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Citrus flavonoids: Molecular structure, biological activity and nutritional properties: A review

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Abstract

Epidemiological studies have shown an inverse relationship between dietary flavonoid intakes and cardiovascular diseases. *Citrus* fruits are the main winter fruits consumed in the Mediterranean diet, so they are the main source of dietary flavonoids. The possible beneficial effects are due, not only to the high amounts of vitamins and minerals, but also to the antioxidant properties of their flavonoids. Dietary flavonoids may help to supplement the body antioxidant defences against free radicals. These compounds' possible beneficial effects are due to their antioxidant activity, which is related to the development of atherosclerosis and cancer, and to anti-inflammatory and antimicrobial activity. The present review summarizes the existing bibliography on biological and pharmacological studies of *Citrus* flavonoids, both *in vitro* and *in vivo*.

Keywords: Citrus fruits; Flavonoids; Antioxidant; Atherosclerosis; Cancer

1. Introduction

The possible beneficial effects of foods are due to micronutrients (for example vitamins and minerals) and to functional food ingredients and antioxidant nutraceuticals, "phytochemical substances". Phytochemicals can be defined as substances found in edible fruits and vegetables that, daily ingested, may exhibit a potential for modulating human metabolism in a manner favourable for the prevention of chronic and degenerative diseases. Nowadays, many studies are carried out on the thousands of phytochemicals that may have important physiological effects. An increased consumption of fruit and vegetables, typical Mediterranean diet foods, may protect against degenerative pathologies, such as cancer and atherosclerosis (Hertog, Hollman, & Katan, 1992; Keys, 1995). Epidemiological studies have shown an inverse relationship between dietary flavonoid intake and cardiovascular diseases (Hertog, Hollman, Katan, & Kromhout, 1993). Among the phytochemicals, flavonoids are widely contained in *Citrus* fruits (Yao et al., 2004). *Citrus* fruits are the principal source of such important nutrients. They contain vitamin C, folate, dietary fibre and other bioactive components, such as carotenoids and flavonoids, which are suggested to be responsible for the prevention of cancer and degenerative diseases (Ejaz, Ejaz, Matsuda, & Chae, 2006).We will consider the biological activity and the healthy effects of *Citrus* flavonoids as antioxidant compounds.

2. The *Citrus* flavonoids

According to their molecular structures, flavonoids are divided into six classes: flavones, flavanones, flavonols, isoflavones, anthocyanidins and flavanols (or catechins) (Fig. 1) (Peterson, Dwyer, & Dsc, 1998). Flavonoids

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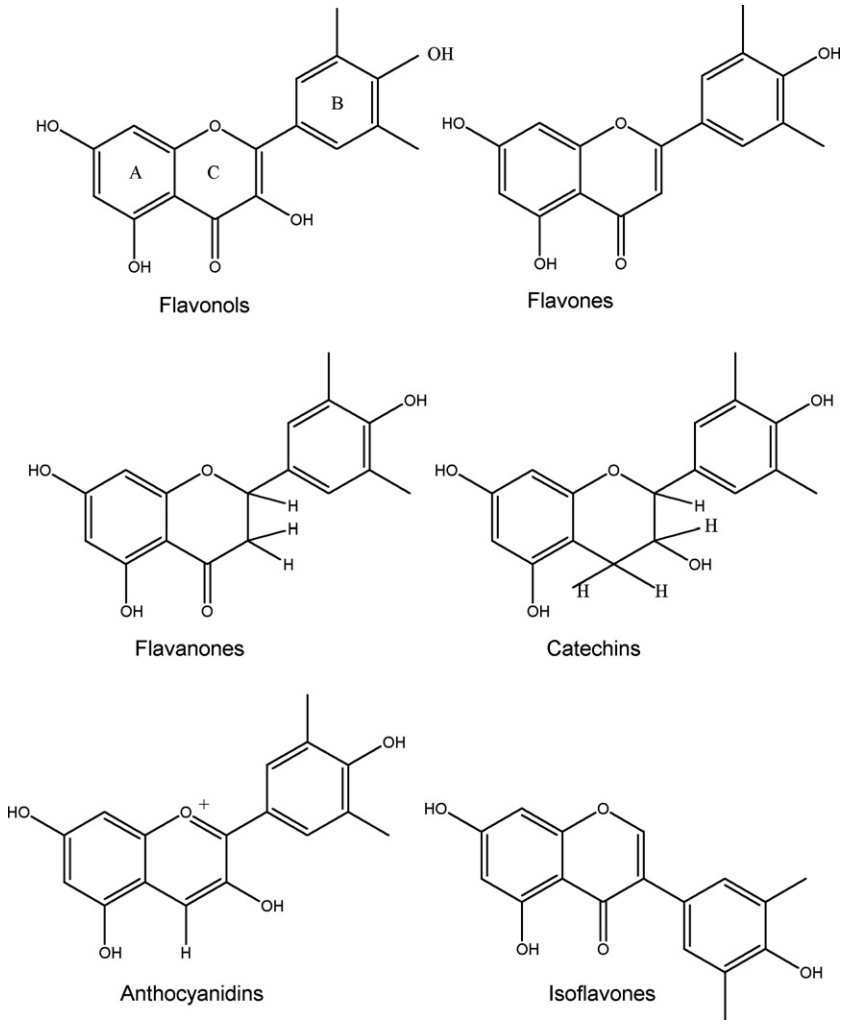


Fig. 1. Molecular structures of flavonoids. The basic structure consists of the fused A and C ring, with the phenyl ring B attached to through its 1′ position to the 2-position of the C ring (numbered from the pyran oxygen).

identified in *Citrus* fruits cover over 60 types, according to the five classes mentioned (Horowitz & Gentili, 1977): flavones, flavanones, flavonols, flavans and anthocyanins (the last only in blood oranges). Table 1 shows the main chemical structures of some flavonoids isolated from *Citrus* fruits, their structures (flavanone, flavone, or flavonol) and their chemical groups.

Citrus flavanones are present in the glycoside or aglycone forms. Among the aglycone forms, naringenin and hesperetin are the most important flavanones (Table 1). Among the glycoside forms, two types are classified: neohesperidosides and rutinosides (Gionfriddo, Postorino, & Bovalo, 1996; Macheix, Fleuriet, & Billot, 1990). Neohesperidosides, flavanones, naringin, neohesperidin and neoeriocitrin consist of a flavanone with neohesperidose (rhamnosyl-α-1,2 glucose) and they have a bitter taste (Table 1), while rutinosides (flavanones, hesperidin, narirutin and didymin) have a flavanone and a disaccharide residue e.g. rutinose (ramnosyl-α-1,6 glucose) and they are without taste (Table 1). Flavanones are usually present in diglycoside form, conferring the typical taste to *Citrus* fruits (Macheix et al., 1990).

Phenolic compounds and flavonoid profiles are detected by HPLC-MS. These compounds can be divided into two groups according to the lag-times: the first eluted are flavanone glycosides while the second group are polymethoxylated flavones (subsequently eluted they are less polar) (Fig. 2) (Mouly, Gaydou, & Auffray, 1998).

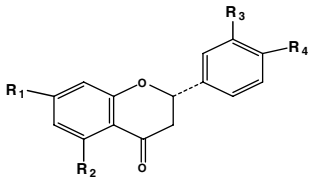
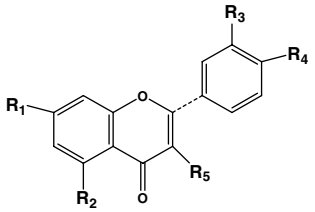
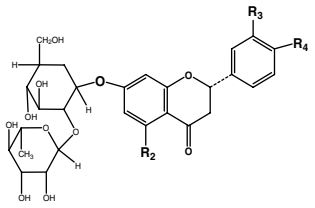
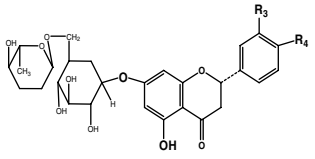
Among flavonoids, the anthocyanins are structurally derived from pyran or flavan and, in particular, oxygen attributes a basic property to this molecule. They can be present as aglycones (anthocyanidins metabolites of flavones) (Fig. 1).

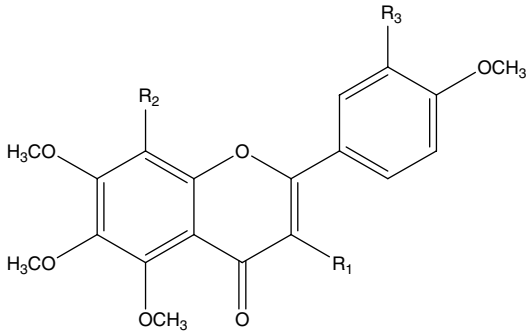
Catechins, leucoanthocyanin and proanthocyanins are in the flavan group, as also are tannins. They can be found in monomer, dimer and polymer forms, respectively monoflavans, biflavans or triflavans (Cook & Samman, 1996).

3. Distribution of *Citrus* flavonoids

Flavonoids are a group of pigments contained in plants and they are responsible for flower and fruit colouration. Flavonoids are present in dietary fruits and vegetables (Macheix et al., 1990). The *Citrus* peel and seeds are very

Table 1
Structural characteristics and molecular weights of Citrus flavanoids in the aglycone and glycoside forms

Compounds	Structural formula	Molecular weight	Molecular formula
Flavanone aglycone forms			
			
Naringenin	$R_1 = OH$; $R_2 = OH$; $R_3 = H$; $R_4 = OH$	271 Da	$C_{15}O_5H_{11}$
Hesperetin	$R_1 = OH$; $R_2 = OH$; $R_3 = OH$; $R_4 = OCH3$	288 Da	$C_{15}O_6H_{13}$
Isosakuranetin	$R_1 = OH$; $R_2 = OH$; $R_3 = H$; $R_4 = OCH3$	285 Da	$C_{16}O_5H_{13}$
Heridictyol	$R_1 = OH$; $R_2 = OH$; $R_3 = OH$; $R_4 = OH$	287 Da	$C_{15}O_6H_{11}$
Flavone and flavonol aglycone forms			
			
Apigenin	$R_1 = OH$; $R_2 = OH$; $R_3 = H$; $R_4 = OH$; $R_5 = H$	270 Da	$C_{15}O_5H_{10}$
Luteolin	$R_1 = OH$; $R_2 = OH$; $R_3 = OH$; $R_4 = OH$; $R_5 = H$	286 Da	$C_{15}O_6H_{10}$
Diosmetin	$R_1 = OH$; $R_2 = OH$; $R_3 = OH$; $R_4 = OCH_3$; $R_5 = H$	288 Da	$C_{15}O_6H_{12}$
Quercetin	$R_1 = OH$; $R_2 = OH$; $R_3 = OH$; $R_4 = OH$; $R_5 = OH$	302 Da	$C_{15}O_7H_{10}$
Kämpferol	$R_1 = OH$; $R_2 = OH$; $R_3 = H$; $R_4 = OH$; $R_5 = OH$	286 Da	$C_{15}O_6H_{10}$
Flavanone neohesperidoside forms			
			
Naringin	$R_2 = OH$; $R_3 = H$; $R_4 = OH$	604 Da	$C_{29}O_{14}H_{32}$
Neohesperidin	$R_2 = OH$; $R_3 = OH$; $R_4 = OCH3$	634 Da	$C_{30}O_{15}H_{34}$
Poncirin	$R_2 = OH$; $R_3 = H$; $R_4 = OCH3$	588 Da	$C_{29}O_{13}H_{32}$
Neoeriocitrin	$R_2 = OH$; $R_3 = OH$; $R_4 = OH$	620 Da	$C_{29}O_{15}H_{32}$
Flavanone rutinside forms			
			
Narirutin	$R_3 = H$; $R_4 = OH$	551 Da	$C_{29}O_{11}H_{27}$
Hesperidin	$R_3 = OH$; $R_4 = OCH_3$	583 Da	$C_{30}O_{12}H_{31}$
Didymin	$R_3 = H$; $R_4 = OCH_3$	567 Da	$C_{30}O_{11}H_{31}$
Eriocitrin	$R_3 = OH$; $R_4 = OH$	571 Da	$C_{29}O_{12}H_{31}$
Diosmin	$R_3 = OH$; $R_4 = OCH_3$	583 Da	$C_{30}O_{12}H_{31}$



R_1	R_2	R_3	
H	H	H	Scutellarein
H	H	OCH_3	Sinensetin
H	OCH_3	H	Tangeretin
OCH_3	H	OCH_3	Hexamethoxyflavone
H	OCH_3	OCH_3	Nobiletin
OCH_3	OCH_3	OCH_3	Heptamethoxyflavone

Fig. 2. Common *Citrus* polymethoxylated flavones.

rich in phenolic compounds, such as phenolic acids and flavonoids. The peels are richer in flavonoids than are the seeds (Yusof, Mohd Ghazali, & Swee King, 1990). Since a *Citrus* fruit is peeled, peel and seeds are not used. It is necessary to estimate these by-products as natural antioxidants in foods (Kroyer, 1986; Pratt & Hudson, 1990).

The 7-O-glycosylflavanones are the most abundant flavonoids in all *Citrus* fruits (Benavente-García, Castillo, Sabater, & Del Rio, 1995; Lewinsohn, Berman, Mazur, & Gressel, 1989); for example, lemon peel is rich in glycosidic flavonoids (Park, Avery, Byers, & Nelson, 1983). Among the neohesperidoside flavanones, naringin, neohesperidin and neoeriocitrin, are mainly present in bergamot, grapefruit and bitter orange juices. Among rutinoside flavanones, hesperidin, narirutin and didymin, are present in bergamot, orange, mandarin and lemon juices (Horowitz, 1986).

Flavanone chemical structures are specific for every species, which renders them markers of adulteration in commercial juices (Marini & Balestrieri, 1995; Mouly, Arzouyan, Gaydou, & Estienne, 1994; Ooghe & Detavernier, 1997).

The seed and peel compositions are not always the same in *Citrus* fruits. For example, the lemon seed mainly contains eriocitrin and hesperidin, while the peel is rich in neoeriocitrin, naringin and neohesperidin. Moreover, the glycosylated flavanone concentrations are different; neoeriocitrin and naringin have similar concentrations in peel while, in seed, eriocitrin is 40 times more abundant than is naringin (Bocco, Cuvelier, Richard, & Berset, 1998). Neohesperidin, naringin and neoeriocitrin are extracted from peel in great amounts. Bitter orange is a very interesting neohesperidin and naringin source; these compounds can be useful for the production of sweeteners. The seeds of bergamot are the most important source of the glycosylated flavanones, naringin and neohesperidin; lemon is rich in eriocitrin and hesperidin. All the other *Citrus* fruits have small amounts of glycosylated naringin (Bocco, Cuvelier, Richard, & Berset, 1997; Yusof et al., 1990). Flavanone glycosyl compositions of peels and seeds are quite unlike those of juices. Naringin has been found in lemon peel and seed and in mandarin seed, but it is not present in the juices of these fruits (Mouly, Arzouyan, Gaydou, & Estienne, 1995; Ooghe & Detavernier, 1997). This glycosylated flavanone is never present in sweet orange juice, and its presence is used to detect adulteration (Mouly et al., 1994). Although flavones and flavonols have been found in low concentrations in *Citrus* tissues, these compounds are studied to evaluate their antioxidant ability (Fig. 2) (Benavente-García, Castillo, Marin, Ortuño, & Del Río, 1997).

Miyake, Yamamoto, Morimitsu, and Osawa (1997) isolated two C-glucosylflavones from the peel of lemon fruit (*Citrus Limon* B_{URM}. f.). They identified 6,8-di-C-β-glycosyldiosmin and 6-C-β-glycosyldiosmin by UV, IR, FAB-MS, ^1H NMR, and ^{13}C NMR analyses.

In lemon juice extracted from several cultivars, there is little difference in the glycosylated flavonoids amounts. Eriocitrin, 6,8-di-C-β-glycosyldiosmin and 6-C-β-glycosyldiosmin are particularly abundant in lemon and lime, while they are almost absent in other *Citrus* fruits (Miyake, Yamamoto, Morimitsu, & Osawa, 1998).

Anthocyanins constitute the colouring compounds of flowers and fruits, but sometimes also of leaves, buds and roots (Herrman, 1976). They are in the epicarp, but they also colour the mesocarp of oranges. The anthocyanin content is strongly dependent on the level of maturation.

Catechins, leucoanthocyanin and proanthocyanins are not *Citrus* fruit specific compounds because they are also found in other vegetables.

4. Absorption and pharmacokinetics of *Citrus* flavonoids

It is essential to establish whether *Citrus* flavonoids are absorbed in the intestine and how they are distributed in the organism, to verify their antioxidant activity *in vivo*.

Some authors have studied the naringin and naringenin metabolic fates in rats after oral administration. Structural studies on the biliary and urinary metabolites showed that both compounds are transformed into the same metabolites. The main biliary metabolites are naringenin-7-glucuronide, naringenin-7-sulfate 4′-glucuronide and naringenin-7-glucuronide 4′-sulfate. The main urinary metabolites are naringenin 4′-glucuronide and naringenin 7,4′-disulfate, as well as naringenin 7-glucuronide. Moreover, all these compounds have been found in plasma, like the main metabolites.

Studies on plasma concentrations, biliary and urinary excretion of metabolites, show that naringenin is absorbed more quickly than is naringin, causing higher metabolite concentrations in plasma, urine and bile. These data indicate that naringin is absorbed like naringenin after hydrolysis (Abe, Katayama, Suzuki, & Yumioka, 1993).

Choudhury, Chowrimootoo, Srai, Debnam, and Rice-Evans (1999) studied interactions in the gastrointestinal tract of flavonoids and the influence of glycosylation on their subsequent metabolism. They examined the urinary recoveries of the flavonoid naringenin-7-glucoside and its aglycone, in the conscious rat model, after oral and intravenous administration. Their findings suggest that, via the oral route, the glycoside group is cleaved by an intestinal enzyme prior to glucuronidation within the epithelium. This is shown by the urinary elimination of the native glycoside and the lack of detection of glucuronide after intravenous administration (Choudhury et al., 1999).

Paganga and Rice-Evans (1997) described a method for identifying and quantifying several dietary flavonoid families in human plasma by applying HPLC analysis. The identification and quantification of phloridzin and rutin have indicated their absorption as glycosides (Paganga & Rice-Evans, 1997). To investigate the absorption and metabolism of flavones, flavonols, flavanones and their glycosides and hydroxycinnamates in the gastrointestinal tract, an isolated preparation of jejunum and ileum from the small intestine and monitoring of the resulting compounds, conjugates and metabolites has been used. Dietary glycosides were also compared with free aglycones to investigate the influence of glycosylation on the fate of these compounds. In order for the glycosides luteolin-7-glycoside, kaempferol-3-glycoside, quercetin-3-glycoside and rutin to appear as glucuronides of their aglycones, the glycosidic groups must first be cleaved by an intestinal enzyme prior to glucuronidation. The studies reported by Spencer et al. (1999) showed that luteolin-7-glycoside, kaempferol-3-glycoside and quercetin-3-glycoside are cleaved by rat jejunal or ileal mucosa, suggesting the presence of a glycosidase and a UDP-glucuronyl transferase to glucuronidate the phenolics before efflux into the serosal fluid. These findings of Spencer et al. (1999) concerning quercetin and kaempferol-3-glycosides contrast with the study of Day et al. (1998) describing a β-glycosidase in cell free extracts of the human small intestine hydrolysing some monoglycosides, e.g. quercetin-4′-glycoside, naringenin-7-glycoside, but not quercetin-3-glycoside, kaempferol-3-glycoside, or the diglycosides, such as rutin and quercetin-3,4′-diglycoside. Prior to this, it had been assumed that the glycosides could not be absorbed from the small intestine and cleavage of the β-glycosidic bond will not occur until the compound reaches the microflora of the large intestine.

Glucuronidation of flavonoids was observed to occur at different, and possibly, multiple hydroxyl groups within the structure. The position of glucuronidation is important in considering the resulting antioxidant potential of the absorbed glucuronides, especially since the reduction potential of the phenolic B-ring is lower than that of the A-ring (Jovanovich, Steenken, Simic, & Hara, 1998). For example, glucuronidation at the 3′- or 4′-OH groups on the flavonoids B-ring would increase the reduction potential and therefore decrease its antioxidant activity. However, if glucuronidation occurs at hydroxyl groups in the flavonoid A-ring, the antioxidant capacity would be less influenced (Spencer et al., 1999). Indeed, recent studies have identified the 5-O-β-glycoside of catechin and epicatechin excreted in the urine of rats and this glucuronidation reaction does not influence their antioxidant activity (Harada et al., 1999; Okushio, Suzuki, Matsumoto, Nanjo, & Hara, 1999).

Perfusion studies on luteolin were done by Shimoi et al. (1998) who reported the absorption of flavonoids from the mucosa to the serosal side, using a rat everted intestine model. However, although they also observed glucuronidation of luteolin and the hydrolysis of luteolin-7-glycoside to luteolin during transfer across the gut, their studies only revealed the presence of two glucuronidated/sulfated metabolites whereas Spencer et al. (1999) identified six distinct luteolin glucuronides. In contrast, no hesperidin (hesperetin-7-rhamnoglycoside), or its hydrolysis or glucuronidation products were detected at any time after perfusion with hesperidin. Both compounds were stable over the course of the experiments (Spencer et al., 1999). Recently, Day, Gee, DuPont, Johnson, and Williamson (2003) proposed two hypotheses on absorption mechanisms of flavonoid glycosides across the small intestine: active uptake of the quercetin glycoside by the sodium-dependent glucose transporter (SGLT1) with subsequent deglycosylation within the enterocyte by cytosolic β-glucosidase, or luminal hydrolysis of the glycoside by lactase phlorizin hydrolase (LPH) and absorption by passive diffusion of the released aglycone. According to these authors, the mechanism of absorption of quercetin-4′-glucoside involves both an interaction with SGLT1 and luminal hydrolysis by LPH, whereas quercetin-3-glucoside appears to be absorbed only following hydrolysis by phlorizin hydrolase (Day et al., 2003).

Miyake et al. (2000) made a study *in vitro*, using the plasma of rats and human intestinal bacteria, in order to estimate eriocitrin metabolism. The β-glycoside bound is resistant to the pancreatic enzyme hydrolysis and the glycoside flavonoids are hydrolysed by the digestive microflora before their absorption, as Kuhnan (1976) had already predicted.

Eriocitrin is metabolised to heridictyol (the aglycone) by *Bacteroides distasonis* or *Bacteroides uniformis*, which are present in the intestinal flora; moreover, heridictyol is converted into 3,4-dihydroxycinnamic acid by *Clostridium butyricum*. The same authors have also analysed, *in vivo*, the eriocitrin digestion products and identified heridictyol and 3,4-dihydroxycinnamic acid in the rats' intestine. Probably, heridictyol is linked to glucuronic acid for the intestinal absorption; actually in the intestine mucous membrane, UDP-glucuronosyltransferase has been found (Da Silva, Piskula, & Terao, 1998). Two types of metabolism can occur with heridictyol: one by the intestinal bacteria (with 3,4-dihydroxycinnamic acid formation) and the other by the liver; heridictyol is metabolised to homoeriodictyol and hesperetin through methoxylation.

Liver is generally considered the main organ involved in flavonoid metabolism (Piskula & Terao, 1998); methylation of hydroxyl groups occurs in this organ, as also does the conjugation with glucuronic acid and/or sulfuric acid (Terao, 1999). In flavonoids with a catechol-containing B-ring, there is also extensive O-methylation catalysed by the action of COMT (Spencer et al., 1999). In particular, according to Miyake et al. (2000), the *o*-dihydroxy structure, in the 3′ and 4′ positions of the B-ring, can represent the most obvious target for eriocitrin metabolism. It seems that O-methylation occurs in positions 3′ and 4′ of the B-ring during the heridictyol metabolic conversion. This methylation causes loss of antioxidant activity, because the group $-OCH_3$ prevents the enol–keto tautomerism necessary for the "scavenger" activity (Fig. 3(c)).

In the plasma, 4 h after the eriocitrin ingestion, heridictyol, homoeriodictyol and hesperetin have been found in the conjugated form. Conjugated metabolites have not been found in urine, but they have been found in plasma. This suggests that conjugated metabolites are deconjugated in kidneys and free metabolites are expelled with the urine. The results of Miyake et al. (2000) indicate that heridictyol is metabolised to homoeriodictyol and hesperetin through methylation in the liver; the *o*-dihydroxy structure in the B-ring is involved in the antioxidant activity. Eriodictyol, homoeriodictyol, hesperetin and eriocitrin are the main metabolites *in vivo* (Miyake et al., 2000). In a new study of Miyake et al. (2006), metabolites in plasma (after ingestion of flavanone glycosides and their aglycones in humans) were shown to exist as the glucuro- and/or sulfo-conjugates of heridictyol, homoeriodictyol and hesperetin. After ingestion of flavanone aglycones, the concentration of metabolites in plasma exhibited a high maximum peak at 1 h. The AUC (area under the blood concentration time curve) level of metabolites of flavanone aglycones was higher than

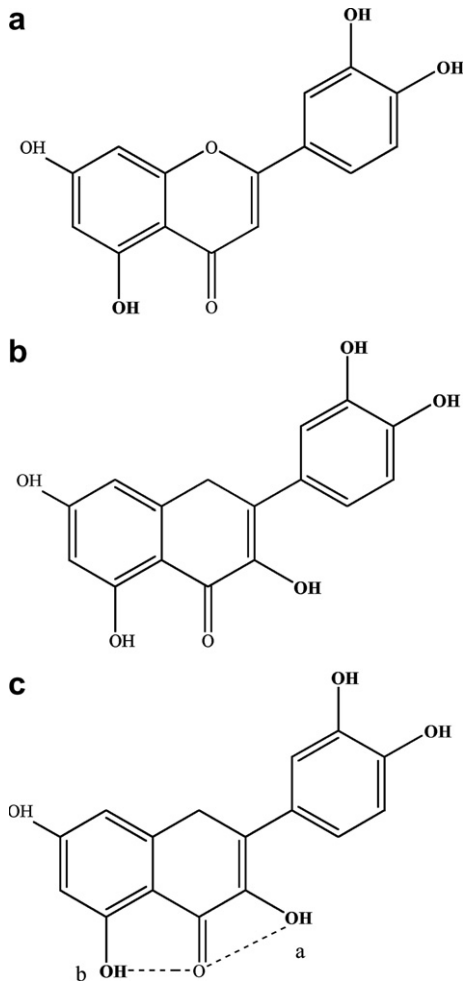


Fig. 3. Functional groups of flavonoid structure with high antioxidant capacity.

that of flavanone glycosides. Flavanone aglycones were suggested to be absorbed faster and in higher amounts than flavanone glycosides (Miyake et al., 2006).

The renal excretion of naringin (naringenin 7-O-β-rhamnoglycoside) has been examined in men by HPLC. Naringenin and its metabolites conjugated with glucuronic acid are detectable in urine for 24 h and the naringenin conjugated metabolites are present also in the plasma samples (Fuhr & Kummert, 1995). Other authors have determined the absorption kinetics, the urinary excretion and circulating metabolites of hesperidin and narirutin. Orange juice was administered to a group of five healthy volunteers; blood and urine for 48 h were examined. Plasma flavanone metabolites appeared 3 h after ingestion of the juice, with a peak between the fifth and seventh hour; the metabolites were not present after 24 h. The circulating hesperetin metabolites were glucuronidated (87%) and sulfo-glucuronidated (13%). The hesperetin and naringenin urinary excretions were similar (Manach, Morand, Gil-Izquierdo, Bouteloup-Demange, & Remesy, 2003).

Other authors studied nobiletin metabolism after oral administration in rats (Fig. 2). This polymethoxylflavone

(found only in the *Citrus depressa* fruits) was present in urine as the demethylated form. The main metabolite was demethylated at the C-4′ position (Yasuda et al., 2003). This is in agreement with the results of previous investigation; the demethylated metabolites were isolated from rat urine and feces after oral administration of tangeretin (Fig. 2). In this study, only about 38% of the tangeretin metabolites were excreted in urine as conjugates to glucuronic acid or sulfate groups, whereas the remaining 60% of the metabolites were excreted as free aglycones. The tangeretin metabolites identified were all products of demethylation and hydroxylation reactions. The C-4′ position seemed to be the primary site for tangeretin demethylation. The 6-position was found to be the second most common site for demethylation (Nielsen, Breinholt, Cornett, & Dragsted, 2000). Murakami et al. (2001) investigated the *in vitro* metabolism of nobiletin in rat liver microsomes and identified the major metabolites as the 3′-demethylation products of nobiletin. However, the results of a study in rats show that the identified nobiletin metabolites are all products of the demethylation reaction. The C-4′ position appears to be the primary site for nobiletin demethylation, since the major metabolite is demethylated at the C-4′ position (Yasuda et al., 2003). The aim of the study of Nielsen et al. (2006) was to demonstrate, in human subjects, that the removal of the rhamnose group to yield the corresponding flavonoid glycoside (hesperetin-7-glucoside) would improve the bioavailability of the aglycone hesperetin. The results of this study demonstrated that the bioavailability of hesperidin was modulated by enzymatic conversion to hesperetin-7-glucoside, thus changing the absorption site from the colon to the small intestine (Nielsen et al., 2006).

Spencer, Abd El Mohsen, and Rice-Evans (2004) has reviewed the uptake and metabolism of flavonoids by liver and gastrointestinal tract cells. It is now well established that the gastrointestinal tract plays a significant role in the metabolism and conjugation of polyphenols before entry into the systemic circulation and the liver. Enterocytes in the jejunum and ileum of the small intestine transfer flavonoids from the luminal side of the gut to the portal vein, during which there is significant glucuronidation of nearly all flavonoids tested by the action of UDP-glucuronosyltransferase enzymes (Spencer et al., 2004).

5. The antioxidant activity of citrus flavonoids

Flavonoids can exercise their antioxidant activity in several ways (Bombardelli & Morazzoni, 1993):

1. antiradical activities: $OH^{\cdot}$ (hydroxyl), O_2, $^1O_2$, $O_2^{\cdot-}$ (superoxide);
2. anti-lipoperoxidation activities ($R^{\cdot}$; $ROO^{\cdot}$; $RO^{\cdot}$);
3. activities of metal chelation.

Flavonoids are powerful antioxidants against free radicals, because they act as "radical-scavengers". This activity is attributed to their hydrogen-donating ability. Indeed, the phenolic groups of flavonoids serve as a source of a readily available "H" atoms such that the subsequent radicals produced can be delocalized over the flavonoid structure (Burda & Oleszek, 2001; Di Majo et al., 2005). The chemical nature depends on structural class, degree of hydroxylation, other substitutions and conjugations and degree of polymerisation (Rice Evans, Miller, & Paganga, 1996; Calabrò et al., 2004). In order to classify a flavonoid as an antioxidant, the following parameters can be tested: (a) the rate constant (k) with different types of radicals, (b) decay kinetics and stability of the aroxyl radical, (c) the stoichiometry of the radical-scavenging reaction.

According to kinetic studies of aroxyl radical formation and decomposition reactions, the antioxidant capacity of a flavonoid is linked to the its particular chemical structure. Three structural groups are important for the evaluation of their antioxidant capacity (Fig. 3) (Bors, Hellers, Michel, & Saran, 1990a, 1990b): (A) the *ortho*-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, responsible for electron dislocation from the B-ring; (C) the presence of both 3-(a)-and 5-(b)-hydroxyl groups (Fig. 3(c)). Obviously, the flavonoid antioxidant capacity is linked to a combination of these chemical and structural elements, for example, glycoside presence or absence (glycosides or aglycones) and the presence of free hydroxyls or the number and position of hydroxyls eventually esterified (Benavente-García et al., 1997; Di Majo et al., 2005).

The antiradical activity of several *Citrus* flavonoids, in comparison with the superoxide anion, has been studied using many methods and with different structural correlations (Darmon, Ferrandiz, Canal, & Mitjavilla, 1990). This activity is influenced by the flavonoid concentration in the reaction environment, increasing from zero to the maximum, or determining the auto-oxidation of the flavonoid itself. The common structural element is the configuration of the C-ring with the 3-hydroxyl group that activates the double bond in position 2,3. Only when the concentration is below 100 μM is the presence of the hydroxyl groups in the B-ring important for antiradical activity. Kaempferol (a flavonol), for example, has no activity against the superoxide ion at 10 μM, but only in the range 60–100 μM.

The absence of the hydroxyl group at position 3 in flavanones and flavones decreases their antioxidant ability, also does the absence of the catechol structure in the B-ring (Sichel, Corsaro, Scalia, Di Bilio, & Bonomo, 1991). However, the double bond at 2,3 makes the structure more reactive, for this reason apigenin is a moderate antioxidant compound, while naringenin has no activity against the superoxide ion.

According to other studies (Cillard & Cillard, 1988; Darmon et al., 1990; Pincemail et al., 1986), flavonoids are excellent "radical-scavengers" of the hydroxyl radical; they seem also to have an activity degree that depends on their structure (Table 1). It is based on their ability to inhibit

the hydroxyl radical and it shows the importance of double substitution also when the hydroxyl groups are esterified with methyl groups. The negative influence of the hydroxyl group in compounds with the mono-substituted B ring at position 3 is meaningful. Moreover, it has been reported that the corresponding 3-O-glucosides are more active than are their aglycones (Pincemail et al., 1986).

Some authors have determined the antioxidant capacity of the polyphenols, the flavonoids, anthocyanins, hydroxycinnamic acids and ascorbic acid contained in juices of some varieties of pigmented oranges (Moro, Sanguinella, Tarocco and Washington) (Rapisarda et al., 1999). All examined orange juices show an antioxidant capacity, due to total phenol amounts and to their ability to interact with the biomembrane; their antioxidant capacity seems to be widely influenced by the anthocyanin concentrations in the pigmented oranges juices. This study shows that the daily phenol intake, as orange juice, may represent an additional protection *in vivo* against cellular biomolecule oxidation. Proteggente, Saija, De Pasquale, and Rice Evans (2003) analysed the phenolic compositions, the ascorbic acid contents and the antioxidant activities of fresh Sicilian orange juices from pigmented (Moro, Tarocco and Sanguinella) and non-pigmented (Oval, Valencia and Navel) varieties of orange (*Citrus sinensis* L. Osbeck). Furthermore, cyanidin-3-glycoside and cyanidin-3-(6″-malonyl)-glycoside were predominant in all the pigmented varieties, but their concentrations were higher in the juices of the Moro variety. Differences between varieties in terms of the flavanone glycoside content, particularly hesperidin, were observed, with the Tarocco juices showing the highest content (Proteggente et al., 2003).

The antioxidative activities of 6,8-di-C-β-glycosyldiosmin and 6-C-β-glycosyldiosmin and flavonoid compounds (eriocitrin, diosmin, hesperidin and narirutin) in lemon fruit were examined using linoleic acid autoxidation, the liposome oxidation system, and the low-density lipoprotein (LDL) oxidation system. 6,8-Di-C-β-glycosyldiosmin and 6-C-β-glycosyldiosmin showed antioxidative activity in these autoxidation systems but exhibited weaker activity than did eriocitrin, its heridictyol of its aglycone (Miyake et al., 1997). Eriocitrin, neoeriocitrin and 6,8-di-C-β-glycosyldiosmin antioxidant activities were higher than those of other compounds.

According to Finotti and Di Majo (2003), all *Citrus* flavonoids have an antioxidant action in a hydrophilic environment while, in a lipophilic environment, some molecules (neohesperidin, hesperetin, didymin and isosakuranetin) show a reduced antioxidant capacity, and others (naringin, narirutin, naringenin, neoeriocitrin, heridictyol) invert their behaviour, becoming prooxidants.

6. *Citrus* flavonoids as compounds with anti-inflammatory activity

Many regulatory enzymes (protein kinase C, phosphodiesterase, phospholipase, lipoxygenase, and cyclooxygenase) control the formation of the biological mediators responsible for the activation of endothelial cells and specialized cells involved in inflammation. Flavonoid inhibition of the immune and inflammation responses can be associated with their inhibition of these enzymes.

Indeed, *Citrus* flavonoids are able to inhibit the kinases and phosphodiesterases essential for cellular signal transduction and activation. They also affect the activation of a number of cells involved in the immune response, including T and B lymphocytes (Manthey, Guthrie, & Grohmann, 2001).

Hesperidin inhibited pleurisy induced by carrageenan, reducing the volume of exudates and the number of migrating leucocytes by 48% and 34%, respectively, of control values (Da Silva, Oliveira, & Lapa, 1994). Hyperthermia induced by yeast in rats was slightly reduced by hesperidin. The results indicated that hesperidin obtained from *Citrus* cultures may have a potential therapeutical use as a mild anti-inflammatory agent, being also useful as a precursor of new flavonoids endowed with this activity (Da Silva et al., 1994). Indeed, other studies, using mouse macrophage cells, have shown that hesperidin has an inhibitory effect on lipopolysaccharide (LPS)-induced over expression of cyclooxygenase-2, inducible nitric oxide synthase (iNOS), over-production of prostaglandin E_2 and nitric oxide (NO) (Sakata, Hirose, Qiao, Tanaka, & Mori, 2003). The effects of nobiletin on the production of cyclooxygenases have been examined. Nobiletin selectively downregulates cyclooxygenase-2 C, but not cyclooxygenase-1 mRNA expression and also interferes with the LPS-induced production of prostaglandin E_2 and the gene expression of proinflammatory cytokines (IL-1alpha, IL-1beta, TNF-alpha and IL-6). In addition, nobiletin downregulates the IL-1-induced gene expression and production of proMMP-1/procollagenase-1 and proMMP-3/prostromelysin-1 in human synovial fibroblasts (Lin et al., 2003). In contrast, production of the endogenous MMP inhibitor, TIMP-1, was augmented by nobiletin. Nobiletin has anti-inflammatory actions similar to those of dexamethasone (Lin et al., 2003).

7. *Citrus* flavonoids in the prevention of atherosclerosis

Szent-Gyorgyi (1938) was the first to consider flavonoids effects on capillary fragility and bleeding, due to the presence of a vitamin that they called "P". The regular consumption of flavonoids reduces the risk of coronary diseases in old men. The flavonoids have anti atherosclerotic activity, inhibiting the formation of atheroma in many steps of its pathogenesis (Hertog et al., 1993).

Cha et al. (2001) has studied the effect of dietary hesperetin on the hepatic lipid content and enzyme activities involved in triacylglycerol synthesis in rats fed diets with or without 1% orotic acid. Orotic acid, an intermediate in pyrimidine nucleotide biosynthesis, administered in high amounts, causes hepatic triacylglycerol accumulation in rats, possibly due to reduced secretion of VLDL, enhanced

triacylglycerol synthesis and reduced oxidation. Hesperetin limited the rise in hepatic triacylglycerol and cholesterol contents induced by orotic acid (Cha et al., 2001). Hesperetin exerts a hypolipidemic effect only when the lipid concentrations are high (Kim, Jeong, Lee, Park, & Choi, 2003). Kurowska and Manthey (2004) studied the hypolipidemic effects and metabolic fates of hesperidin, naringin and formulations containing polymethoxylated flavones, mainly tangeretin, in hamsters with diet-induced hypercholesterolemia. Diets containing 1% polymethoxylated flavones significantly reduced serum total and very low-density lipoprotein (VLDL) + LDL cholesterol and either reduced or tended to reduce serum triacylglycerols. Moreover, according to the authors, elevated levels of polymethoxylated flavones metabolites in the liver might be directly responsible for their hypolipidemic effects *in vivo*.

A Japanese study also showed an inverse relationship between flavonoid intake and total cholesterol concentration in plasma (Choi, Yokozawa, & Oura, 1991).

Citrus flavonoids have a platelet anti-aggregation and anti-adhesive activity. Methoxylated flavonoids (nobiletin, tangeretin) are much more active than are hydroxylated compounds and they show an activity similar to acetylsalicylic acid (Beret & Cazenave, 1988; Robbins, 1976).

Other authors have pointed out that adhesion and platelet aggregation inhibition depend on the aggregation induction and part of the chemical flavonoid structure (Manach et al., 1996). *In vitro* studies showed that flavonoids bound to platelet membranes may therefore have an accumulative effect over time (Van Wauwe & Goossens, 1983). The induced platelet aggregation by arachidonic acid is inhibited more effectively by fisetin, kaempferol and quercetin than by myricetin. The aggregation induced by ADP is less influenced by flavonoids, except myricetin. Quercetin, fisetin and myricetin show a higher inhibitory effect on the aggregation induced by collagen (Tzeng, Ko, Ko, & Teng, 1991). These different effects suggest that many complexes mechanisms are involved in the inhibition of platelet aggregation (Beret, Anton, & Cazenave, 1986).

Other studies have shown that flavonoids are active antithrombotic agents, inhibiting cyclooxygenase and lipooxygenase activities, with consequent decrease of thromboxane A_2 and of leucotriene series 4 production (Alcaraz & Ferrandiz, 1987; Tzeng et al., 1991). Some flavonoids inhibit cyclooxygenase or lipooxygenase enzymes, while others block both of them (Landolfi, Mower, & Steiner, 1984).

Flavones have a considerable anti-thrombotic activity, because they act as "radical-scavengers", so they maintain the right prostacyclin and NO endothelium levels (Gryglewski, Korbut, Robak, & Swies, 1987). It has also been shown that several flavonoids provoke a reduction of ischemic damage, interfering with the activity of macrophagic NOS (Nijveldt et al., 2001).

The hypocholesterolemic effects of dietary citrus juices in rabbits were investigated. Orange juice and grapefruit juice influence cholesterol metabolism in rabbits with high LDL-cholesterol levels, induced by feeding a semi-purified, cholesterol-free, casein diet. In animals, the experimental diet (for 3 weeks) reduced serum LDL-cholesterol by 43% and 32%, respectively ($p < 0.05$) (Kurowska, Borradaile, Spence, & Carroll, 2000). Other studies have shown that naringenin at a 0.1% dietary level lowered levels of plasma cholesterol and hepatic levels of triacylglycerol and cholesterol in rats fed a high-cholesterol diet, accompanying a decrease in the activity of 3-hydroxy-3-methylglutaryl–coenzyme A (CoA) reductase and acyl-CoA:cholesterol acyltransferase (Lee et al., 1999; Lee et al., 2003).

However, it has been demonstrated grapefruit juice, unlike other citrus fruit juices, interacts with some drugs. It has several effects on cyclosporin, statins and some calcium antagonists (Kane & Lipsky, 2000). For some drugs, such as felodipine, nicardipine and halofantrine, increased drug concentrations have been associated with an increased frequency of dose-dependent adverse effects (Bailey, Malcolm, Arnold, & Spence, 1998; Charbit, Becquemont, Lepere, Peytavin, & Funck-Brentano, 2002; Lundahl, Regardh, Edgar, & Johnsson, 1997; Uno et al., 2000). The major mechanism for grapefruit–drug interaction is inhibition of the drug-metabolising enzyme cytochrome P-450 3A4 (CYP450 3A4) in the small intestine, resulting in a significant reduction of the presystemic metabolism of drugs (Bailey et al., 1998). Dosage adjustments to maintain drug concentrations within their therapeutic windows, are necessary (De Castro, Mertens-Talcott, Rubner, Butterweck, & Derendorf, 2006).

8. *Citrus* flavonoids in the prevention of cancer

8.1. General

Dietary flavonoids have been considered to be chemopreventive or anticancer agents (Elangovan, Sekar, & Govindasamy, 1994; Hertog et al., 1993; Hirano, Gotoh, & Oka, 1994; Kandaswami, Perkins, Soloniuk, Drzewiecki, & Middleton, 1991). Anticancer effects may also be exhibited through selective cytotoxicity, antiproliferative actions and apoptosis. *Citrus* flavonoids exert their anticancer effects through a number of these diverse mechanisms (Manthey et al., 2001). Flavonoids can be potentially involved in carcinogenesis:

1. DNA damage (induction),
2. tumor development (promotion),
3. invasion (proliferation).

8.2. Antimutagenic effects

Flavonoids can protect DNA by their ability to absorb ultraviolet light (Stapleton & Walbot, 1994). Recent experiments on a UV irradiated model of plasmidic DNA indicate a protecting effect of naringenin and rutin against UV-induced damage of DNA (Kooststra, 1994). Flavonoids neutralize free radicals that promote mutations,

when they are generated near DNA. This "radical-scavenger" property is responsible for a protecting effect in body γ-ray-irradiated mice (Shimoi, Masuda, Furogori, Esaki, & Kinae, 1994). Indeed, naringin plays an important role in regulating antioxidative capacity by increasing superoxide dismutase and catalase activities and by up-regulating the gene expressions of superoxide dismutase, catalase, and glutathione peroxidase in cholesterol-rich diet-fed rabbits (Jeon et al., 2001). Since naringin blocks H_2O_2-induced cytotoxicity and apoptosis, naringin might affect H_2O_2-induced expression of an apoptosis-associated gene or protein as one of its pharmacological actions (Kanno, Shouji, Asou, & Ishikaw, 2003).

Flavonoids can also protect the DNA by interacting directly with the tumoral agents, as in the induced chromosomal aberrations by bleomycin (Heo et al., 1994).

8.3. Inhibition of tumor development

Flavonoids can act by affecting tumor promotion at the beginning of carcinogenesis by an increase of the detoxification processes. In particular, *Citrus* flavonoids inhibit ornithine decarboxylase induction of skin tumor promotion, increasing the inorganic phosphate ^{32}P incorporation rate in the phospholipid membrane and activation of protein kinase C (Manach et al., 1996). Despite the large number of studies on this topic, the intracellular mechanism involved is not clear. Hesperetin and naringenin were tested for their abilities to inhibit the development of breast cancer induced by 7,12-dimethylbenz[*a*]anthracene in female rats. This experiment showed that tumor development was delayed in the groups fed with orange juice or with the naringin-supplemented diet. This study provides evidence of anticancer properties of orange juice and indicates that *Citrus* flavonoids are effective inhibitors of human breast cancer cell proliferation *in vitro* (So, Guthrie, Chambers, Moussa, & Carroll, 1996).

Flavonoids could affect cellular metabolism in various ways; they can act in the cellular membrane and on intracellular enzymes. Flavonoids frequently inhibit glycolysis, the most active metabolic pathway in tumoral cells (Manach et al., 1996). They depress production of lactate in leucemia cell lines or in Ehrlich tumor cells (Suolinna, Buchsbaum, & Racker, 1975). These effects are probably due to inhibition of the transport of lactate or to the membrane ATPases (Belt, Thomas, Buchsbaum, & Racker, 1979; Shoshan & MacLennan, 1981). Inhibition of the Na/K ATPase pump could negatively influence the ionic gradient and therefore energetic metabolism, the synthesis of the proteins and DNA replication, by pH reduction of the cells (Hirano, Oka, & Akiba, 1989).

Flavonoids can affect enzymic activity involved in the transduction of the mitogenics (kinase, phospholipase, phosphodiesterase) and they regulate critical proliferation enzymes. *Citrus* flavonoids can potentiate the drug therapies effects against cancer. For example, quercetin markedly increases the effect of adriamycin in a multidrug-resistant MCF-7 human breast-cancer cell line (Scambia et al., 1994) and on cells of colon MCT-15 (Critchfield, Welsh, Phang, & Yeh, 1994). Naringenin stimulates DNA repair following oxidative damage in human prostate cancer cells. Gao et al. (2006) determined the induction of mRNA expression over time after oxidative stress, followed by naringenin administration of three major enzymes in the DNA base excision repair pathway: 8-oxoguanine-DNA glycosylase 1, apurinic/apyrimidinic endonuclease and DNA polymerase β. 8-Oxoguanine-DNA glycosylase 1 and DNA polymerase β mRNA expression in cells after 24-h exposure to naringenin were significantly increased compared with control cells without this *Citrus* flavonoid. The cancer-preventive effects may be due, in part, to stimulation of DNA repair by naringenin which, by stimulating base excision repair processes, may prevent mutagenic changes in prostate cancer cells (Gao et al., 2006).

8.4. Inhibition of tumoral cell proliferation

Citrus flavonoids can inhibit invasion, by rat malignant cells, in cardiac and hepatic tissue of syngenetic rats (Bracke et al., 1989). The mechanism of anti-metastasis and anti-invasive activities of *Citrus* flavonoids are based on cell mobility inhibition (Bracke et al., 1989, 1991). *In vitro*, flavonoids inhibit several human neoplastic cellular line proliferations: lymphoid and myeloid leukaemias (Larocca et al., 1990), gastric carcinoma (Yoshida et al., 1990), ovarian carcinoma (Scambia et al., 1990), prostate carcinoma (Peterson & Barnes, 1993) and squamous-cellular carcinoma (Kandaswami et al., 1991); in this last case, it has been observed that the lipophilic polymethoxylated flavonoids (nobiletin and tangeretin) inhibit tumoral cell development in a dose-dependent manner; moreover, when the incubation is extended, these two flavonoids cause the death of cells at 8 μg/ml. These data indicate, therefore, that the polymethoxylated flavonoids can be considered anticancer substances (Kandaswami et al., 1991). Hydroxycinnamates, glycosylated flavonoids and the polymethoxylated flavones have shown inhibitory activity on several tumoral cell line proliferations (Manthey & Guthrie, 2002).

In a recent study, the growth-inhibitory action of nobiletin in mouse fibroblast clone A-31 and various human gastric cancer cells has been evaluated and the mechanism of its antiproliferation effect has been studied, too. Nobiletin showed direct cytotoxicity on TMK-1, MKN-45, MKN-74 and KATO-III in a concentration-dependent manner but no suppressive effect on clone A-31. A striking observation from these data was that TMK-1 was the most sensitive line to the nobiletin-mediated loss of viability, and that sensitivity was greatest at the lowest dose. The same authors have also investigated whether the nobiletin-mediated loss of cell viability in TMK-1 is a result of apoptosis. This essay demonstrated the presence of apoptotic TMK-1 cells (Yoshimizu et al., 2004).

Naringenin inhibited proliferation of HT-29 colon cancer cell lines at concentrations of 0.71–2.85 mmol. This equivalent concentrations (0.71–2.85 mmol) of naringenin found to be effective in plasma are daily provided by drinking between 2 and 3 l of grapefruit juice. This, however, assumes that all naringenin available in the juice is absorbed, without first-pass metabolism. Erlund, Meririnne, Alfthan, and Aro (2001) showed that the bioavailability of naringenin from grapefruit juice was much less than anticipated, and so the effective volumes of grapefruit juice required would be greatly increased. It may therefore be more practical to give naringenin in capsular form. (Erlund et al., 2001; Frydoonfar, McGrath, & Spigelman, 2003).

9. *Citrus* flavonoids as compounds with antimicrobial activity

Some studies have shown flavonoid antimicrobial activity (Huet, 1982). There is an important structure-activity relationship that influences antiviral activity (Kaul, Middleton, & Ogra, 1985). The antiviral activity appears to be associated with non-glycosidic compounds, and hydroxylation at the 3-position is apparently a prerequisite for antiviral activity. Naturally occurring 4′-hydroxy-3-methoxyflavones possess antiviral activity against rhino- and poliomyelitis viruses (Middleton, Kandaswami, & Theoharides, 2000). The anti-picornavirus activity of the methoxyflavones was associated with the 4′-hydroxyl and 3-methoxyl groups. These methoxyflavones, poly-substituted in the A-ring, show a higher antiviral activity than do mono-substituted compounds (De Meyer et al., 1991). Quercetin and hesperidin inhibit the infectivity and replication of herpes simplex, poliovirus, parainfluenzal virus and syncytial virus, while some flavonoids contained in the grapefruit, although important, like naringenin, do not introduce this activity (Kaul et al., 1985). Several polymethoxylated flavones were found to strongly inhibit bacterial lipopolysaccharide-induced expression of TNF-α, whereas flavonoid glycosides were inactive (Manthey, Grohmann, Montanari, Ash, & Manthey, 1999). In particular, hesperidin has significant inhibitory activities on inflammation, because it is able to reduce both LPS-elicited and infection-induced TNF-α production and inhibit infection-induced lethal shock, which resembles clinical cases. Hesperetin, the aglycone of hesperidin, has a moderated antimicrobial activity against *Salmonella typhi* and *S. typhimurium* (Kawaguchi et al., 2004).

10. Conclusion

The studies performed both *in vitro* and *in vivo* have shown that *Citrus* flavonoids play an important role in the prevention of degenerative and infective diseases. Flavonoids are a widely distributed group of polyphenolic compounds, called "nutraceutical substances", with anti-cancer, antiatherogenic, antimicrobial and anti-inflammatory properties.

The beneficial effects of the dietary *Citrus* fruits can in particular be attributed, not only to the vitamin C, folate, dietary fibre and carotenoids, but also to the antioxidant activity of their flavonoids. This property is linked to particular chemical structures. Three structural groups are important for the evaluation of their antioxidant capacity: the *ortho*-dihydroxy (catechol) structure in the B-ring, the 2,3-double bond in conjugation with a 4-oxo function and the presence of both 3-(a)-and 5-(b)-hydroxyl groups. A diet rich in grain, legumes, vegetables and fresh fruit, such as *Citrus* fruits and juices, e.g. the Mediterranean diet, has beneficial effects on human health.

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