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REVIEWS

Uses and Properties of *Citrus* Flavonoids

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Flavonoids are a widely distributed group of polyphenolic compounds with health-related properties, which are based in their antioxidant activity. These properties have been found to include anticancer, antiviral, antiinflammatory activities, effects on capillary fragility, and an ability to inhibit human platelet aggregation. The antioxidant capacity of any flavonoid will be determined by a combination of the *O*-dihydroxy structure in the B-ring, the 2,3-double bond in conjugation with a 4-oxo function and the presence of both hydroxyl groups in positions 3 and 5. Flavanones, flavones, and flavonols are the flavonoids present in *Citrus*, and although flavones and flavonols have been found in low concentrations in *Citrus* tissues, in relationship to flavanones, these types of compounds have been show to be powerful antioxidants and free radical scavengers. Some *Citrus* flavonoids can be used directly as repellents or toxins or be used in plant improvement programs to obtain more resistant crops. In addition, some *Citrus* flavonoids and their derivates, in the field of food technology, are principally known for their ability to provide a bitter or sweet taste and as bitterness inhibitor.

Keywords: *Free radicals, antioxidant; anticarcinogenic; antiinflammatory; platelet aggregation; antiallergic; analgesic; antimicrobial; food additives*

INTRODUCTION

It is widely accepted that fruits and vegetables have many healthful properties. There is a considerable amount of epidemiological evidence revealing an association between those who have a diet rich in fresh fruit and vegetables and a decrease risk of cardiovascular diseases and certain forms of cancer (Salah et al., 1995). It is generally assumed that the active dietary constituents contributing to these protective effects are the antioxidant nutrients, although more recent work has highlighted the additional role of the polyphenolic components of the higher plants (Hertog et al., 1993), which may act as antioxidants or agents of other mechanisms that contribute to their anticarcinogenic or cardioprotective actions. These compounds have applications in food stabilization due to their ability to protect against peroxidation of oxygen sensitive foods.

Flavonoids are a widely distributed group of polyphenolic compounds characterized by a common benzo-γ-pyrone structure, that have been reported to act as antioxidants in various biological systems (Morel et al., 1993; Salah et al., 1995; Wang and Zheng, 1992). Flavonoids are present in a wide variety of edible plants, especially in *Citrus* species. Four types of flavonoids (flavanones, flavones, flavonols, and anthocyanins, the last only in blood oranges) occur in *Citrus* (Horowitz and Gentili, 1977), and more than 60 individual flavonoids have been identified. Flavanones are the most abundant, but the highly methoxylated flavones exhibit

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higher biological activity even though they occur in much lower concentrations.

In this paper we study mainly the health-related properties of flavonoids, which are based in their antioxidant activity. These properties have been found to include anticancer, antiviral, and antiinflammatory activities, effects on capillary fragility, and an ability to inhibit human platelet aggregation. In addition, we study the role of *Citrus* flavonoids and their derivates in the field of food technology, in which they are principally known for their ability to provide a bitter or sweet taste and to inhibit bitterness.

ANTIOXIDANT ACTIVITY

Free radical formation is associated with the normal natural metabolism of aerobic cells. The oxygen consumption inherent in cell growth leads to the generation of a series of free radicals of oxygen, which are the most abundant and characteristic species in the phenomenon known as "oxidative stress". The interaction of these species with molecules of a lipid nature produces new radicals: hydroperoxides and different peroxides.

This group of radicals (superoxide, hydroxyl, and lipoid peroxides) may interact with biological systems in a clearly cytotoxic manner. These species interact with such life essential molecules as nucleic acids and proteins, producing oxidative reactions involving alterations and protein exchange, a fundamental process whose efficacy is subordinate to the functional activity of potential repair system (Saez et al., 1994).

Research into the mechanisms by which these free radicals can be blocked and/or scavenged is, therefore, of interest. In this respect, phenolic compounds, particularly flavonoids, have been shown to posses an important antioxidant activity toward these radicals, which is principally based on their structural characteristics. Together with an ability to capture electrons, these characteristics import great stability to the flavonoid radical formed, by means of a tautomeric dislocation, which prevents the propagating chain reactions of these oxygen free radicals.

A. Formation of Radicals Involved in Cellular Oxidative Processes. The different radicals responsible for the cell oxidation processes are the following: singlet oxygen ($^1O_2$); superoxide anion ($O_2^{\bullet -}$); hydroxyl radical ($^\bullet OH$); and peroxyl radical (R-OO$^\bullet$).

Singlet oxygen can be formed by photosensitization (Khan, 1985) with several sensitizer compounds. These reactions involve energy transfer from the excited triplet state of the sensitizer (^{3}Sens*) to molecular oxygen (Tournaire et al., 1993).

The remaining oxygenic reactive species are formed by a sequential electron reduction mechanism, by means of which molecular oxygen gives rise to superoxide radical, hydrogen peroxide, and hydroxyl radical successively.

The electron supply mechanism may arise from causes that are endogenous or exogenous to the medium under consideration. Included in the former are the mitochondrial and microsomal electron transport chain, phagocytic cell action mechanisms, and autoxidation reactions of different compounds or their generation as products or intermediates of cell oxidative metabolism (oxidases/oxygenases). Among the exogenous causes are solar radiation, photosensitization by visible light, ionizing radiation, thermic shocks, environmental contaminants, drug biotransformations, and the induction of redox cycles.

The hydroxyl radical is the most cytotoxic of all those so far described, and its formation from hydrogen peroxide (formed by reduction of the superoxide anion) may follow two mechanisms: the Haber–Weis and Fenton models.

In the first model the hydrogen peroxide is reduced to hydroxyl radical, hydroxyl anion, and molecular oxygen by the superoxide anion itself, while in the Fenton model a transition metal reduces the hydrogen peroxide.

Finally, the polyunsaturated fatty acids present in cell membranes are easily oxidized by both enzymatic and oxidative peroxidation via free radical chain reactions (Aust and Svingen, 1982). Initiation of lipid peroxidation can be induced by free radicals (superoxide, hydroxyl) and singlet oxygen produced in biological systems (Foote, 1976; Mead, 1976; Manson, 1982; Pryor, 1976; Pryor et al., 1982; Torel et al., 1986).

To the potential and aggressive cytotoxic action of these organic radicals must be added the mutagenic and carcinogenic capacity of these electronically inert species with their ability to interact with and alter genetic material.

It has been shown that lipid peroxidation can be inhibited by flavonoids acting as strong radical scavengers and singlet oxygen quenchers. It has also been proposed that flavonoids react with peroxy radicals, thus bringing about the termination of radical reactions.

When this wide range of reactions is encompassed in an overall autoxidation scheme of polyunsaturated fatty acids, the different points at which the flavonoids may exercise their antioxidant action may be observed (Bombardelli and Morazzoni, 1993):

1. antiradical activity ($^\bullet OH$, hydroxyl)
2. antilipoperoxidant activity (R$^\bullet$, alkyl; ROO$^\bullet$, peroxy; RO$^\bullet$, alcoxy)
3. antioxygen activity (O_2, $^1O_2$)
4. antiradical activity ($O_2^{\bullet -}$, superoxide)
5. metal chelating activity

The antioxidative capacity of a given substance depends of two principles: a high absolute reactivity against different radicals and a relatively high stability of the intermediately formed "antioxidant radical" (Bors et al., 1990a).

All the above mentioned radicals are oxidizing species and are assumed to form aroxyl radicals with phenolic compounds (Erben-Russ et al., 1987). Spectral observations of radical reactions with flavonoids are generally aided by the strong absorption characteristics of both the parent compound and their respective aroxyl radicals (Saran et al., 1987). The stability of the radical formed after scavenging may be explained by a very reactive secondary radical probably propagating rather than interrupting a chain reaction. Effective antioxidants have been shown to react in a 1:2 stoichiometry (Boozer et al., 1955), with one antioxidant molecule reacting with two radical species, the second reaction being a radical–radical recombination process. This type of reaction has been observed for aliphatic peroxyl radicals reacting with phenolic and arylamine antioxidants (Erben-Russ et al., 1987; Boozer et al., 1955) and α-tocopherol (Boozer et al., 1955; Tsuchiya et al., 1983; Winterle et al., 1984). Obviously, with the increasing stability of an antioxidant-derived aroxyl radical, a recombination reaction becomes more and more likely (Bors et al., 1990b).

Taking into consideration all the data discussed so far, the following parameter considerations may be used

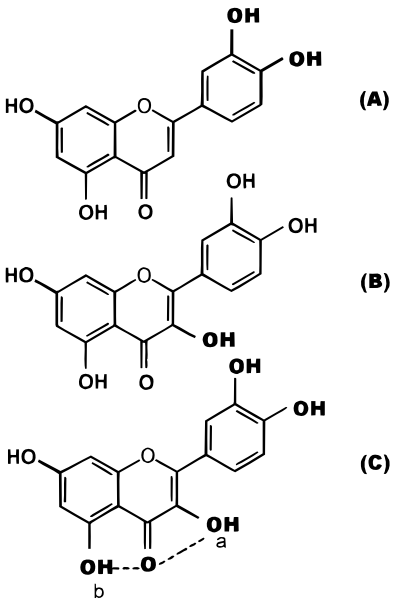

Figure 1. Functional groups of flavonoid structure with high antioxidant capacity.

to classify a certain flavonoid as an effective antioxidant: (a) the rate constants (k) with different types of radicals, (b) the stability and decay kinetics of the flavonoid-aroxyl radical, and (c) the stoichiometry of the radical-scavenging process.

B. Relationship between Flavonoid Structure and Radical-Scavenging Activity. According kinetic studies of the aroxyl radical formation and decomposition reactions, the antioxidant capacity of a flavonoid is closely linked to the particular structure of these polyphenols. Three structural groups are important for determining their radical scavenging and/or antioxidative capacity (Figure 1) (Bors et al., 1990a,b): (A) the *O*-dihydroxy (catechol) structure in the B-ring, which confers greater stability to aroxyl radicals, possibly through hydrogen bonding, and which participates in electron dislocation; (B) the 2,3-double bond in conjugation with a 4-oxo function, which are responsible for electron dislocation from the B-ring; and (C) the presence of both 3-(a)- and 5-(b)-hydroxyl groups (Figure 1C) for maximal radical-scavenging capacity and strongest radical absorption. From a kinetic standpoint, the 3- and 5-hydroxyl groups are equivalent because of their hydrogen bonds with the 4-keto group. Despite this general consideration, the presence or absence of the 5-hydroxyl group may have a decisive influence on the flatness of the flavonoid, introducing an stereoisomeric component into the electron dislocation capacity and thus into the stability of the flavonoid aroxyl radicals.

Obviously, the antioxidant capacity of any flavonoid will be determined by a combination of these structural elements. However, this capacity will not be similar or show the same degree of effectiveness toward each of the above mentioned radicals but will depend on the different action mechanisms which take place in each particular case. These mechanisms are influenced by structural factors other than those described, such as the presence or absence of glycosidic moieties in the flavonoid skeleton (glycosides or aglycons), the glycosylation site, and number and position of the free hydroxyls and of the sterified hydroxyls, etc.

Citrus Flavonoids. Most *Citrus* species accumulate substantial quantities of flavonoids during the development of their different organs (Castillo et al., 1992, 1993; Benavente-Garcia et al., 1993). All the flavonoids described in *Citrus* sp. can be classified into these groups:

- flavanones
- flavones
- flavonols

Recent studies on the quantitative distribution of these flavonoids in *Citrus* have shown that the 7-*O*-glycosylflavanones are the most abundant flavonoids in all species of the genus, whose aglycones are intermediates in the biosynthetic pathway (Benavente-Garcia et al., 1995; Lewinsohn et al., 1989). Although flavones and flavonols have been found in low concentrations in *Citrus* tissues, these types of flavonoids have been show to be powerful antioxidants and free radical scavengers. Table 1 shows some of the most studied flavonoids isolated in *Citrus*, their structure (flavanone, flavone, or flavonol), and their substitution groups. Table 2 relates these *Citrus* flavonoids with the functional groups involved in their antioxidant capacity (Figure 1).

Table 1. Principal Flavonoids Isolated in *Citrus*, with Structure and Substitution Pattern

flavonoid	*Citrus* sp.	C-ring structure[a]	substitution pattern
naringin	*C. paradisi*	FLA	5,4′-OH
	C. aurantium		7-*O*-Neo[b]
neoeriocitrin	*C. aurantium*	FLA	5,3′,4′-OH
			7-*O*-Neo
hesperidin	*C. sinensis*	FLA	5,3′-OH, 4′-OMe
			7-*O*-Rut[b]
diosmin	*C. sinensis*	FLO	5,3′-OH
	C. limonia		4′-OMe
			7-*O*-Rut[b]
rutin	*C. limonia*	FOL	5,7,3′,4′-OH
			3-*O*-Rut
naringenin	*C. paradisi*	FLA	5,7,4′-OH
eriodictyol	*C. aurantium*	FLA	5,7,3′,4′-OH
hesperetin	*C. sinensis*	FLA	5,7,3′-OH
			4′-OMe
apigenin	*C. paradisi*	FLO	5,7,4′-OH
luteolin	*C. Limonia*	FLO	5,7,3′,4′-OH
	C. aurantium		
diosmetin	*C. sinensis*	FLO	5,7,3′-OH
			4′-OMe
kaempferol	*C. paradisi*	FOL	5,7,3,4′-OH
quercetin	*C. limonia*	FOL	5,7,3,3′,4′-OH
tangeretin	*C. aurantium*	FLO	5,6,7,8,4′-OMe
	C. paradisi		
	C. limonia		

[a] FLA, flavanone; FLO, flavone; FOL, flavonol. [b] Neo, neohesperidoside; Rut, rutinoside.

Table 2. Functional Groups Involved in the Antioxidant Activity of *Citrus* Flavonoids

	type of antioxidant structure (as in Figure 1)				
flavonoid	A	B	C(a)	C(b)	others
naringin				X	4′-OH
neoeriocitrin	X			X	
hesperidin				X	3′-OH, 4′-OMe
diosmin		X		X	3′-OH, 4′-OMe
rutin	X	X	X[a]	X	
naringenin				X	4′-OH
eriodictyol	X			X	
hesperetin				X	3′-OH, 4′-OMe
apigenin		X		X	4′-OH
luteolin	X	X		X	
diosmetin		X		X	3′-OH, 4′-OMe
kaempferol		X	X	X	4′-OH
quercetin	X	X	X	X	
tangeretin		X			5,6,7,8,4′-OMe

[a] Glycosylated in 3-OH.

Flavonoid Quenching Capacity of Singlet Oxygen. Flavonoids have been reported to act as quenchers of singlet oxygen (Sorata et al., 1984; Matsuura, 1970; Takahama et al., 1974). To establish a structure−activity relationship, the rate constant of the chemical reactions of these flavonoids with singlet oxygen and their rate constants of physical quenching were determined by Tournaire et al. (1993) by kinetic measurements and near-IR singlet oxygen luminescence.

The chemical activity increased in the order naringenin < eriodictyol < tangeretin < luteolin < kaempferol < quercetin. The lowest flavanone activity must be emphasized. This, together with other evidence, suggests that the structural factors considered in Table 2 have the following increasing order of importance, A < C(b) < C(a) < B, where the basic element is the combination between the conjugation of the B-ring to the 4-oxo structure via a 2,3-double bond with the presence of the 3-hydroxyl group.

However, the physical activity (quenching) increased in the order naringenin < tangeretin < kaempferol < luteolin ≤ eriodictyol ≤ quercetin. This order points to an order of increasing structural effectiveness of C(b) < C(a) < B ≪ A. Obviously, flavonoids with a catechol B-ring structure are the best physical quenchers of singlet oxygen among these compounds. The importance of 3′,4′-dihydroxy structure can be seen by comparing eriodictyol (1.4×10^6 M^{-1} s^{-1}) and naringenin (5.0×10^4 M^{-1} s^{-1}).

The combination of both activities makes it possible to establish the true potential of each flavonoid, although the same order is not always evident since it is the experimental conditions which determine the value of the chemical and physical reaction constants and, with them, the total relative reactivity. Whatever the case, this value is basically dominated by the kinetic constant of physical quenching, which is, in general, higher than the chemical constant.

Citrus Flavonoids as Superoxide Scavengers. The superoxide anion scavenging activity of several *Citrus* flavonoids has been studied using several methods and with different structural correlations. Such activity is influenced significantly by the flavonoid concentration of the medium, increasing from zero to maximum activity or even bringing about the flavonoid autoxidation (Darmon et al., 1990). Nevertheless in this study the dominant structural element is the C-ring configuration, particularly the presence of a 3-hydroxyl group activating the 2,3-double bond. Only when the concentration is less than 100 μM is the presence of hydroxyl groups in the B-ring important to the scavenger properties of these compounds. When the scavenger's efficiency index is defined versus superoxide anion at different concentrations, it can be seen that kaempferol shows no activity at 10 μM but has a value above 60 at 100 μM. Quercetin, on the other hand, has an efficiency index of 24 at 10 μM and is autoxidized at 100 μM (Darmon et al., 1990). The determination of half-inhibition concentrations (IC_{50}, μM) of quercetin on superoxide anion generation shows a value of 56 ± 3.5 (Yuting et al., 1990), which agrees with the above mentioned data.

When the reaction rate constant with superoxide anions is calculated (Bors et al., 1990b), the negative influence of the catechol B-ring structure on this scavenging capacity becomes clear due to the generation of hydrogen peroxide in the reaction medium:

$$\text{Flav-B-ringdiOH} + O_2^{\bullet -} \rightarrow \text{Flav-B-ringOO}^{\bullet -} + H_2O_2$$

Hydrogen peroxide is a strong oxidant and constitutes a fundamental element in propagation of the cell oxidation reactions.

The absence of 3-hydroxyl group from flavanones and flavones weakens their scavenging capacity and sometimes increases the superoxide anion test signal, even in the absence of the catechol B-ring structure (Sichel et al., 1991). However, the presence of the 2,3-double bond renders the flavonoid structure more reactive, for which reason apigenin is a moderate scavenger while naringenin shows practically no activity.

Hydroxyl Radical Scavenging Activity. Flavonoids are excellent hydroxyl scavengers and, according to the studies of other authors (Darmon et al., 1990; Cillard and Cillard, 1988; Pincemail et al., 1985), they too seem to possess a degree of effectiveness which depends on their structure since, in growing order of activity, they are as follows:

$$\text{FOL-4′-OH} \ll \text{FLO-4′-OH} \leq \text{FLA-4′-OH} \leq \text{FLA-3′,4′-OR} \leq \text{FLO-3′,4′-OR} < \text{FOL-3′,4′-OR}$$

$$R = H \text{ or } CH_3$$

This order, which is based on their capacity to inhibit hydroxyl radical, shows the importance of the binary substitution model in B-ring even when the hydroxyls are esterified with methyl groups. The negative influence of the hydroxyl group in position 3 in monosubstituted B-ring compounds is significant. Moreover, it has been shown that the 3-*O*-glycosides are more active than their corresponding aglycones (Pincemail et al., 1985). The inhibition of hydroxyl radical generation is markedly inhibited by increasing flavonoid concentration above a threshold level, such increases depending on the individual flavonoid and reaction medium.

The reaction rate constants of kaempferol and quercetin (at either end of above mentioned order) show similar values [$(40-45) \times 10^8$ M^{-1} s^{-1}] (Erben-Russ et al., 1987). The greater activity of compound with B-ring catechol structure is due to the stability of the flavonoid radical generated in the process, as shall be seen below (Bors et al., 1990b).

Antilipoperoxidant Activity. A number of papers show that these are strong scavengers of lipoid radicals among flavonoids (Wang and Zheng, 1992; Morel et al., 1993; Torel et al., 1986; Bors et al., 1990a,b; Cillard and Cillard, 1988; Pincemail et al., 1985; Das and Pereira, 1990). The distinction between antiradical and antilipoperoxidant activity proposed by Pincemail et al. (1985) seems reasonable because the reactivity of *Citrus* flavonoids was occasionally different for the different free radicals species, and this could be the cause for the differing behavior toward the lipid peroxidative process (Bombardelli and Morazzoni, 1993). The antilipoperoxidant activity of flavonoids depends in a complex way on various factors, among them the nature of the organic substrate susceptible to oxidation, operational conditions and even the method used to evaluate this potential.

One of the most studied substances is linoleic acid and its autoxidation process. The antioxidant activity of flavonoids is related, in this case, to an inhibition of the formation of *trans*,*trans* hydroperoxide isomers of this acid. This inhibition exhibits the great H atom donating

Table 3. Inhibition by Flavonoids of Malondialdehyde Synthesis Using the Method Described in Ratty and Das (1988)

flavonoid at 50 μM	% MDA synthesized
control	100
rutin	28
quercetin	38
eriodictyol	38
kaempferol	45
neoeriocitrin	63
hesperetin	118
diosmin	131
naringin	167

ability of flavonoids to peroxy radical, thus terminating the chain radical reaction. In this case, the antioxidant efficiency increases in the order quercetin < rutin < luteolin < kaempferol. This order suggests the greater importance of the B-ring substitution pattern. In addition, the catechol structure of the B-ring seems less active and the absence of hydroxyl group in position 3 in flavonoids with this catechol structure seems to have a positive (Torel et al., 1986; Cillard and Cillard, 1988). The reaction rate constants of the two elements at the extremes of the above sequence, quercetin and kaempferol, show values of $(0.15-0.18) \times 10^8$ M^{-1} s^{-1} and $(0.34-0.42) \times 10^8$ M^{-1} s^{-1}, respectively (Erben-Russ et al., 1987; Bors et al., 1990b).

Inhibition studies on thermal autoxidation of palmitic acid showed similar results with the following order of effectiveness being established: naringenin < rutin < naringin < luteolin < apigenin < quercetin < kaempferol. This order clearly reflects the greater activity of monohydroxylated flavonoids compared with dihydroxylated flavonoids (kaempferol vs quercetin, apigenin vs luteolin). However, in the case of compounds with a catechol structure, the presence of a hydroxyl group in position 3 increases the antilipoperoxidant activity thanks to the 2,3-double bond (Das and Pereira, 1990).

Some authors have suggested that this activity of the dihydroxylated compounds in the B-ring is partially based in the good correlation between their iron-chelating ability and the antioxidant effect. Thus, the antiperoxidative capability of several flavonoids could be ascribed to concomitant activities of scavenging free radicals and of chelating iron (Morel et al., 1993; Havsteen, 1983; Afanas'ev et al., 1989).

It must be pointed out that in most of these studies the flavanones hesperidin and naringin, which are the most abundant in *Citrus* tissues, show an almost total absence of antilipoperoxidant activity (Wang and Zheng, 1992). However, other studies point to the important hypolipidemic effects of hesperidin, which may be related to its antioxidant capacity (Fraga et al., 1987; Lonchampt et al., 1989; Monforte et al, 1995).

Our experiment concerning stearic acid autoxidation (data not published) (Table 3) using the malondialdehyde methods (Ratty and Das, 1988) seems to confirm the importance of the structural elements: A (catechol B-ring) and B-C(a) (2,3-double bond associated with the 3-hydroxyl group).

Stability of Flavonoid Aroxyl Radicals. The aroxyl radical species of flavonoids has a molecular structure capable of an extensive electron dislocation, which is a prerequisite for radical stabilization, generating multiple mesomeric structures (Bors et al., 1990b).

The decay rate constants of flavonoid aroxyl radicals generated by their interrelation with other radicals show that all the most stable aroxyl radicals, without exception, contain the 3′,4′-catechol B-ring substitution pattern. All other phenolic compounds form far less stable aroxyl radicals.

Several *Citrus* flavonoids show the following values of second-order decay rate constants (value $\times 10^6$ M^{-1} s^{-1}): apigenin (170) < kaempferol (140) ≪ quercetin (3.4) < eriodictyol (0.4) < luteolin (0.2) (Bors and Saran, 1987).

ANTICARCINOGENIC PROPERTIES

Recently plant flavonoids have attracted attention as potentially important dietary cancer chemoprotective agents (Hertog et al., 1993). In addition, the possible antitumor action of certain flavonoids has also generated interest (Kandaswami et al., 1991; Hirano et al., 1994; Elangovan et al., 1994).

Flavonoids, due to their antioxidant properties and their ability to absorb UV light, may act in all stages of the carcinogenic process: damage to the DNA (or initiation step), tumor growth (or promotion step), and invasion (or proliferative step). We shall examine the role of *Citrus* flavonoids in each of those steps.

A. Antimutagenic Effects. Due to their absorption of ultraviolet light, flavonoids can protect DNA from damage. This effect is one of the physiological functions attributed to flavonoids in the plant kingdom (Stapleton and Walbot, 1994), although it may be generalized to animal cells, particularly those of mammals. Recent experiments in template plasmid DNA irradiated with UV-B light, showed the protective effect of naringenin and rutin against UV-induced DNA damage (Kooststra, 1994). In parallel, flavonoids are able to quench free radicals which may promote mutations when they are generated in the vicinity of DNA. This radical scavenging ability, in a direct or endogenous enzyme-mediated manner, is responsible for the protective effect of flavonoids in whole-body γ-ray irradiated mice (Shimoi et al., 1994).

Flavonoids may also protect DNA by interacting directly with carcinogens that have escaped detoxification processes, as occurs in the chromosome aberrations induced by bleomycin (Heo et al., 1994). These results showed that in vitro or in vivo treatment of lymphocytes with galangin, a flavonoid metabolic derivative, suppressed the induction of chromosome aberrations by bleomycin in a galangin, dose-dependent manner.

B. Antiproliferative Effects. In vivo studies on inhibition of experimental induction of tumors have been essentially effected on flavonols. The intake of quercetin in experimental diets lowered the incidence of colon tumors in azoxymethanol treated rats (Deschener et al., 1993) as well as fibrosarcoma in mice induced by 20-methylcolanthrene (20-MC) (Elangovan et al., 1994). Subcutaneous injections of 20-MC produced 100% tumor incidence and the onset of tumors within 7 weeks while flavonoid-treated mice produced tumors in the 9th week, and the tumor incidences in mice treated with quercetin- and luteolin-mixed diets were 52% and 60%, respectively. Subcutaneous administration of 20-MC along with the flavonoid compounds (quercetin, luteolin) was found to have a significant effect on tumor expression.

Test-diet treated animals showed a reduction in lipid peroxides and cytochrome P450. In vitro [^{3}H]thymidine incorporation showed that flavonoids inhibited DNA synthesis in fibrosarcoma cells. The possible mode of action of this compound may be through their influence on the initiation and promotion phases of the carcinogenic processes coupled with enhancement of

the detoxification process. Similarly, *Citrus* flavonoids inhibit processes that are believed to represent nonspecific markers of tumor promotion: epidermal ornithine decarboxylase induction, accelerated incorporation of ^{32}P inorganic phosphate into membrane phospholipid, and activated protein kinase C (Manach et al., 1996).

In vitro, flavonoids display an antiproliferative effect on various human neoplasic cell lines, for example myeloid and lymphoid leukaemia cells (Larocca et al., 1990), gastric cancer cells (Yoshida et al., 1990), ovarian cancer cells (Scambia et al., 1990), prostate cancer cells (Peterson and Barnes, 1993), and squamous cell carcinoma (Kandaswami et al., 1991). Lipophilic *Citrus* polymethoxylated flavonoids (such as nobiletin and tangeretin) inhibited the cell growth of squamous cell carcinoma in a dose-dependent manner. At 8 μg/mL these two flavonoids appeared to cause cell death as incubation was prolonged (Kandaswami et al., 1991).

The intracellular mechanism involved in such effects are still poorly understood in spite of the great number of studies on this subject. Flavonoids affect cell metabolism in various ways, either at the cell membrane level or the intracellular enzymes. Flavonoid effects frequently include an inhibition of glycolysis, which is interesting because this metabolic pathway is generally very active in tumor cells (Manach et al., 1996). Flavonols depress lactate production in leukaemia cell lines, or Erlich ascite tumor cells (Soulinna et al., 1975). These effects could be due to the inhibition of lactate transport as well as various membrane ATPases (Belt et al., 1979; Shoshan and MacLennan, 1981). The inhibition of Na/K ATPases might disturb the cellular ionic gradient and hence energy metabolism, as well as other major cell functions, such as protein synthesis and DNA replication, due to the acidification of the cell pH (Hirano et al., 1989).

The flavonoids may affect the activity of various enzymes involved in the transduction of mitogenic signals (kinases, phospholipases, phosphodiesterases) and regulate other enzymes which are critical for cell growth and proliferation.

Finally, *Citrus* flavonoids may strengthen the effects of other cancer therapies. For example, quercetin greatly increases the growth-inhibitory activity of adriamycin (ADR) on MCF-7 ADR-resistant human breast cancer (Scambia et al., 1994) and MCT-15 colon cells (Critchfield et al., 1994).

C. Inhibition of Carcinogenic Cell Invasion. The prognosis of human cancers is partially correlated with tumor growth but is mainly determined by the invasiveness of tumors and the metastatic capability of the tumor cell population (Attaway, 1992). Malignant tumors not only grow but also invade the surrounding normal tissues. In this process the interaction of the tumor cell population with the extracellular matrix (ECM) is believed to be crucial for invasion, since the activities of invading cells such adhesion, proteolysis, and motility are influenced by ECM molecules (Liota et al., 1988). The currently used techniques of chemotherapy and irradiation attempt to slow the rate of growth and proliferation, although these treatments have little anti-invasive activity. *Citrus* flavonoids can inhibit the invasion of chick heart fragments and syngenic mice liver by malignant mouse (MO_4) tumor cells (Bracke et al., 1989). The mechanism of *Citrus* flavonoid anti-metastatic and anti-invasive activity is not via the ECM or cell surface glycoprotein (laminin) or via the cytoplasmatic microtubule complex, so it is assumed that the activity found is brought on by the inhibition of cell motility (Bracke et al., 1989, 1991).

CARDIOVASCULAR PROPERTIES

Certain flavonoids have been shown to effect cells of the vessel wall, blood platelet function, leukocyte function, blood coagulation, blood rheology, and ultimately thrombosis. Several studies indicates that certain flavonoids may have a protective and therapeutic effect in coronary heart disease.

A. Effect on Capillary Fragility. The effect of flavonoids on bleeding and capillary fragility was first reported by Szent-Gyorgyi in 1938, who considered *Citrus* flavonoids to have vitamin activity, which he named vitamin P.

Capillary damage includes increased permeability, seepage of blood and plasma constituents into the tissues, followed by an inflammatory reaction. These illnesses can now be treated by many drugs, which are based on flavonoids, principally those derived from hesperidin and rutin, mainly diosmin or hesperidin methylchalcone and hydroxyethylrutosides, respectively, which act primarily on the microvascular endothelium to reduce hyperpermeability and edema. In patients with chronic venous insufficiency or diabetes, hydroxyethylrutosides improve microvascular perfusion and microcirculation and reduce erythrocyte aggregation. The preparations also alleviate symptoms in patients with severe hemorrhoids (Waldworth and Faulds, 1992). Diosmin produces a significant decrease in venous capacitance, venous distensibility, and venous emptying time (Geroulakos and Nicolaides, 1994). Other pharmacological activities of diosmin are (1) interference with edema mechanisms and (2) interference with hyperpermeability induced by bradykinin or ischemia in rats and increased lymphatic flow in anaesthetized dog (Labrid, 1994).

Some investigators have related the increase in vascular tone observed in vivo after treatment with these drugs to the inhibition of amine reuptake where the flavonoids act as antagonists of plasma membrane amine transporters (Sher et al., 1992). The vasodilatory mechanism of flavonoids seems to be the inhibition of protein kinase C (Duarte et al., 1993).

B. Effect on Platelet Aggregation. *Citrus* flavonoids show an antiadhesive and antiaggregation action against red cell clumping (Robbins, 1974). Methoxylated flavonoids (nobiletin, tangeretin, etc.) are much more active than hydroxylated compounds, and their action might be similar to that of acetylsalicylic acid which has been shown to inhibit platelet aggregation. More recently, studies have been shown that both tangeretin and nobiletin inhibit platelet aggregation in the 30 μM range for ADP and collagen induced aggregation, comparing favorably with quercetin and fisetin, although neither naringin nor hesperidin was active even at 200 μM (Beret and Cazenave, 1988).

Other authors have reported that flavonoids are effective inhibitors of platelet adhesion, aggregation, and serotonin secretion although the degree of inhibition depends on the type of inducer and on the flavonoid structure (Manach et al., 1996). Fisetin, kaempferol, or quercetin at 30 μM inhibits the platelet aggregation induced by arachidonic acid, whereas myricetin is effective only at concentrations greater than 150 μM. The aggregation induced by ADP is less affected by flavonoids, except by myricetin. Quercetin, fisetin, and

myricetin show a more pronounced inhibitory effect against collagen-induced aggregation (Tzeng et al., 1991). These differing effects of flavonoids suggest that several mechanisms are involved; for example, inhibition of the enzyme phosphodiesterase could be one such mechanism through its effect on cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) (Beret et al., 1986). Cyclic nucleotides are known to be involved in several fundamental processes in the body including blood platelet aggregation. Another mechanism might involve inhibition of cycloxygenase, with a consequent depression of thromboxane A2 synthesis (Tzeng et al., 1991). Inhibition of the intracellular mobilization of Ca^{2+} and of its influx across the plasma membrane could also play a role (Ozaki et al., 1993).

C. Effect on Coronary Heart Disease. In vitro, flavonoids inhibit the oxidation of low-density lipoprotein (LDL) and reduce thrombotic tendencies. Flavonoid intake has been inversely and significantly associated with death from coronary heart disease and showed an inverse relation with the incidence of myocardial infarction (Hertog et al., 1993). There is evidence that free-radical oxidation of LDL plays an important part in atherogenesis. Flavonoids, as mentioned above, are scavengers of free radicals such as superoxide anions and peroxy radicals and will thus interrupt radical chain reactions.

In vitro some flavonoids, including *Citrus* flavonoids, inhibit the oxidative modification of LDL by macrophages, mainly by inhibiting the generation of hydroperoxides and protecting the α-tocopherol present in lipoprotein oxidation. It is possible that flavonoids reduce the rate of oxidized compound, thus inhibiting the growth of atherosclerotic complications (Hertog et al., 1993). On the other hand, *Citrus* flavonoids exert an apparent regulatory action on erythrocyte aggregation and concentration (hematocrit), the two major factors affecting blood viscosity and flow (Robbins, 1976). The methoxylated compounds exhibit a highly significant antiadhesive action on erythrocytes and inhibit erythrocyte sedimentation rate (ESR), while hydroxylated glycosides accelerate aggregation and ESR. The aggregating effect of flavonoids has been interpreted as a mechanism whereby erythrocyte concentration is reduced since clumped cells are sequestered and removed from circulation. Such action appears to be selective and similar to that of polylysine which preferentially aggregates old erythrocytes with less charge than young erythrocytes which have higher negative charges (Robbins, 1976).

The hematological and serum chemical profiles on patients with coronary heart disease show significantly higher serum cholesterol, hematocrit, and ESR. In such patients the intake of methoxylated flavonoids would appear to be beneficial, exerting a dietary control of the blood high viscosity syndrome.

ANTIINFLAMMATORY, ANTIALLERGIC, AND ANALGESIC ACTIVITY

The possible activity of flavonoids in antiinflammatory and antiallergic responses were well documented by Gabor (1986). Recent studies on *Citrus* flavonoids (Galati et al., 1994; Tordera et al., 1994; Huet, 1982) have shown the antiinflammatory dose-dependent activity of hesperidin, diosmin, and other flavonoids and their influence on the metabolism of arachidonic acid and histamine release. These flavonoids significantly inhibit lysosomal enzyme secretion and arachidonic acid release from membranes by inhibiting lipoxygenase, cycloxygenase, and phospholipase A_2.

The inhibition of arachidonic acid release in the inflamed cells would provide less arachidonic substrate for the lipooxygenase and cycloxygenase pathways, leading to a lesser quantity of endoperoxides, prostaglandins, prostacycline, and thromboxanes on the one hand and hydroperoxy- and hydroxyeicosatrienoic acids and leukotrienes on the other (Gabor, 1986). Such an effect confirms the decrease in histamine release, and in fact, histamine is known to act in the first stage of the inflammatory process (Middleton, 1986).

Diosmin behaves as a powerful protective agent against inflammatory disorders. Diosmin reduced edema formation and inhibited the synthesis for prostaglandin E2 (78.5%), prostaglandin F2 (45.2%), and thromboxane B2 (59.5%). Intravenous injection of diosmin reduced hyperglycemia induced by injection of alloxan in rats. This effect was linked to its ability to scavenge active oxygen radicals, as demonstrated in vitro using human neutrophils or mouse peritoneal macrophages (Jean and Bodinier, 1994).

Another flavonoid activity related with histamine release is antiallergic activity. Middleton et al. (1981), who studied the effect of quercetin on ragweed antigen-induced basophil histamine release in subjects with hay fever, reported a dose-dependent inhibitory effect and an antagonistic effect of calcium in the inhibition of histamine release by quercetin. Other *Citrus* flavonoids (hesperidin, tangeretin, and nobiletin) exhibited slight to moderate activity (Middleton and Drzewiwcki, 1982). However, a significant ability of hesperidin to inhibit histamine release from pertinent mast cells of rats has recently been reported (Matsuda, 1991), suggesting that hesperidin is an effective component with an antiallergic action.

ANTIMICROBIAL ACTIVITY

One of the properties of flavonoids most related with their physiological action in plants is their antifungal and antiviral activity (Huet, 1982).

An important structure−activity relationship exists, which strongly influences the activity of a flavonoid against viruses (Kaul et al., 1985). Quercetin and hesperetin actively inhibited the infectivity and/or replication of herpes simplex type viruses, polio viruses, parainfluenza type viruses, and syncytial viruses, although the quantitatively important grapefruit flavonoid, naringin, totally lacked this ability (Kaul et al., 1985). Quercetin caused a concentration-dependent decrease in the infectivity of each of the above viruses. The fact that neither dialysis nor ultracentrifugation could dissociate the viruses from quercetin after 1 h of interaction suggests the formation of quercetin−virus complexes which may have lost the ability to induce infection.

The antipicornoviral activity of the methoxyflavone structure was dependent on the 3′-methoxy and the 4′-hydroxy function in the parent flavone structure. 4′-Hydroxy-3′-methoxyflavones with a polysubstituted A-ring exhibited higher antiviral activity than the corresponding monosubstituted compounds.

In summary, research into the antiviral activity of naturally occurring plant flavonoids has barely scratched the surface. It is possible that a compound exists in nature that has the correct structure relationship and

pharmacokinetic properties to make it a better antiviral agent than synthetic drugs now in use.

ECOLOGICAL FUNCTION

The cause−effect relationship between flavonoid ingestion and the behavior of certain animals has been described in studies which are now considered classical (Bradbury and White, 1953). This group of polyphenols plays an important role in the relationship of plants with the environment and in particular with those animals that eat them.

Monophagic insects are chemically attracted to certain plants by some compound or compounds, among which volatiles are important alongside others which determined palatability. For example, in the case of the silk worm, it is isoquercetin which is principally responsible for the worming interaction with mulberry. In other cases, such as that of *Scolity mediterraneus* (a pest which feeds on the peel of *Prunus* sp.), taxifolin, pinocembrin, and dihydrokaempferol have been shown to be involved. With regard to the effectiveness of one type of compound or another in stimulating the feeding animal, it seems that flavonoid glycosides are better than aglycones, with quercetin 3-rutinoside (rutin) stimulating the feeding behavior of *Manduco sexta* in tobacco to a greater extent than the aglycon, quercetin (De Boer and Hanson, 1987). Flavonoids can stimulate a plant-eater relationship not only from an ingestion viewpoint but also, as in the case of the butterflies *Papilus* sp, with regard to egg-laying, and it has been demonstrated that naringin, hesperidin, rutin, and narirutin stimulate this activity in *Papilus* sp. which feed off *Citrus* sp.

In addition to being an attractant (animals), flavonoids can also repel or even be toxic to others; rutin and isoquercitrin, for example, and inhibit larval worm growth in the fruit (Duffey and Isman, 1981) and heart of the tobacco plant (Elliger et al., 1980). With respect to mammals, tangeretin produces an increase in the neonatal death rates of rat (Stout et al., 1964) and eupatoretrin has a growing cytotoxic effect as the degree of hydroxylation and methylation (Kupchan et al., 1969).

Studies of the effect of flavonoids on aphids (Dreyer and Jones, 1981) have shown that eriodictyol, homoeriodictyol, luteolin, and, to a lesser extent, vitexin and naringenin have a repellent effect while flavanones and flavones-*O*-glucosides do not. Curiously, neohesperidin and neohesperidin dihydrochalcone (an artificial derivate of neohesperidin) have a repellent effect that is ten times less than that of the natural phloridzina.

Plant varieties which have been selected for their palatability and nutritional qualities for humans are usually more susceptible to diseases. The use of synthetic pesticides is expensive and can cause environmental problems and leave residues in the crops themselves or soil. Since certain products may be toxic to insects but not mammals, which they can attract or repel, growing interest has been expressed in the possibility of controlling pest by environmentally friendly means. Flavonoids are an ideal source of such since they can be used directly as repellents or toxins or be used in plant improvement programs to obtain more resistant crops which themselves may have a repellent or selectively toxic effect.

The use of flavonoids in the above way or as complementary substances in integrated pest management opens up new and interesting fields of study for scientists and agronomists. As an example, one may cite the use of quercetin glucosides to combat pests such as *Spodoptera* sp. and *Heliothis* sp., which are commonly found in intensive cultivation of crops such a strawberries and tomatoes. These pests do not die directly through the ingestion of the quercetin glucosides; rather, the first and second larval stages are prolonged, which means that they are more susceptible to attack by parasites or predators in integrated pest management systems and are exposed to certain specific pesticides for longer times.

OTHER PROPERTIES

Other properties of flavonoids, which are not related with their antioxidant capacity, mainly involve their capacity to modify the flavor and/or taste of different compounds and preparations used in the food and cosmetics industry.

Among the flavonoids and derivates occurring in the *Citrus* genus, compounds with widely differing properties exist. Some have a very bitter taste, others are tasteless, while others still, such as the dihydrochalcones (Horowitz and Gentili, 1969), are extremely sweet.

Such differences in taste between flavonoids have, as in the case of their antioxidant properties, a structural basis. One of the most important structural differences when establishing flavonoid taste is the type of glycosidic chain: rutinoside (rhamnopyranose-α-1,6-glucopyranose) and neohesperidoside (rhamnopyranose-α-1,2-glucopyranose). The first produces tasteless and the second bitter compounds (Horowitz and Gentili, 1969), so that the neohesperidoside flavanones naringin, poncirin, and neohesperidin are bitter, while the rutinoside flavanones isonaringin, neoponcirin, and hesperidin are tasteless.

Another very important structural factor is the C-ring of the flavonoid structure. Oxidation of this ring to convert the flavanones, naringin and neohesperidin, into their corresponding flavones, rhoifolin and neodiosmin, leads to loss of bitterness. This fact suggests that the planar structure of flavones, with higher conjugation, suppresses the taste responses, while the less conjugated, nonplanar flavanones favor then (Horowitz and Gentili, 1969).

Another type of reaction which alters the C-ring of flavanones is their conversion into chalcones and dihydrochalcones (Horowitz and Gentili, 1963). These compounds are extremely sweet when derived from neohesperidoside flavanones (naringin dihydrochalcone and neohesperidin dihydrochalcone) but tasteless when they come from rutinoside flavanones (isonaringin dihydrochalcone and hesperidin dihydrochalcone). Even among the neohesperidoside dihydrochalcones there is a gradation of taste, the presence of a free hydroxyl in the B-ring being necessary for a greater degree of sweetness (Horowitz and Gentili, 1969).

Yet other *Citrus* flavonoids exist which, although tasteless, can alter the taste of fruit juice and other food products. For example, the addition of the flavone neodiosmin to citric juices can significantly reduce the perception threshold of the bitterness produced by limonin (Guadagni et al., 1976).

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