEGF) and matrix metalloproteinase-2 (MMP-2) (*112*).

As described in previous sections, four types of flavonoids occur in *Citrus* species, flavanones, flavones, flavonols, and anthocyanins, and more than 60 individual flavonoids have been identified. Flavanones are the most abundant. Generally, the flavones exhibit higher biological activity even though they occur in much lower concentrations (*1*). However, there has been no comparative study in which the different anti-angiogenic activities of these compounds are analyzed from a structural point of view; only specific studies on some of the four flavonoid families mentioned have been carried out or studies in which the activity of some members (related or not) of each type are compared.

VEGF release from mammary adenocarcinoma (MDA) human breast cancer cells was measured by enzyme-linked immunosorbent assay (ELISA) (*113*). Of the compounds tested, several *Citrus* flavonoids showed significant inhibitory activity at the micromolar concentration in MDA human breast cancer cells. The rank order of inhibitory potency was naringin > rutin > apigenin > kaempferol > chrysin. Considering this order and from a structural point of view, there are several observations that should be made: first, that the glycosylated flavonoids induced the greatest response to treatment at the lowest concentration (naringin, 7-*O*-glycosilated, and rutin, 3-*O*-glucosilated); the flavanone structure (naringin) seemed to be the most active of those studied, ahead of the flavonol (rutin and kaempferol) and the flavone (apigenin and chrysin). However, this conclusion may not be valid because no glycosylated flavone was included in the assay. However, it does seem likely that the activity to inhibit VEGF activity increases with the number of free hydroxyls in the B ring of the flavonoid structure. Despite these results, the same order of efficacy should not perhaps be extrapolated to potential *in vivo* applications, given that the characteristic metabolism of these compounds leads in one of the initial stages to the enzymatic hydrolysis of the glycosidic radical.

A derivative of naringin, the most abundant flavanone in grapefruit, 8-prenylnaringenin, inhibits angiogenesis induced by basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), or the synergistic effect of the two cytokines in combination, with an IC_{50} value between 3 and 10 μM (*114*).

Flavonols have been proposed to act as chemopreventive agents in numerous epidemiological studies and have recently been shown to inhibit angiogenesis and the proliferation of tumor and endothelial cells *in vitro*. Quercetin (and its derivatives) is the most widely studied flavonol as a chemoprotective agent. Quercetin was found to inhibit several steps of angiogenesis, including the proliferation, migration, and tube formation of human microvascular dermal endothelial cells in a dose-dependent manner (*115*). The effect of quercetin on endothelial cell proliferation was confirmed *in vitro* [human umbilical vein endothelial cells (HUVEC) model] and *in vivo* (chicken chorioallantoic membrane assay). These findings suggest that quercetin has an anti-angiogenic potential and that this effect may be related to a decrease in the expression and activity of matrix metalloproteinase-2.

Screening using a reporter under the control of the hypoxia-response element (HRE) identified several flavonols that inhibit the activation of HRE under hypoxic conditions (*116*). A stable transformation of CHO cells (clone A4-4) was established by the transfection of HIF-1-dependent luciferase (5xHRE/pGL3/VEGF/E1b) and neomycin-resistant genes. The flavonols, quercetin (3′,4′-hydroxy) and isorhamnetin (4′-hydroxy-3′-methoxy), showed an effective dose of 3−9 μM to inhibit the increase in reported activity in CHO A4-4 cells; however, their respective 3-*O*-glycosides, isoquercitrin and rutin, and isorhamnetin-3-*O*-glucoside and 3-*O*-rutinoside, were not active.

The anti-oxidant activity of flavonols has been suggested to contribute to several health benefits. Four flavonols, myricetin (M) (3′,4′,5′-hydroxy), quercetin (Q) (3′,4′-hydroxy), kaempferol (K) (4′-hydroxy), and galangin (G), were examined for their anti-oxidant activity and cytotoxicity on HUVECs and for the potential anti-angiogenic and cell adhesion effects (*117*). The relative anti-oxidant capacity of these flavonols in cell-culture medium (cell-free system) and their intracellular anti-oxidant activity were M = Q > K = G, which correlated, respectively, to the presence of 3, 2, 1, and 0 moieties of −OH on their B ring. The higher the number of −OH moieties on the B ring,

the less toxic the flavonol to HUVEC, while the LD_{50} was determined as M (100 μM) > Q (50 μM) > K (20 μM) > G (10 μM). These flavonols at approximately 0.5 LD_{50} doses suppressed VEGF-stimulated HUVEC tubular structure formation by M (47%) > Q (37%) > K (15%) > G (14%), which was not linearly associated with their number of −OH moieties. However, the degree by which activated U937 monocytic cell adhesion to HUVEC was suppressed by flavonoids was indeed associated with the number of −OH moieties on the B ring. This was clearly the case when U937 cells were pretreated with these flavonols. In contrast, the numbers of −OH moieties had no apparent influence on the adhesion or expression of adhesion molecules when activated HUVECs were pretreated with these flavonols. The presence of different numbers of −OH moieties on the B ring of the flavonols may play an important role in their potency for biological action in cases such as angiogenesis and immune-endothelial cell adhesion.

In the past decade, several studies have reported that certain structurally related flavone-aglycones are potent inhibitors of cell proliferation and *in vitro* angiogenesis. Indeed, 3-hydroxyflavone, 3′,4′-dihydroxyflavone, 2′,3′-dihydroxyflavone, fisetin, and, especially apigenin and luteolin inhibited the proliferation of normal and tumor cells, as well as *in vitro* angiogenesis, at half-maximal concentrations in the low micromolar range (*118, 119*).

The flavone luteolin (3′,4′-hydroxy) is a potent inhibitor of HIF-1α activity (activation of HRE under hypoxic conditions) at a dose of 3−9 μM in CHO A4-4 cells, while its respective 7-*O*-glycosides, luteolin-7-*O*-glucoside and 7-*O*-rutinoside, were not active (*116*). In addition, luteolin inhibited tumor growth and angiogenesis in a murine xenograft model. This flavone inhibits VEGF-induced *in vivo* angiogenesis in the rabbit corneal assay. In addition, luteolin inhibits both the VEGF-induced survival and proliferation of human umbilical vein endothelial cells (HUVECs) with an IC_{50} value of about 5 μM. Luteolin inhibits VEGF-induced phosphatidylinositol 3′-kinase (PI3K) activity in HUVECs, and this inhibition was seen to be critical for both the antisurvival and antimitotic affects of the compound. Indeed, luteolin abolished VEGF-induced activation of Akt, a downstream target of PI3K, conveying both survival and mitotic downstream signals. The fact that the overexpression of a constitutively active form of Akt rescued HUVECs only from the antisurvival effects of luteolin suggests that luteolin targeted mainly the survival signals of the PI3K/Akt pathway (*23*). Apigenin is a potent inhibitor of cell proliferation and angiogenesis, but the mechanisms leading to the pathological anti-angiogenic effects are still unclear. The effect of apigenin on endothelial and smooth-muscle cells in an *in vitro* model was studied by Trochon et al. (*120*). Apigenin markedly inhibited the proliferation and, to a lesser degree, the migration of endothelial cells and capillary formation *in vitro*, independent of its inhibition of hyaluronidase activity. In contrast, it strongly stimulated vascular smooth-muscle-cell proliferation. The authors showed that apigenin inhibits endothelial-cell proliferation by blocking the cells in the G(2)/M phase as a result of the accumulation of the hyperphosphorylated form of the retinoblastoma protein. Apigenin stimulation of smooth-muscle cells was attributed to the reduced expression of two cyclin-dependent kinase inhibitors, p21 and p27, which negatively regulate the G(1)-phase cyclin-dependent kinase.

In a HUVEC model, apigenin further decreased TIMP-1 expression to below basal levels and completely abolished TIMP-2 expression. VEGF and bFGF stimulation significantly induced urokinase-type plasminogen activator (uPA) expression, most strikingly the level of 33 kDa uPA, and increased the expression of plasminogen activator inhibitor (PAI)-1. Apigenin effectively blocked the generation of 33 kDa uPA and further decreased the activity of 55 kDa uPA and the expression of PAI-1 below the basal level. These data suggest that this flavone inhibits *in vitro* angiogenesis, in part by preventing VEGF/bFGF-induced MMP-1 and uPA expression and activation of pro-MMP-2 and modulating their inhibitors, TIMP-1 and TIMP-2 and PAI-1 (*121*).

More recent research has considered novel explanations for the anti-angiogenic activity of apigenin and other flavones, suggesting that it inhibits angiogenesis by inhibiting HIF-1α and VEGF expression. Thus, apigenin inhibited the hypoxia-induced expression of vascular endothelial growth factor (VEGF) mRNA in human umbilical artery endothelial cells. Apigenin also suppressed the expression of erythropoietin mRNA, which is a typical hypoxia-inducible gene, through the degradation of hypoxia-inducible factor 1α (HIF-1α) (*122*).

Ovarian cancer is one of the most common causes of cancer death among women. Apigenin inhibited VEGF expression at the transcriptional level through expression of HIF-1α. Apigenin inhibited the expression of HIF-1α and VEGF via the PI3K/AKT/p70S6K1 and HDM2/p53 pathways. Apigenin inhibited *in vitro* tube formation by endothelial cells (*123*).

Apigenin inhibited A549 lung cancer cell proliferation and VEGF transcriptional activation in a dose-dependent manner (*124*). In an attempt to understand the mechanism of apigenin-inhibited VEGF expression, the authors found that apigenin inhibited VEGF transcriptional activation through the hypoxia-inducible factor 1 (HIF-1) binding site and specifically decreased HIF-1α but not HIF-1β subunit expression in the cells. In the efforts to understand the signaling pathway that mediates VEGF transcriptional activation, it was found that apigenin inhibited AKT and p70S6K1 activation (*124*). *In vivo*, apigenin significantly inhibited tumor growth in nude mice. In conclusion, the inhibition of tumor angiogenesis was associated with the decrease of HIF-1 and VEGF in tumor tissues. When all of these data are taken together, they show that apigenin suppresses tumor angiogenesis through HIF-1 and VEGF expression (*124, 125*).

Described previously, the flavone apigenin has strong cytostatic and anti-angiogenic effects *in vitro*. Engelmann et al. (*126*) investigated its efficacy against experimental Lewis lung carcinomas (LLC), C-6 glyomas, and DHDK 12 colonic cancers *in vivo*. Tumor-bearing mice received 50 mg/kg per day apigenin in three different galenical formulations for 12 days at 8 h intervals. Only weak effects of apigenin on the size and number of new tumor blood vessels of both established and newly transplanted tumors were recorded, although intratumoral necrosis was high (45 ± 15 versus 20 ± 7% (control), $p <$ 0.05%). These results contrast sharply with the high *in vitro* sensitivity of LLC, C-6, DHDK 12, and endothelial cells to apigenin, in which case complete growth suppression occurs at concentrations below 30 g/mL. A very recent study reports the activities of quercetin and its main circulating conjugates in human [quercetin-3′-sulfate (Q3′S) and quercetin-3-glucuronide (Q3G)] on *in vivo* angiogenesis induced by VEGF and examines the effects of these molecules on cultured endothelial cells (*127*). The authors found opposing effects of quercetin and its metabolites on angiogenesis. While quercetin and Q3G inhibited VEGF-induced endothelial cell functions and angiogenesis, Q3′S per se promoted endothelial cell proliferation and angiogenesis. The inhibitory effect elicited by Q3G was linked to inhibition of the ERK1/2 phosphorylation elicited by VEGF. The activation of endothelial cells by Q3′S was associated to stimulation of VEGF receptor-2 and to downstream signaling activation

(phosphatidylinositol-3 kinase/Akt and nitric oxide synthase pathways), ultimately responsible for ERK1/2 phosphorylation. These data indicate that the effects of circulating quercetin conjugates on angiogenesis differ and depend upon the nature of the conjugate. Q3G and Q3′S are the two major conjugates in plasma, but their ratio is dependent upon several factors, so that inhibition or activation of angiogenesis could be subtly shifted as a result of metabolism *in vivo*.

These data point to the fact that any new studies on the chemoprotective effects of flavonoids should be carried out both *in vitro* and *in vivo*, including a study of the effectiveness of the main metabolites of the flavonoid in question. Similarly, the effective bioavailability of each flavonoid needs to be determined, establishing the dose necessary to obtain positive effects obtained *in vitro*. The low or null toxicity of *Citrus* flavonoids will make such a study worth while, and hopefully, the molecular targeting of the VEGF by flavonoids will prove to be a useful and novel strategy for chemoprevention and/or treatment of cancerous diseases.

CARDIOVASCULAR PROPERTIES

Reactive oxygen species (ROS) are highly reactive molecules that are constantly produced by enzymatic reactions in cells. In normal physiological conditions, ROS are produced at low levels, which are necessary for maintaining normal cell functions, and the endogenous anti-oxidant defense systems of the body have the capacity to avert any harmful effects. However, several established risk factors for cardiovascular disease have been linked to excessive generation of ROS, known as a state of oxidative stress. For instance, in animal models of hiperlipidemia (*128, 129*), hypertension (*130–132*), and diabetes (*133, 134*), elevated levels of vascular superoxide anion production have been found. Moreover, clinical studies have demonstrated that hypercholesterolemia and diabetes in humans are also associated with increased vascular superoxide anion generation (*135*). All these data strongly suggest that increased oxidative stress is involved in the pathophysiology of cardiovascular disease.

Several mechanisms have been proposed to explain how excessive production of ROS leads to vascular pathology. First, ROS are able to promote the oxidation of low-density lipoprotein (LDL) (*136, 137*). Uptake of oxidatively modified lipoproteins by macrophages transforms these cells into foam cells, which are a key component of atherosclerotic plaques (*138, 139*). Second, superoxide anion rapidly inactivates endothelium-derived nitric oxide (NO), a molecule with intrinsic anti-atherogenic properties, leading to endothelial dysfunction, which is a hallmark of early atherosclerosis (*140*). Moreover, the reaction between superoxide anion and NO generates peroxynitrite ($ONOO^-$), which has been found to be cytotoxic to endothelial and vascular smooth muscle cells through a broad range of biological actions, such as lipid oxidation and mitochondrial DNA damage (*141, 142*). Third, ROS have been shown to be involved in increased expression of certain vascular pro-inflammatory genes that are pertinent to atherogenesis (*143, 144*), such as monocyte chemoattractant protein-1 (MCP-1), vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1) (*145–150*). In addition, evidence has shown that many intracellular signal transduction molecules are sensitive to changes of intracellular redox status induced by ROS formation. For example, intracellular Ca^{2+} mobilization (*151, 152*), protein phosphorylation via altered balance of protein kinase and phosphatase activity (*153–155*), and activation of transcription factors, such as nuclear factor κB (NFκB) (*156, 157*), are all subject to modulation by oxidative stress. These ROS-mediated actions lead to direct regulation of cell function and gene expression.

Many epidemiological studies have shown that regular flavonoid intake is associated with a reduced risk of cardiovascular diseases (*158*). In the coronary heart disease, the protective effects of flavonoids include mainly antithrombotic, anti-ischemic, anti-oxidant, and vasorelaxant (*159*). It is suggested that flavonoids decrease the risk of coronary heart disease by three major actions: (A) improving coronary vasodilatation, (B) decreasing the ability of platelets in the blood to clot, and (C) preventing LDLs from oxidizing.

A. Vasorelaxant and Vasoprotective Effects. There is compelling evidence that the endothelium is critical to normal coronary vascular function and that endothelial dysfunction, generally indicated by an impairment of endothelium-dependent vasodilatation, is an important component of coronary artery disease (CAD). Endothelial cells synthesize and release a number of factors, including prostacyclin, NO, endothelium-derived hyperpolarizing factor (EDHF), and endothelin, which are important in the regulation of vascular tone and the control of platelet and leukocyte adhesion, aggregation, and migration. NO appears to be the critical factor in the preservation of normal coronary vascular functions, and there is a well-established correlation between CAD and an impairment of NO activity.

Vascular endothelial cells control vascular tone and modulate blood flow to organs by synthesizing and releasing the vasoactive autocoids endothelium-derived relaxing factor (EDRF), which is synonymous with NO and prostacyclin (PGI2) (*160, 161*). Among these, NO probably plays a more important role. In vascular endothelium, NO is produced by a constitutively expressed enzyme known as endothelial nitric oxide synthase (eNOS), which converts L-arginine to L-citrulline (*162, 163*). In addition to endothelium-dependent vasodilatation, NO also has a number of other critical functions in the vascular system, including inhibition of platelet aggregation, inhibition of endothelial cell adhesion molecule expression, prevention of vascular smooth-muscle cell migration and proliferation, and prevention of intravascular coagulation and thrombosis (*164–168*). Therefore, NO is an important factor in the maintenance of normal vascular homeostasis and the protection of vessels from injuries induced by atherogenic processes, such as smooth-muscle cell proliferation, platelet aggregation, monocytes adhesion, and oxidative modification of LDL (*163, 169*).

In vitro studies show that flavonoids may exert multiple actions on the NO-guanylyl cyclase pathway, endothelium-derived hyperpolarizing factor(s), and endothelin-1 and protect endothelial cells against apoptosis. *In vivo*, flavonoids prevent endothelial dysfunction and reduce blood pressure, oxidative stress, and end-organ damage in hypertensive animals. Moreover, some clinical studies have shown that flavonoid-rich foods can improve endothelial function in patients with hypertension and ischemic heart disease (*170*).

The effects of individual flavonoids on the relaxation of isolated arteries from rats have been investigated in many studies. The relaxation responses of flavonols, flavones, flavanols, flavanones, anthocyanins, and isoflavones have all been assessed in an isolated animal vessel model. These studies show that flavonoids can cause vasorelaxation at physiological concentrations. The relaxation observed is largely endothelium- and NO-dependent, although other mechanisms also appear to be involved (*171–173*).

In vitro experiments in isolated rat aorta have revealed that chrysin was able to induce endothelium- and NO-dependent vasorelaxation, mediated by the prevention of superoxide-anion-

induced inactivation of endothelium-derived NO and also the potentiation of guanosine 3,5-cyclic monophosphate (cGMP)-induced vasodilatation. In addition, it has been recently reported that chrysin exerts antihypertensive effects and reduces left ventricular hypertrophy and endothelial dysfunction in spontaneously hypertensive rats (*174*). Endothelium-mediated effects of chrysin in mesenteric vascular beds are related to endothelial NO production and a L-NAME-sensitive increase on vascular smooth-muscle cGMP levels, and the release of an endothelium-derived hyperpolarizing factor (EDHF) could also be involved in such effects (*174*). In this sense, a diet rich in quercetin show increases in NOS activity, NO production, and cyclic GMP content in rat aorta in a resting state, and these changes are endothelium-dependent. The absence of an increase in endothelial nitric oxide synthase (eNOS) expression indicates that the mechanism of action of flavonoids is not transcriptional (*175*). Others authors have concluded that flavonoids, such as apigenin or kaempferol, may increase eNOS activity at the same time as inhibitors of inducible nitric oxide synthase (iNOS) because of the inhibition of iNOS gene transcription. The question arises whether flavonoids may prove beneficial in conditions in which regulatory, protective eNOS activity is overwhelmed by excessive iNOS induction, such as endotoxic shock or atherosclerosis (*176*).

Inhibition of iNOS by *Citrus* flavonoids *in vitro* has also been found in LPS-activated RAW 264.7 cells (*177, 178*). In this model, flavones, such as apigenin, luteolin, or quercetin, inhibited NO production. In contrast, flavanones, such as naringenin, did not demonstrate significant inhibition. These results clearly indicated that a C-2,3 double bond might be important and that the potency of inhibition depended upon the substitution patterns of the flavonoid molecules. The inhibitory activity of flavonoids was not due to direct inhibition of iNOS enzyme activity because they did not reasonably inhibit iNOS activity but reduced iNOS enzyme expression. All of these results clearly demonstrated that certain flavonoids inhibit NO production in lipopolysaccharide-activated RAW 264.7 cells, and their inhibitory activity might be due to the reduction of iNOS enzyme expression (*177*).

Other recent studies on the potential vasorelaxant, antioxidant, and cyclic nucleotide phosphodiesterase (PDE) inhibitory effects of the *Citrus*-fruit flavonoids naringenin and hesperetin in intact rat aortic rings have shown that their vasorelaxant effects seem to be basically related to the inhibition of different PDE isoenzymes (*179, 180*).

B. Antithrombotic Properties. Platelets are implicated in hemostasis, thrombosis, and inflammatory processes. Platelet aggregation is the critical event occurring during the initiation of coronary thrombosis, and flavonoids have been reported to modulate platelet function, thus reducing the risk of clot formation (*181*).

Upon vascular damage, platelets adhere to exposed subendothelium, become activated, and secrete biologically active ligands, including adenosine diphosphate (ADP), serotonin, and thromboxane A2 (TxA2) (*182*). TxA2 is a potent vasoconstrictor and platelet agonist that, at micromolar concentrations, causes platelets to change their discoid shape to spiculated spheres and leads to the recruitment and deposition of circulating cells at the site of injury and the expansion of the thrombus.

Certain dietary flavonoids have been shown *ex vivo* to inhibit platelet function (*183*). *In vitro*, diverse mechanisms of inhibition of platelet-signaling pathways have been hypothesized, and some studies have related the flavonoid structure to their inhibitory potential. Thus, flavonoids have been shown to impair enzymes involved in cellular signaling as cyclo-oxygenases and lypoxygenases, phosphodiesterases, tyrosine kinases, and phospholipases (*1, 14, 17, 18*). They have also been postulated to have anticoagulant activity by inhibition of NAD(P)H:quinine acceptor oxidoreductase (*184*), an enzyme inhibited by oral anticoagulants, or by interfering with phosphatidylserine exposure (*185*). Other mechanisms reported for their antiplatelet effects rely on their anti-oxidant properties (*186*). Quercetin has been shown to inhibit collagen-induced responses of platelets through selective blockade of the glycoprotein VI signaling pathway (*187*).

Table 3. Dose-Dependent Inhibition of ^{125}I-BOP Binding to Washed Platelets by Ligand Analogues or Flavonoids[a]

	K_i (μmol/L)
U46619	0.127 ± 0.007
SQ29548	0.0040 ± 0.012
apigenin	4.0 ± 2.7
genistein	2.1 ± 0.7
luteolin	3.1 ± 0.6
quercetin	35.6 ± 29.2
catechin	160.5 ± 34.6
rhoifolin	119.3 ± 27.1
rutin	227.0 ± 115.9

[a] Platelets were incubated with 7.5 pmol/L ^{125}I-BOP, in the absence or presence of other TxA2 receptor ligands or flavonoids. The K_i value defines the concentration of competing ligand that binds to half the binding sites at equilibrium in the absence of a radioligand or other competitors. Lower K_i values correspond to higher affinity of compounds for the ^{125}I-BOP binding sites in platelets. The K_i values were derived from inhibition curves analyzed with LIGAND software (drug option). Results are mean ± standard deviation (SD) from at least two experiments with different platelets.

The effect of distinct structural types of flavonoids on agonist-induced platelet responses have been investigated (*181*). A powerful action of these compounds as antagonists of the TxA2 receptor has been identified, exposing a probable common mechanism for their inhibitory actions on platelet function. The study of certain structure−activity relationships reveals key chemical substituents on the flavonoid molecule, greatly affecting their affinity for the thromboxane receptor (*181*).

From the platelet aggregation and secretion studies, two *Citrus* flavonoids, apigenin and luteolin, effectively inhibited TxA2-mediated responses. Thus, these flavonoids abrogate aggregation and dense granule secretion elicited by arachidonic acid, resulting in an inhibition of reactivity similar to that caused by aspirin. This effect is, however, reversible and independent of an impaired TxA2 synthesis, suggesting minor effects on cyclo-oxygenases or thromboxane synthase. Furthermore, these flavonoids displace ^{125}I-BOP binding to platelets, consistent with these agents acting as competitors for the TxA2 receptor (**Table 3**). It is noteworthy that there is agreement between the strength of these flavonoids in interfering with radioligand binding to TxA2 receptors and their inhibition of platelet aggregation and secretion, thus suggesting a direct relationship between these two effects. Thus, results of this study confirm the antiplatelet action of certain flavonoids, which selectively inhibit the amplification action of TxA2 at the receptor level.

Findings of this study that apigenin and luteolin interact with the TxA2 receptor with high affinity (**Table 3**) provide evidence that not all flavonoids have similar pharmacological actions. The interaction of selective flavonoids with the TxA2 receptor, a member of the G protein-coupled receptor superfamily, suggests that these three compounds might share antiplatelet actions that are dependent upon a similar chemical structure. The study of certain structural characteristics reveals that

Table 4. Effect of Apigenin, Diosmetin, and Silymarin on NO Production, TNF-α Production, and Cell Viability in N-11 Microglia Activated with 750 μg/mL BSA−AGEs[a]

	NO		TNF-α		cell viability in percent (at EC_{50} for NO)	
	EC_{50}		EC_{50}		Alamar Blue	MTT
compound	(μM)	(μg/mL)	(μM)	(μg/mL)	(%)	(%)
apigenin	14		8		>85	>85
diosmetin	19		39		>85	>85
silymarin		8		10	>85	>85

[a] Extracts that contained a minimum of 95% purity were measured in micromolars. Plant extracts that were complex mixtures are measured in μg/mL. Alamar Blue and MTT values refer to percentage viability at the highest concentration (50 μg/mL or 100 μM) compared to the positive control.

flavones (apigenin and luteolin) with the higher affinity for the TxA2 receptor (lower K_i) share a characteristic conjugation between A, C, and B rings with the presence of a lactone structure. Of note is that this lactone-like structure is also present within the TxA2 molecule, which may account for competition between these compounds for the same receptors. Tight binding of flavonoids to the TxA2 receptor also seems influenced by certain features affecting their steric and electrostatic properties. Indeed, glycosylation (rhoifolin and rutin) might enlarge the flavonoid size, thus complicating binding to the receptor, while methylation (dimethylapigenin and diosmetin) might decrease affinity for TxA2 receptors because of the change in the electrical charge of the drug. Moreover, the double bond in C2−C3 and/or keto group in C4 are considered important for the binding to the TxA2 receptor, because the lack of such ring substituents (catechin) results in a decreased affinity of these compounds for the receptor. Substitution in C3 (quercetin versus luteolin) significantly decreases the affinity of flavonoids for the TxA2 receptor. Thromboxane production, together with ADP generation, is necessary for platelet aggregation induced by low doses of collagen (*188*). Consistent with the data above, the two flavonoids exhibiting the highest affinity for the TxA2 receptor (apigenin and luteolin) efficiently inhibited collagen-induced aggregation. Nevertheless, flavonoids induced a mild decrease, similar to aspirin, in the secretion from platelet-dense granules upon activation with collagen, and these compounds also reduced the extent of TxA2 secretion in collagen-stimulated platelets. This is in agreement with previous observations indicating a requirement of TxA2 receptor signaling for aggregation induced by low-dose collagen-induced aggregation, with that signaling being, however, only partially required for secretion and TxA2 production but dependent upon aIIbb3-mediated outside−in signaling (*188*). Moreover, the fact that the effect of flavonoids that exhibit higher affinity for the TxA2 receptor in the inhibition of aggregation is stronger in collagen than in arachidonic acid and U46619-stimulated platelets suggests that TxA2 receptor blockade by flavonoids seems to be an additional mechanism to those that have been proposed, through which some of these compounds might inhibit the collagen-signaling pathway.

In conclusion, some flavonoids have been found to have biological effects attributable to TxA2 receptor antagonism. Key elements on the flavonoid molecule have been identified as being important to exert this effect. While many of the effects attributed to flavonoids have unexplained mechanisms, our results support that a certain ability of these compounds on their antiplatelet effect is receptor antagonism, mainly on thromboxane receptors. Further studies are expected to analyze the probable capacity of flavonoids to bind to other platelet receptors and determine their activity in the presence of plasma proteins to conclude whether pharmacological supplementation or dietary intake of these compounds might prompt beneficial effects in the prevention or treatment of thrombotic events.

C. Effect on Coronary Heart Disease (CHD). One of the mechanisms to trigger CHD is a greater production of LDL cholesterol. A number of studies have shown that consumption of fruit and vegetables is associated with a reduction of elevated LDL cholesterol levels (*189, 190*). Dauchet et al. (*191*) conducted a prospective study to assess the relationship between the frequency of fruit (including *Citrus* fruit) and/or vegetable consumption and CHD risk in France and Northern Ireland. They followed up 8087 men aged 50−59 years, free of CHD, for over 5 years, and found a favorable relationship between the frequency of *Citrus* fruit consumption and the lower rates of acute coronary events in both France and Northern Ireland. However, other fruit intake was only associated with lower rates of acute coronary events in Northern Ireland but not in France. There are a few other epidemiological studies that have specifically analyzed the effect of the consumption of *Citrus* fruit on cardiovascular events. In the nurses' health study and the health professionals' follow up study, based on 42 148 men and 84 251 women, respectively, Joshipura et al. (*192*) showed a reduction of 12% in the risk of myocardial infarction events in the top quintile of *Citrus* fruit consumption as compared to the bottom quintile.

The lipid transport system involves lipoproteins that transport cholesterol and triglycerides from sites of absorption and synthesis to sites of use. The lipoprotein surface coat contains the free cholesterol, phospholipids, and apolipoproteins, thus permitting these particles to be miscible in plasma as they transport their hydrophobic cargo. Apolipoprotein B (apo-B) is the principal protein of the cholesterol-carrying LDL and is the determinant for cellular recognition and uptake of LDL by the high-affinity LDL receptor. Binding of apo-B to LDL receptors results in internalization and degradation of LDL, promoting the clearance of LDL from plasma and regulating intracellular cholesterol handling and biosynthesis. Acceleration of atherosclerosis is principally correlated with an elevation of LDL or β fraction, which is rich in cholesterol but poor in triglycerides.

Increased LDL and especially oxidized LDL are recognized as risk factors in CHD. De Whalley et al. (*193*) showed that certain flavonoids were potent inhibitors of the modification of LDL by mouse macrophages, with IC_{50} values in the micromolar range (1−2 μM for quercetin). Flavonoids also inhibited the cell-free oxidation of LDL mediated by $CuSO_4$. The flavonoids appeared to act by protecting LDL against oxidation caused by the macrophages, because they inhibited the generation of lipid hydroperoxides and protected α-tocopherol, a major lipophilic anti-oxidant carried in lipoproteins, from being consumed by oxidation in the LDL. Thus, the flavonoids protected α-tocopherol (and possibly other endogenous anti-oxidants) in LDL from oxidation, maintained their levels for longer periods of time, and delayed the onset of lipid peroxidation. While the mechanisms by which flavonoids inhibit LDL oxidation are not certain, the following possibilities have been advanced. First, they may reduce the generation or release of free radicals in the macrophages or may protect the α-tocopherol in LDL from oxidation by being oxidized by free radicals themselves. Second, flavonoids could regenerate active α-tocopherol by donating a hydrogen atom to the α-tocopheryl radical; the latter is formed when it transfers its own OH hydrogen atom to a lipid peroxyl radical to terminate the chain reaction of lipid peroxidation.

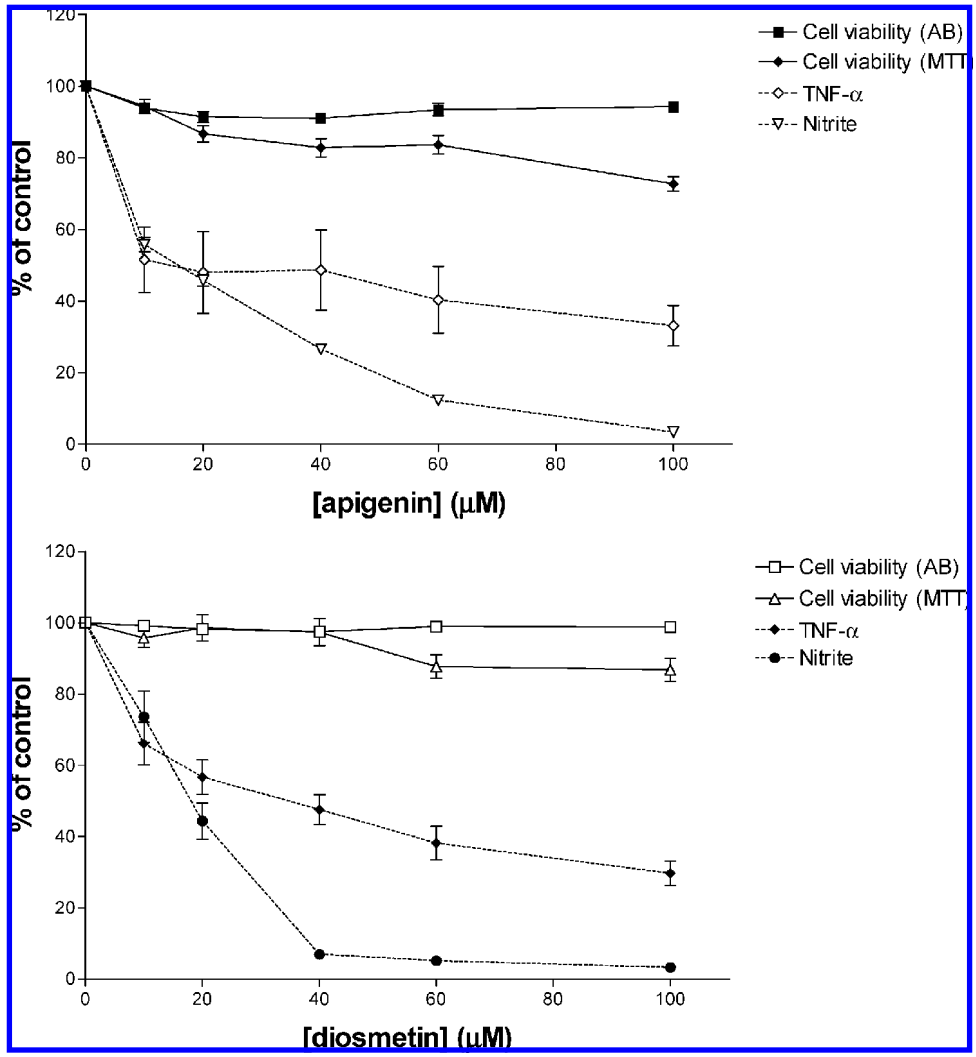

Figure 6. Dose effect of apigenin and diosmetin on BSA−AGE-induced NO and TNF-α production as well as cell viability in N-11 microglias (mean ± error of the mean). Data are taken from three different experiments (each performed in triplicate).

Table 5. Effects of Several Flavonoids on NO Production and Cell Viability in Microglia (N11) Activated with LPS[a]

	NO production		cell viability	
	IC_{50}		Alamar Blue	MTT
compound	(μg/mL)	(μM)	(%)	(%)
apigenin	5	15	125	125
diosmetin	6	19	120	140
diosmin	58	90	ND	100
hesperetin	32	100	90	80
hesperidin	NA	NA	ND	100
naringenin	12	40	60	80
naringin	43	70	70	60

[a] Cell viability is expressed as a percentage of the control at the highest concentration of plant extracts or compounds tested (50 μg/mL or 100 μM). Abbreviations used are NA, 50% inhibition of NO not achieved at the highest concentration tested; ND, no data.

Third, flavonoids may sequest metal ions, such as iron and copper, thereby diminishing the engendered free radicals in the medium.

Citrus flavonoids also can act as regulators of apolipoprotein B (apoB) (*194–196*). The ability of *Citrus* aglycone, hesperetin, and naringenin to modulate apoB secretion and cellular cholesterol homeostasis was determined in the human hepatoma cell line, HepG2 (*194*). ApoB accumulation in the media decreased in a dose-dependent manner following 24 h incubations with naringenin (up to 82%) or hesperetin (up to 74%). Decreased apoB secretion was associated with a reduced cellular cholesteryl ester mass. Cholesterol sterification was decreased, dose-dependently, up to 84% at flavonoid concentrations of 200 μM. Neither flavonoid demonstrated selective inhibition of either form of acyl CoA:cholesterol acyltransferase (ACAT) as determined using CHO cells stably transfected with either ACAT1 or ACAT2. However, in HepG2 cells, ACAT2 mRNA was selectively decreased (−50%) by both flavonoids, whereas ACAT1 mRNA was unaffected. In addition, naringenin and hesperetin decreased both the activity (from −20 to −40%) and expression (from −30 to −40%) of microsomal triglyceride transfer protein (MTP). Both flavonoids caused a 5−7-fold increase in LDL receptor mRNA, which resulted in a 1.5−2-fold increase in uptake and degradation of ^{125}I-LDL (*194*).

The authors conclude that both naringenin and hesperetin decrease the availability of lipids for assembly of apoB-containing lipoproteins, an effect mediated by (1) reduced activities of ACAT1 and ACAT2, (2) a selective decrease in ACAT2 expression, and (3) reduced MTP activity. Together with an enhanced expression of the LDL receptor, these mechanisms may explain the hypocholesterolemic properties of the *Citrus* flavonoids.

More recent studies show that naringenin inhibits apoB secretion primarily by inhibiting microsomal triglyceride transfer protein and enhances LDL receptor (LDLr)-mediated apoB-

containing lipoprotein uptake (*195*). The other *Citrus* flavonoid, tangeretin, shows a marked reduction in apoB secretion in HepG2 cells incubated with 72.8 μM tangeretin. This effect was rapid, apoB-specific, and partly reversible. The reduction was also observed under lipid-rich conditions and found to be insensitive to proteasomal degradation of nascent apoB (*196*).

Other dietary experiment studies on rats have determined that hesperidin, the main *Citrus* flavanone, increased HDL cholesterol, while it lowered LDL, plasma triglycerides, and total lipids (*197*).

The protective role of flavonoids in cardiac ischemia may also relate to their ability to inhibit mast cell secretion. Mast-cell-derived mediators may be involved in cardiovascular inflammation, which is now considered a key factor in CAD (*198*). Moreover, IL-6 was recently shown to be a key factor in CAD. IL-6 is known to be released from mast cells. IL-6 is released from the heart in acute CAD (*198*).

In general, flavonoids could potentially influence disease states in which lipid peroxidation products are intricately involved, especially vascular disorders and CAD. The anti-inflammatory and mast cell inhibitory actions of flavonoids provide new evidence of their possible ability to modulate inflammation, which is increasingly implicated in CAD (*198*).

In summary, flavonoids may be protective against CAD by influencing several processes, such as (1) decrease in LDL oxidation, (2) increase in HDL levels, (3) reduction of cardiac mast-cell-mediator release, and (4) decrease in cardiovascular inflammation.

ANTI-INFLAMMATORY ACTIVITY IN DEGENERATIVE DISEASES

Inflammation is typically characterized by increased permeability of endothelial tissue and influxes of blood leukocytes into the interstitium, resulting in edema. Many different biological mediators influence each step of the inflammation cascade, and typically, anti-inflammatory agents exhibit therapeutic properties by blocking the actions or syntheses of these mediators. While inflammation is a normal response toward tissue injury, it is often uncontrolled in chronic autoimmune diseases, such as rheumatoid arthritis and Chrohn's disease, or when linked to allergic response as asthma and anaphylactic shock. In these cases, anti-inflammatory compounds are therapeutically administered to control the inflammation response. Plants rich in certain flavonoids have been traditionally used for their anti-inflammatory properties, and recently, attention has been given to isolated flavonoids, including those in *Citrus*, as potential anti-inflammatory agents (*12*).

The approach to use bioflavonoids as a general anti-inflammatory and anti-aging therapy for humans is controversial. Low bioavailability and loss of function because of metabolic processing are the two main arguments against the efficacy of dietary supplementation with plant flavonoids (*199*). However, there is now sufficient evidence from *in vivo* research and clinical trials to support their use. Not only are they readily available at lower cost than many anti-inflammatory drugs, but they also generally lack the side effects or intolerance observed with these drugs.

The anti-inflammatory properties of the *Citrus* flavonoids, hesperidin, and its flavone analogue, diosmin, have been studied. Several key studies have shown that the anti-inflammatory properties of diosmin and hesperidin are due to its inhibition of the synthesis and biological activities of different pro-inflammatory mediators, mainly the arachidonic acid derivatives, prostaglandins E_2 and F_2 and thromboxane A_2 (*12*).

Biosynthesis of the inflammatory arachidonic-acid-derived mediators involves the actions of phospholipase A_2 and key oxidative enzymes, such as cyclooxigenase and lipooxygenase. These arachidonic-acid-derived mediators are critical to neutrophil activation and, therefore, are involved in stimulating the production of tissue-damaging ROS at sites of inflammation. Flavonoid inhibition of the critical reactions catalyzed by phospholipase A_2, cyclooxigenase, and lipooxygenase has been demonstrated in a number of *in vitro* studies (*12*).

Recently, several degenerative diseases and particularly brain diseases have been related with the ROS-mediated inflammatory process. Inflammatory responses and their mediators may play a central role in the pathogenesis of various neurodegenerative diseases that involve chronic activation of microglial cells, such as Alzheimer's disease, Parkinson's disease, and acquired immunodeficiency syndrome-related dementia (*200*).

Microglias are a type of neuroglia that support, nurture, and protect the neurons maintaining homeostasis of the fluid that bathes neurons. Microglia act as macrophages in central nervous system (CNS); they migrate to the area of injured nervous tissue and engulf and destroy microbes and cellular debris (*201*). Stimulated microglia produce diverse inflammatory mediators, such as NO and tumor necrosis factor. There is growing evidence that toxic mediators produced by activated microglial cells might be involved in the pathogenesis of various neurodegenerative diseases (*202–204*). Thus, in CNS, the production of toxic inflammatory mediators by activated microglial cells must be strictly regulated to avoid harmful effects. Potential mechanisms for the downregulation of activated microglia may include the deactivation or elimination of activated cells.

Activated microglia and astroglia, characteristic hallmarks of AD, are able to generate free radicals, including superoxide and NO (*205*). Roles proposed for NO in the pathophysiology of the CNS are increasingly diverse and range from intercellular signaling through necrotic killing of cells and invading pathogens to the involvement of NO in apoptosis. High levels of NO, synthesized by iNOS, may be cytotoxic, most likely because of peroxynitrite formation (*206*).

It has been shown previously that membrane permeable antioxidants, such as α-lipoic acid and estradiol, are able to scavenge AGE-induced oxygen free radicals, which have been proposed to act as second messengers in redox-sensitive signal transduction pathways (*207*). Thus, anti-oxidants in general, including food-derived polyphenols, may be able to downregulate AGE-induced downstream signals, such as cytokine release and NO production via downregulation of iNOS expression.

Our group has recently shown that *Citrus* flavonoids, apigenin and diosmetin, are effective in attenuating LPS-induced NO and TNF release at concentrations in the low micromolar range and proposed their use in the treatment of septic shock (*208*). Therefore, we have also studied the effect of selected *Citrus* flavonoids, apigenin and diosmetin (among other compounds), on AGE-induced NO production and TNF-α release in microglia.

The flavonoid apigenin has already been shown to inhibit iNOS and COX-2 expression in LPS-activated RAW264.7, with an IC_{50} of 15 μM (*209, 210*). BSA−AGE-induced NO and TNF-α production were both inhibited by apigenin in a dose-dependent manner, with an EC_{50} values of approximately 14 and 8 μM, respectively (**Table 4**). The Alamar Blue assay indicates that the cell viability is consistently around 100% at all concentrations of apigenin, while the MTT assay shows a small decrease in cell viability at 100 μM (**Figure 6**).

The flavonoid diosmetin highly abundant in *Citrus* fruits, mainly lemon, has a similar structure to apigenin (**Figure 1**);

therefore, it was expected to be similarly potent. Indeed, diosmetin also dose-dependently downregulated BSA−AGE-induced NO and TNF-α production, with EC_{50} values of 19 and 39 μM, respectively (**Table 4**). This dramatic inhibition of NO and TNF-α production is not caused by cell death, because Alamar Blue and MTT assays indicate that the number of viable cells remain above 90% at all diosmetin concentrations (**Figure 6**).

We had previously compared 29 commercially available polyphenol-containing plant extracts and pure compounds for their ability to prevent LPS-induced upregulation of NO production. In N-11 murine microglia, IC_{50} values for apigenin and diosmetin were 15 and 19 μM, respectively (**Table 5**). Structure−activity relationships of the flavonoids demonstrated three distinct principles (**Table 5**): (i) flavonoid aglycones are more potent than the corresponding glycosides (diosmetin versus diosmin); (ii) flavonoids with a 4′-OH substitution in the B ring are more potent than those with a 3′-OH-4′-methoxy substitution (apigenin versus diosmetin); and (iii) flavonoids of the flavone type (with a C2═C3 double bond) were more potent than those of the flavanone type (with a C2−C3 single bond) (apigenin versus naringin).

Our results show that all extracts studied inhibit BSA−AGE-induced NO production, whereas TNF-α was only reduced by apigenin, diosmetin, and silymarin but not by the other compounds (*208*). This indicates that the interference of the two latter with NO production is most likely not due to inhibition of redox-active signaling but rather by some unknown pathway or direct scavenging of NO. It might also be possible that these extracts contain a mixture of pro-inflammatory and anti-inflammatory compounds.

From our results, apigenin and diosmetin based on their general activity and low toxicity are excellent candidates for such trials under the condition that their bioavailability depending upon the mode of delivery allows for the buildup of low micromolar concentrations in the target tissue. Becauss this therapeutic principle might not only be relevant for Alzheimer's disease but also for other inflammatory conditions involving AGEs, particularly those related to diabetes and renal failure, these plant-derived polyphenolic anti-oxidant could be used as potent therapeutic anti-inflammatory drugs for a variety of AGE-mediated chronic inflammatory diseases.

ABBREVIATIONS USED

ACAT, acyl CoA:cholesterol acyltransferase; bFGF, basic-fibroblast-like growth factor; COX-2, ciclooxigenase-2; Cdks, cyclin-dependent kinases; CAD, coronary artery disease; CHD, coronary heart disease; ERK1/2, extracellular signal-regulated kinase-1/2; HUVEC, human umbilical vein endothelial cells; HIF, hypoxia-inducible factor; HRE, hypoxia-response element; PI3K, inositol-1,4,5-triphosphate-3-kinases; ICAM-1, intercellular adhesion molecule-1; IL-8, interleukin 8; LLC, Lewis lung carcinomas; MDA, mammary adenocarcinoma; MMP, metalloproteinase; MTP, microsomal triglyceride transfer protein; MAP kinase, mitogen-activated protein kinase; MCP-1, monocyte chemotactic protein-1; NO, nitric oxide; NOS, nitric oxide synthase; NFκB, nuclear factor κB; PDE, phosphodiesterase; PAI, plasminogen activator inhibitor; PGE, prostaglandin; PC, prostate cancer; PKC, protein kinase C; Q3G, quercetin-3-glucuronide; Q3′S, quercetin-3′-sulfate; ROS, reactive oxygen species; TIMP, tissue inhibitor of metalloprotease; TGF-β, transforming growth factor-β; TxA2, tromboxane A2; TNF-α, tumor necrosis factor-α; uPA, urokinase-type plasminogen activator; VCAM-1, vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor.

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