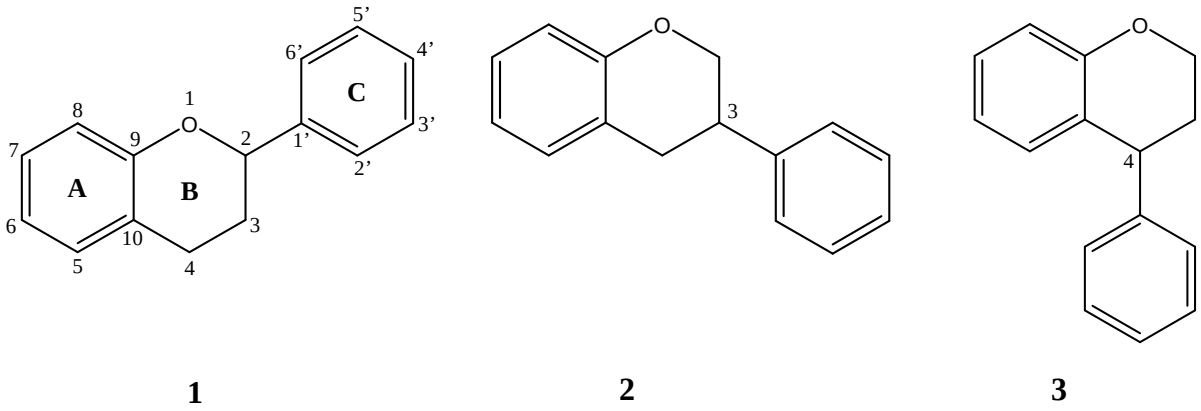
Les Polyphénols d'Agrumes: Les Flavanones

It is now well accepted that a low consumption of fatty foods, regular physical activity and a high consumption of plant-derived foods help maintain a good health status. In particular, there is an association between an increased level of fruits and vegetables in the diet and a reduced risk of some life-threatening diseases such as cardiovascular diseases and cancer (Parr & Bolwell, 2000). There is growing acceptance that many phenolic secondary metabolites (polyphenols) present in foodstuffs may exert beneficial effects in the prevention of these degenerative diseases (Del Rio *et al.*, 2010). Over the last few decades, the worldwide consumption of citrus fruits and juices has been increasing, thereby stimulating the research on the most abundant bioactive citrus phenols, i.e. FLAVANONES.

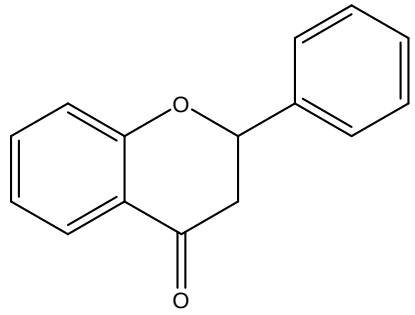
1.1. Chemistry and Classification:

Polyphenols are classified into two major classes: Flavonoids and NonFlavonoids. The later one includes the structurally simple molecules such as phenolic acids (hydroxybenzoic acids and hydroxycinnamic acids) and stilbenes, and complex molecules comprising of stilbene oligomers, tannins and lignins (Cheynier, 2005). The former, the most studied subclass of polyphenols, represents about more than 9000 identified compounds (Marten and Mithöfer, 2005; Pietta, 2000). Flavonoids commonly share the same generic structure, the flavan nucleus, consisting of two aromatic rings (A and B) linked by an oxygen-containing pyran ring (C). Differences in the linkage of aromatic ring (B) to the benzopyran (chroman) moiety (A and C) allow to distinguish between flavonoids (2-phenylbenzopyrans), isoflavonoids (3-benzopyrans), and neoflavonoids (4-benzopyrans) (Fig. 01). The 2-phenylbenzopyrans are further divided into two groups depending on the presence of a hydroxyl group at position C-3 of C-ring. These include: 3-hydroxyflavonoids, which contain a hydroxyl group (flavonols, flavanols, anthocyanidins, dihydroflavonols), and 3-deoxyflavonoids, which are short of a hydroxyl group (flavanones and flavones). Flavones differ from flavanones by a C2-C3 double bond (Fig. 02) (Marais *et al.*, 2006). The flavanone class encompasses an array of compounds with simple and complex structures referring to their *O*- and/or *C*-substitutions (hydroxy, methoxy, methylenedioxy, *C*-methyl, *C*-hydroxymethyl, *C*-formyl groups), isoprenoid substituents (noncyclic isoprenoid group, furano or dihydrofurano rings, dimethylpyrano or dimethyldihydropyrano rings), *C*-benzyl groups, stilbene and anastatin moieties, conjugations to phenolic acids, and diarylheptanoid attachments (Veitch and Grayer, 2006).

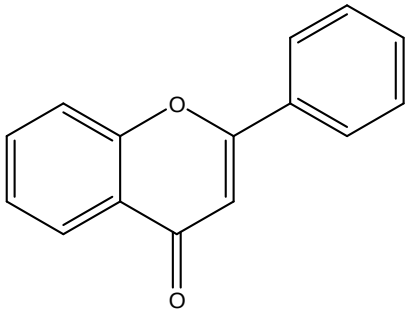


1 **2** **3**

Figure 01: Basic structures of Flavonoids **1**, Isoflavonoids **2**, and Neoflavonoids **3**.



Flavanone



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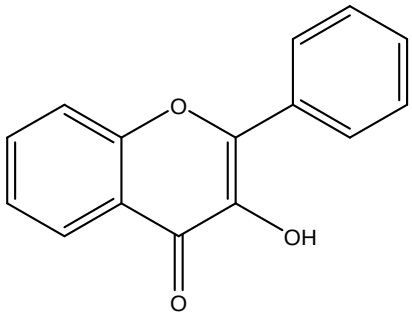
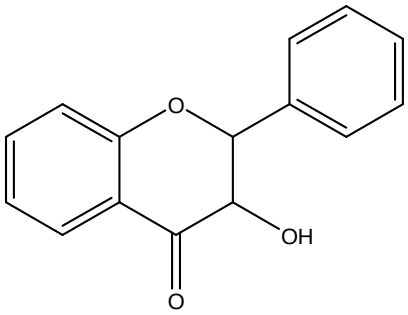
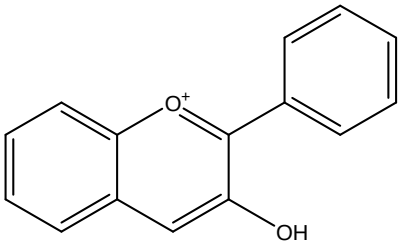


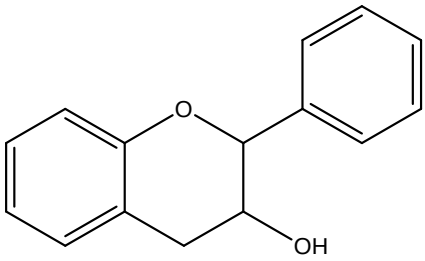
Flavonol



Dihydroflavonol



Anthocyanidin



Flavanol

Figure 02: Basic structures of Flavonoid subclasses.

1.2. Biosynthesis of Flavanones in Plants:

Due to the diverse physiological functions in plants and beneficial nutritional effects, flavonoids are now attractive targets for genetic engineering strategies with aim to produce plants having high nutritional value by modifying the flavonoids biosynthesis. In most of the plant species, the flavonoid biosynthetic pathway has been almost completely elucidated. In general, the biosynthesis of flavonoids is initiated by two precursors named Malonyl-CoA and *p*-Coumaroyl-CoA which are originated from carbohydrate metabolism and phenylpropanoids pathway, respectively. After the condensation of three molecules of malonyl-CoA with one molecule of *p*-coumaroyl-CoA, yellow coloured chalcones are formed which consist of two phenolic groups attached by an open three carbon bridge. This enzymatic initiated step is catalysed by chalcone synthase. The unstable chalcone form is normally isomerised by the enzyme chalcone isomerase to form the corresponding flavanone. Flavanones are the backbone of this biosynthesis pathway as based on them all other flavonoid classes are generated like flavones, isoflavones, flavanols, flavonols and anthocyanidins (Fig 03) (Schijlen *et al.*, 2004; Marten and Mithöfer, 2005). Moreover, in citrus species, UDP-glucose flavanone-7-*O*-glucosyltransferase (UFGT) and UDP-rhamnose flavanone glucoside rhamnosyltransferase (UFGRT) sequentially convert the flavanone aglycones into their glucosides and rhamnoglucosides (Lewinsohn *et al.*, 1989).

This biosynthetic pathway is highly exploited by agronomists, plant pathologists, soil scientists, and biologists to study the role of phenolic compounds in different plant physiological functions such as insect-plant interaction (Simmonds, 2001), pigmentation (Mato *et al.*, 2000), heavy metal tolerance (Keilig and Ludwig-Müller, 2009), disease resistance and UV-scavenging (Cooper-Driver and Bhattacharya, 1998). Recently, Fowler and Koffas (2009) have reviewed the biotechnological production of flavanones by using various microorganisms. On the other hand, some works deal with trying to produce lower levels of flavanones in plants. For example, an *Agrobacterium*-mediated genetic transformation approach has been used to reduce the naringin contents (due to its bitter taste) in *Citrus paradisi* Macf. (grapefruit). A decrease in leaf naringin levels was obtained by targeting the chalcone synthase (CHS) and chalcone isomerase (CHI) genes (Koca *et al.*, 2009).

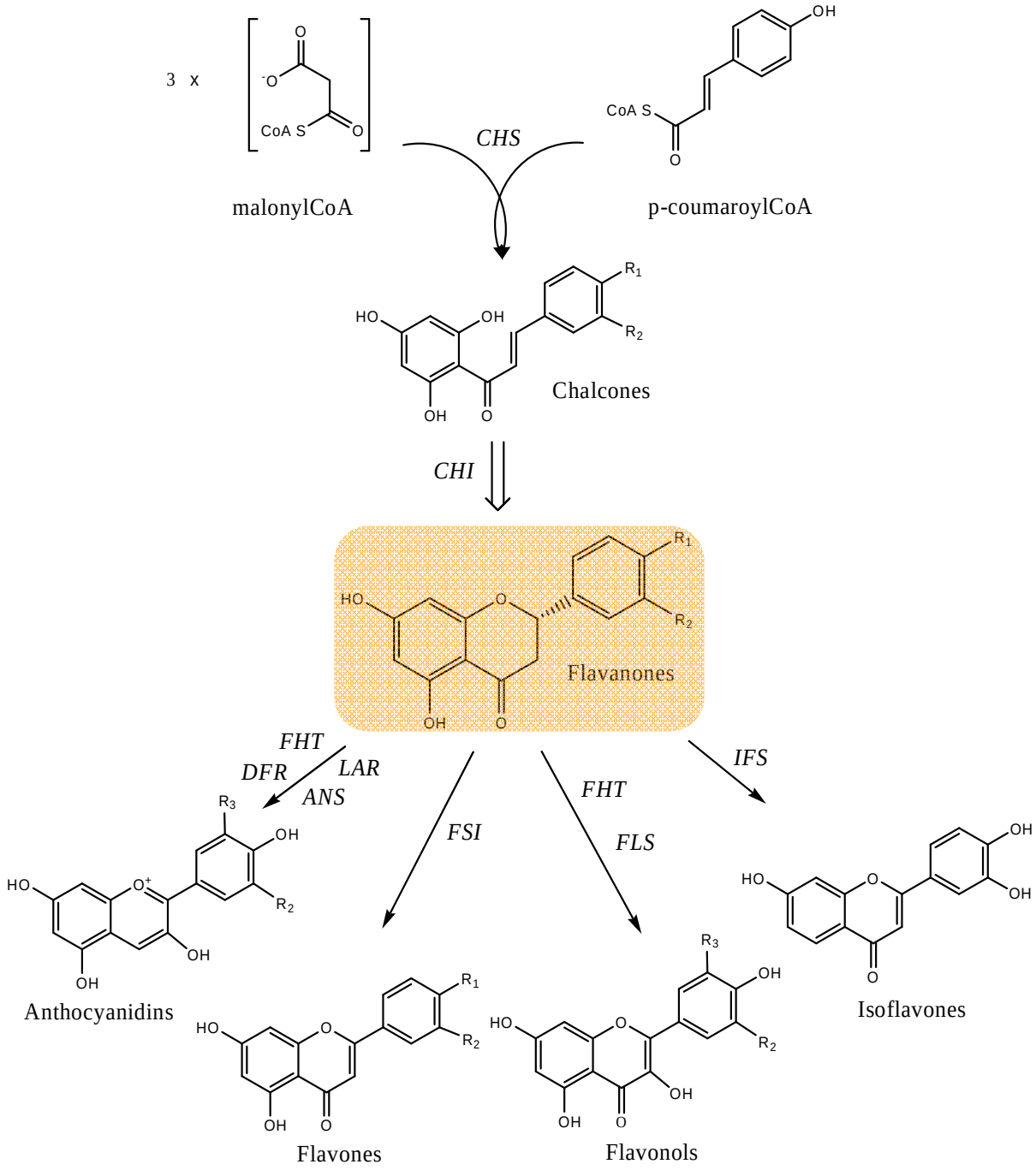


Figure 03: Biosynthesis of flavonoids.
R is generally OH or OMe, although other substitutions can be occurred at these positions.
CHS: Chalcone synthase; *CHI*: Chalcone isomerase; *FHT*: Flavanone 3-hydroxylase; *DFR*: Dihydroflavonol 4′-reductase; *LAR*: Leucoanthocyanidine 4-reductase; *ANS*: Anthocyanidin synthase; *FSI*: Flavone synthase; *FLS*: Flavonol synthase; *IFS*: 2-Hydroxyisoflavone synthase.

1.3. Diversity and Distribution of Flavanones:

1.3.1. Introduction

Fruits and vegetables are rich sources of micronutrients such as vitamins and antioxidants. Among these phytochemicals, flavanones are widely distributed in about 42 higher plant families especially in Compositae, Leguminosae and Rutaceae (Iwashina, 2000). A few decades ago, flavanones were only considered minor flavonoids (see Bohm in the three volumes of "The Flavonoid Advances in Research", Ed. J. B. Harborne, published between 1975 and 1994), like chalcones, dihydrochalcones, dihydroflavonols and aurones. However, during the last 15 years, the total number of known flavanones has become so large that they now appear among the major flavonoid classes like flavones, isoflavones, flavanols, flavonols and anthocyanidins (see Veitch and Grayer in "Flavonoids – Chemistry, Biochemistry and Applications", Eds Andersen and Markham, 2006).

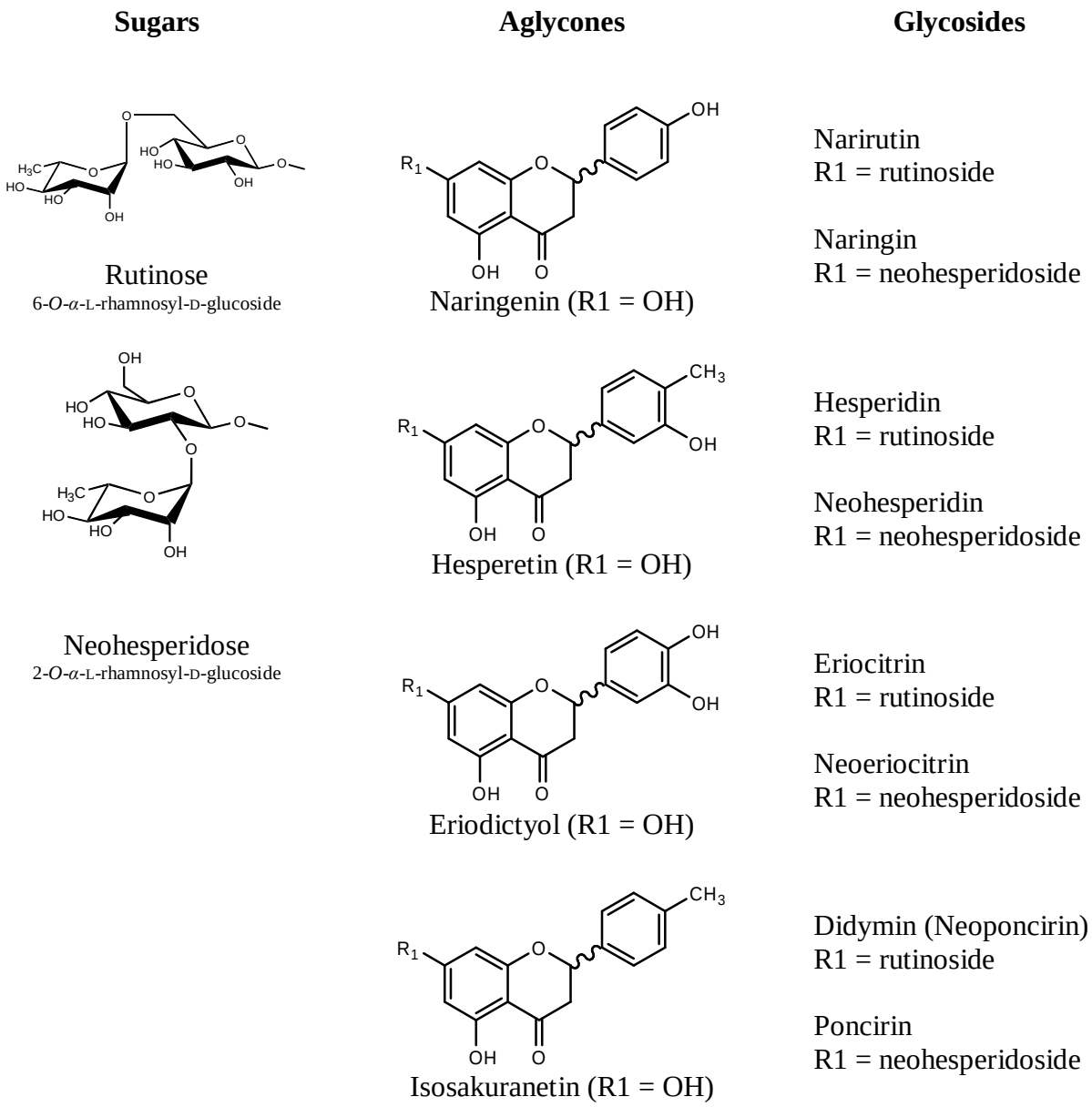
Based on the criterion of flavanone content, citrus plants belonging to the Rutaceae family appear especially important. Depending on the plant type, flavanones can be found in all plant parts, above- and below-ground, from vegetative part to generative organs: stem, branches, bark, flowers, leaves, roots, rhizomes, seeds, fruits, peels etc. Beside the aglycone forms, flavanones are also present along with their conjugates. They can be classified into several subgroups depending on their *O*-substitution (OH and OMe), *C*-methylation, *C*-prenylation and *C/O*-glycosylation (Veith & Grayer, 2008). Up to now about 350 flavanone aglycones and 100 flavanone glycosides have been discovered in nature (Iwashina, 2000). The glycosidic forms represent a significant proportion of the conjugated flavanones (Fig 04).

It is worth noting that the highest concentrations of flavanones are found in peel as compared to the fleshy part of citrus fruit (Nogata *et al.*, 2006). Of the plant flavanones, the naringenin and hesperetin aglycones and their glycosides are of particular interest because of their high prevalence in foods.

1.3.2. Naringenin

Naringenin (5,7,4′-trihydroxyflavanone) is found in high concentrations in citrus fruits while low concentrations are also found in tomatoes and their products (Erlund, 2004). Naringenin can be found as aglycone and / or as glycosides. Among the latter, naringin and

Figure 4: Some common flavanone aglycones and their respective glycosides



narirutin are especially abundant. Naringin (naringenin-7-neohesperidoside) is the conjugate of naringenin with neohesperidose (rhamnosyl-α-1,2 glucose) and has a bitter taste due to its glucose moiety. Naringin is the major flavonoid of grapefruit and sour orange, which present different naringin contents depending on their varieties (Table 01). Other citrus species like sweet orange, tangelo, lemon and lime exhibit low quantities of naringin. Another major naringenin glycoside, narirutin (naringenin-7-rutinoside) displays a rutinose (rhamnosyl-α-1,6 glucose) moiety and is most abundant in grapefruit although less than naringin. Significant levels of narirutin are also detected in tangor, sweet orange, tangerine and tangelo (Peterson *et*

al., 2006a & 2006b). The naringenin chalcone is found in higher quantities in tomato peels, which also have some other flavanone chalcones (Iijima *et al.*, 2008).

Table 1: Flavanones glycosides in different citrus varieties

Citrus type	Narirutin	Naringin	Hesperidin	Neohesperidin	Eriocitrin	Neoeriocitrin	Didymin	Poncirin
Grapefruit	4.90	16.60	2.78	1.4	0.45	0.35	0.07	0.17
Grapefruit red and pink	3.34	13.87	0.27	0.42	0.00	0.00	0.00	0.00
Grapefruit white	5.36	16.90	3.95	0.25	0.16	0.05	0.09	0.20
Lemon	0.80	0.18	15.78	0.00	9.46	0.00	0.17	0.00
Lime	0.23	0.00	15.64	0.00	1.38	0.04	0.00	0.00
Sour orange	0.08	18.83	0.00	11.09	0.53	14.01	2.89	0.00
Sweet orange	2.33	0.17	15.25	0.00	0.28	0.04	0.45	0.00
Tangelo	2.42	5.60	4.21	13.56	1.69	1.11	0.60	0.00
Tangerine (mandarin)	2.70	0.00	19.26	0.00	0.02	0.00	1.11	0.00
Tangor	7.10	0.00	15.42	0.00	1.01	1.77	0.00	0.00

Mean values are in mg aglycone / 100 g juice or edible fruit (without rind, pith and seeds)
Value are taken from Peterson *et al.*, 2006a & 2006b

1.3.3. Hesperetin

As naringenin, hesperetin (4′-methoxy-5,7,3′-trihydroxyflavanone) and its glycosides are also mainly present in citrus fruits. The aglycone is less dominant in nature than the glycosides. The most widely distributed glycosides of hesperetin are hesperidin and neohesperidin, which are conjugates with rhamnosyl-α-1,6-glucose and rhamnosyl-α-1,2 glucose, respectively. Hesperidin (hesperetin-7-rutinoside) is present in higher extents in lemons, limes, sweet oranges, tangerine and tangor species of citrus fruits (Cano *et al.*, 2008), while neohesperidin (hesperetin-7-neohesperidoside) is absent in them. Significant amounts of both also occur in grapefruits while tangelo and sour orange are especially rich in neohesperidin (Peterson *et al.*, 2006a & 2006b).

1.4. Extraction of flavanones:

Epidemiological studies have suggested the beneficial effects of citrus fruits (rich in flavanones) against many degenerative diseases like cardiovascular diseases and some cancers (Benavente-Garcia *et al.*, 1997; Tripoli *et al.*, 2007). These positive influences on human health has significantly increased the citrus consumption in the last few years and it is continuously increasing with an estimated world production of citrus fruits up to 82 million tons in the session 2009–2010, among which the major commercially important orange fruits accounts for about 50 million tons (USDA, 2010). The domestic and industrial use of these large quantities of citrus fruits, especially for the production of juice, results in the accumulation of high amounts of by-products such as peel, seed, cell and membrane residues which account for about half of the fruit weight. These by-products can be used for the production of molasses, pectins, essential oils, limonene and cattle feed (Bocco *et al.*, 1998; Jeong *et al.*, 2004; Li *et al.*, 2006a, 2006b). In addition, citrus by-products are a good source of phenolic compounds, especially the characteristic flavanone glycosides which mainly include naringin, hesperidin, narirutin, and neohesperidin. Currently, their extraction from citrus peels has attracted considerable scientific interest to use them as natural antioxidants mainly in foods to prevent the rancidity and oxidation of lipids (Anagnostopoulou *et al.*, 2006; Peschel *et al.*, 2006; Zia-ur-Rehman, 2006). Indeed, in recent years, a lot of research has focused on plants and their by-products to extract natural and low-cost antioxidants that can replace synthetic additives such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), which might be liver-damaging, carcinogenic (Ak & Gülçin, 2008) and more generally toxic (Moure *et al.*, 2001).

Up to now, several conventional extraction techniques have been reported for the extraction of phenols from citrus peels like solvent extraction (Anagnostopoulou *et al.*, 2006; Jeong *et al.*, 2004; Li *et al.*, 2006a; Manthey & Grohmann, 1996; Xu *et al.*, 2007; Zia-ur-Rehman, 2006), hot water extraction (Xu *et al.*, 2008), alkaline extraction (Bocco *et al.*, 1998; Curto *et al.*, 1992), resin-based extraction (Calvarano *et al.*, 1996; Kim *et al.*, 2007), enzyme-assisted extraction (Li *et al.*, 2006b), electron beam- and c-irradiation-based extractions (Kim *et al.*, 2008; Oufedjikh *et al.*, 2000) and supercritical fluid extraction (Giannuzzo *et al.*, 2003). These conventional or more innovative extraction techniques may either cause the degradation of the targeted compounds due to high temperature and long extraction times as in solvent extractions, or pose some health-related risks due to the unawareness of safety

criteria during irradiation. Furthermore, enzyme-assisted extraction is limited due to problems of enzyme denaturation.

With the increasing energy prices and the drive to reduce CO_2 emissions, chemical and food industries are challenged to find new technologies in order to reduce energy consumption, to meet legal requirements on emissions, product/process safety and control, and for cost reduction and increased quality as well as functionality. Separation technology (such as extraction, distillation, and crystallization) is one of the promising innovation themes that could contribute to sustainable growth of chemical and food industries. For example, existing extraction technologies have considerable technological and scientific bottlenecks to overcome: often requiring up to 50% of investments in a new plant and more than 70% of total process energy used in food, fine chemicals and pharmaceutical industries. These shortcomings have led to the consideration of the use of new "green" techniques in extraction, which typically use less energy and the low costs, such as microwave extraction, ultrasound extraction, ultrafiltration, flash distillation and controlled pressure drop process (Chemat *et al.*, 2009).

With the development of the ‘‘Green Chemistry” concept during the last few years, environment-friendly techniques are becoming more and more attractive. The extraction of bioactive compounds under ultrasound irradiation (20–100 kHz) is one of the upcoming extraction techniques that can offer high reproducibility in shorter times, simplified manipulation, reduced solvent consumption and temperature and lower energy input (Chemat, Tomao, & Virot, 2008). During sonication, the cavitation process causes the swelling of cells or the breakdown of cell walls, which allow high diffusion rates across the cell wall in the first case or a simple washingout of the cell contents in the second (Vinatoru, 2001). It will be important to quote that bursting of cavitation bubbles may cause a temperature of 5000°C and the pressure of 1000 atm. However, this extremely high amount of heat produced cannot significantly affect the bulk conditions because the bubbles are very tiny and the heat is dissipated to the medium in very short period of time (Luque-Garcia & Luque de Castro, 2003). UAE highly depends on the destructive effects of ultrasonic waves. Besides the solvent, temperature and pressure, better recoveries of cell contents can be obtained by optimising ultrasound application factors including frequency, sonication power and time, as well as ultrasonic wave distribution (Wang & Weller, 2006). Optimisation of ultrasound-assisted extraction (UAE) has been described recently to extract hesperidin from Penggan

(*Citrus reticulata*) peel (Ma *et al.*, 2008a), phenolic acids and flavanone glycosides from Satsuma Mandarin (*Citrus unshiu* Marc) peel (Ma *et al.*, 2009; Ma *et al.*, 2008b) and total phenolic contents from Penggan peel (Ma *et al.*, 2008a). Some other examples showing the efficiency of UAE in comparison to conventional or other innovative techniques are presented in Table 1 of chapter 2. In these works, methanol came up as a suitable extraction solvent to reach good yields of the above-mentioned phenolic compounds. However, environmentally benign and non-toxic food grade organic solvents like ethanol, n-butanol and isopropanol are recommended by the US Food and Drug Administration for extraction purposes (Bartnick *et al.*, 2006). Using these food grade solvents, UAE was found more efficient for the extraction of polyphenols from orange peel wastes than conventional solvent extraction (Khan *et al.*, 2010). Moreover, in a 'green chemistry' approach, extraction without solvent has been developed using the technique of Microwave Hydrodiffusion and Gravity (Zill-e-Huma *et al.*, 2009).

1.5. Synthesis of Flavanones

1.5.1. Aglycones

Up to now, the most common pathway for the synthesis of flavanone aglycones is the aldol condensation of 2-hydroxyacetophenones with benzaldehydes (Claisen–Schmidt condensation reaction). The reaction is usually performed under heating using acidic or alkaline conditions. The chalcones initially formed undergo cyclisation to their respective flavanones under the same conditions (Krbechek *et al.*, 1968; French *et al.*, 2010). The condensation is still under study to develop efficient and environment-friendly conditions. For instance, strongly alkaline sodium hydroxide and ethoxide were replaced by Mg-Al hydrotalcites (Climent *et al.*, 1995). Furthermore, different derivatives of chalcones and flavanones were also prepared by aldol condensation (Hsieh *et al.*, 1998). Currently, the emphasis is on developing new catalysts that could be effective in aldol condensations and alternative methods (Chandrasekhar *et al.*, 2005). Recently, the introduction of Li was shown to increase the surface basicity and catalytic activity of MgO in the synthesis of flavanone aglycones (Cortes-Concepcion *et al.*, 2010). An alternative method of Claisen-Schmidt condensation was also proposed to prepare flavanone aglycones and their derivatives (Shi *et al.*, 2010). The method was more straightforward than the Claisen-Schmidt condensation and the overall yield was similar (figure 05).

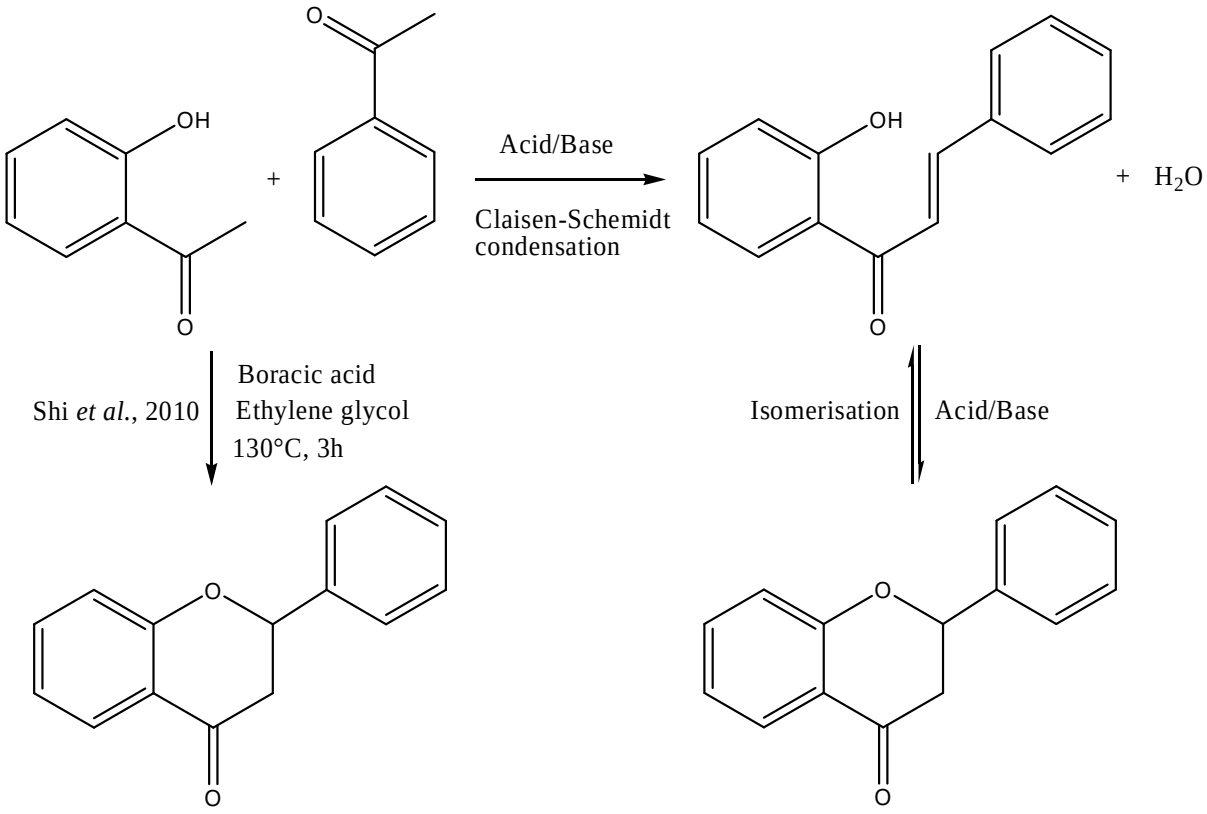


Figure 05: Flavanone synthesis via the Claisen–Schmidt condensation of 2′-hydroxyacetophenone with benzaldehyde followed by the isomerization of the 2′-hydroxychalcone intermediate formed versus the method proposed by Shi et al., 2010.

1.5.2. Chalcones

The scarcity of flavanone chalcones in Nature is primarily due to their instability, as in neutral medium, they undergo cyclisation to the corresponding flavanones. However, chalcones can be simply prepared by opening of the C-ring of flavanones using strongly alkaline conditions (Miles and Main, 1985). The reaction starts by the removal of a weakly acidic hydrogen atom from the flavanone C3 to yield an enolate anion, which opens up into a chalcone anion (Andújar *et al.*, 2003). Upon quick acidification, the chalcone precipitates and can be isolated as a solid. This procedure was successfully used for the preparation of the naringin chalcone (Gonzàlez *et al.*, 2002) and 2′,6′-dihydroxy-4,4′-dimethoxychalcone (Miles and Main, 1985).

1.5.3. Glycosides

The most prevalent flavanone derivatives are the 7-*O*-*β*-glycosides. The selective glycosylation of 7th position OH group flavanone can be performed by using the well known methods of Koenigs and Knorr (silver carbonate and quinoline) (Zemplén and Bognàr, 1943; Oyama and Kondo, 2004) or the method of Zemplén and Farkas (10% aq. sodium or potassium hydroxide and acetone used for synthesis of hesperedin) (Zemplén and Farkas, 1943). With some modifications, these methods are still in use not only for glycosylation of phenolic compounds (Esaki *et al.*, 1994) but also for glucuronidation (Moon *et al.*, 2001). A simple route to flavanone 7-glucoside is the partial hydrolysis of naringin and hesperedin using formic acid in cyclohexanol (Fox *et al.*, 1953). The enzymatic synthesis of flavanone glycosides was also described (Kometani *et al.*, 1996) as well as the synthesis of amino derivatives for use as scaffolds in drug discovery (Hanessian and Kothakonda, 2005) and metal complexes to increase the antioxidant and anti-inflammatory activities (Pereira *et al.*, 2007).

1.5.4. Glucuronides

A better knowledge of the biochemical mechanisms by which dietary flavanones exert their potential health effects requires investigations on appropriate cell models (e.g., endothelial or smooth muscle cells) with the authentic circulating metabolites, of which glucuronides make the largest contribution, instead of the commercially available glycosides and aglycones that are frequently used as a first approach despite the limited biological significance. As an alternative to the expensive, inconvenient and low yielding extraction of conjugates from biological fluids, chemical synthesis appears as the most direct strategy to obtain substantial amounts of these metabolites for bioavailability and in vitro cell studies. Hence, there is a growing interest for the synthesis of polyphenol glucuronides as standards for identification and titration of in vivo metabolites and as biologically pertinent compounds for cell studies aiming at elucidating the potential health effects of polyphenols. Several works have been published about the chemical synthesis of polyphenol glucuronides.

For instance, the popular procedure, based on the Lewis acid-activated coupling of methyl-2,3,4-tri-*O*-acetyl-1-*O*-(trichloroacetimidoyl)-*α*-D-glucuronate (Tomas-Barberan & Clifford, 2000) with partially protected polyphenols, was applied to the synthesis of isoflavone 7-*O*-*β*-D-glucuronides (Al-Maharik & Botting, 2006), quercetin 3-*O*-*β*-D-glucuronide (Needs & Kroon, 2006) and a series of hydroxycinnamic acid *O*-*β*-D-glucuronides (Galland *et al.*, 2008). Catechin *O*-*β*-D-glucuronides were also prepared with

methyl-2,3,4-tri-*O*-acetyl-1-*O*-bromo-α-D-glucuronate as the glucuronyl donor (Gonzàlez-Manzano *et al.*, 2009). Recently, the synthesis of a flavanone glucuronide (persicogenin 3′-*O*-β-D-glucuronide) was carried out with methyl-2,3,4-tri-*O*-acetyl-1-*O*-(trifluoroacetimidoyl)-α-D-glucuronate, followed by a final deprotection step involving pig liver esterase (PLE) for the hydrolysis of the methyl ester of the glucuronyl residue (Boumendjel *et al.*, 2009). A synthesis of quercetin 3-*O*-β-D-glucuronide was also performed by regioselective oxidation of the corresponding 3-*O*-β-D-glucoside (phenolic OH groups protected as benzyl ethers) using TEMPO/NaOCl/NaBr under phase transfer conditions (Bouktaib *et al.*, 2002). Recently, the synthesis of four flavanone glucuronides (naringenin 4′- and 7-*O*-β-D-glucuronides and hesperetin 3′- and 7-*O*-β-D-glucuronides) based on a regioselective protection of the flavanone nucleus was reported (Khan *et al.*, 2010).

1.6. Bioavailability of Flavanones

1.6.1. Introduction

The oral bioavailability of a given nutrient describes its fate once ingested: intestinal absorption, transport in the general circulation, delivery to tisssues, metabolism and excretion. In spite of the high consumption of citrus fruits and juices worldwide, the bioavailability of flavanones is still incompletely known. Their daily intake has not been estimated in different populations but could be quite high compared with the average flavonol intake (25 mg/day) in several European countries (Manach *et al.*, 2003). For instance, the mean dietary intake in Finland has been evaluated to be 8.3 mg/day and 28.3 mg/day for naringenin and hesperetin, respectively (Manach *et al.*, 2003; Erlund, 2004).

After oral intake, flavanone monoglycosides and diglycosides are hydrolysed in the small intestine and in the colon, respectively, and the released aglycones or phenolic acids are converted into their respective glucuronides, sulphates and sulphoglucuronides during their passage across the small intestine and liver. Finally, the bioactive forms (metabolites) are distributed through plasma at various cell sites and significant quantities can also be found in urinary excretions (Matsumoto *et al.*, 2004). The fate of flavanones after ingestion is summarized in figure 6.

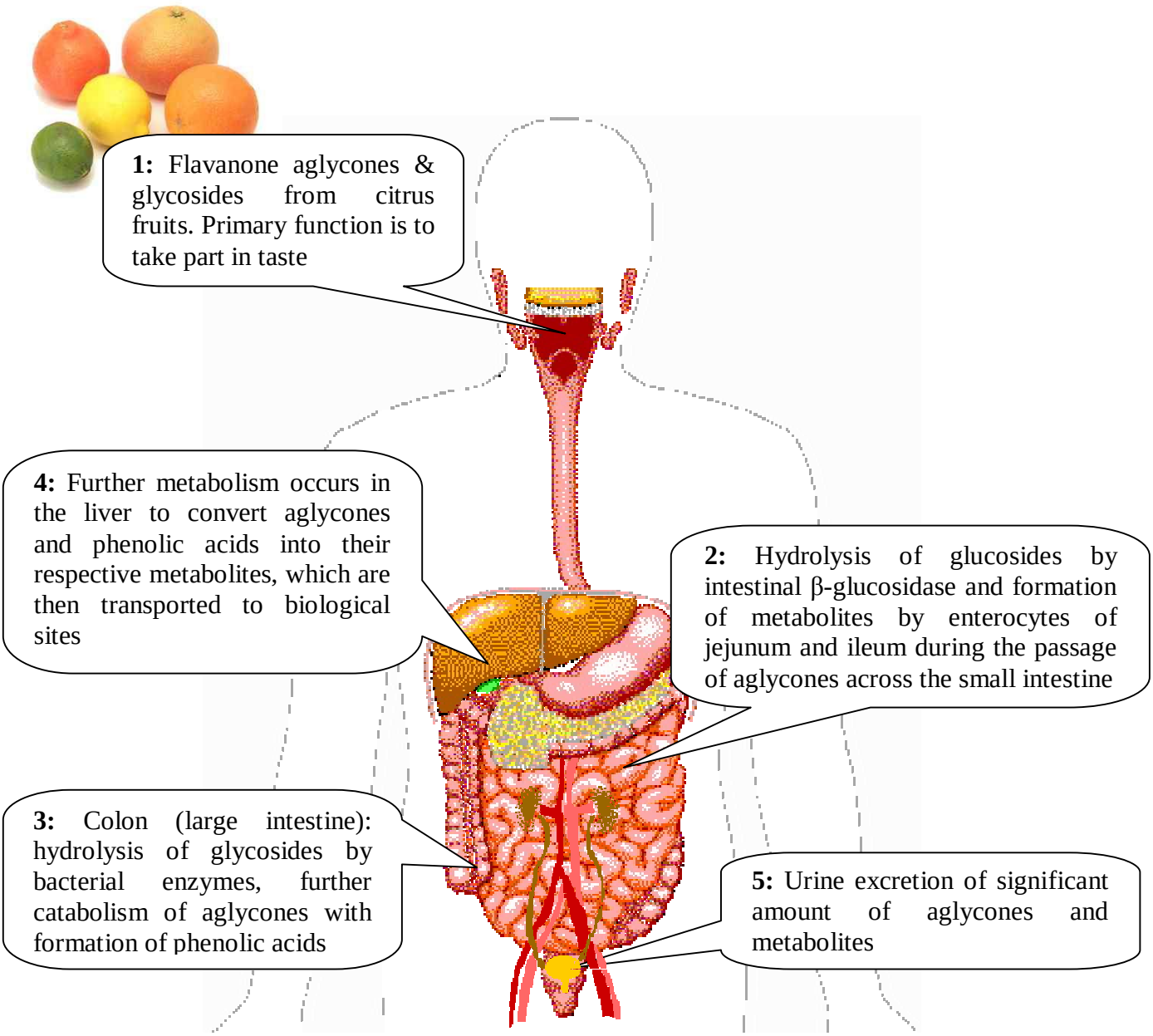


Figure 6: Metabolic fate of flavanones

1.6.2. Metabolism of flavanones and their metabolites

A great part of the bioavailability studies has been devoted to naringenin, hesperetin and their glycosides. Improvements in methods for analyzing flavanone metabolites in human plasma and urine have made possible to estimate flavanone bioavailability in humans.

The the first step in flavanone metabolism is the extensive deglycosylation of flavanone glycosides within the intestinal epithelium by human and bacterial enzymes like β-glucosidase, rhamnoglucosidase, rutinoglucosidase etc. Investigations in rats demonstrated

that the deglycosylation of naringenin-7-glucoside occurred early in the small intestine (Choudhury *et al.*, 1999) while that of naringenin-7-rhamnoglucosides occurred in the colon (large intestine). Indeed, naringenin conjugates (glucurono- and /or sulfo conjugates) appeared within 3h in the plasma of rats fed with naringenin or its 7-glucoside whereas no naringenin metabolites were still detected in rats fed with naringenin-7-rhamnoglucoside. However, 10h after ingestion, similar naringenin concentrations were found regardless of the diet, which clearly showed the delayed intestinal absorption of naringenin rhamnoglucosides (Felgines *et al.*, 2000). It was confirmed in humans that hesperidin and naringin are absorbed in the distal part of the intestine (cecum). Once deglycosylated, the aglycones are glucuronated and/or sulphated during their transfer from the luminal side of the gut to the portal vein by the action of UDP-glucuronosyltransferase and sulphotransferase enzymes (Manach *et al.*, 2003). In cecum, the intestinal microflora not only cleaves the glycosidic bonds but also degrades the aglycones into phenolic acids such as *p*-hydroxyphenylpropionic acid (*p*-HPPA), *p*-coumaric acid (*p*-CA), and *p*-hydroxybenzoic acid (*p*-HBA) (Felgines *et al.*, 2000; Manach *et al.*, 2003). Likewise, eriocitrin (eriodictyol-7-rutinoside) is metabolised by intestinal microflora (Bacteroides distasomis or B. uniformis) to eriodictyol, which is then converted into 3,4-dihydroxycinnamic acid by Clostridium butyricum (Miyake *et al.*, 2000). After intestinal absorption, metabolites, aglycones and phenolic acids reach the liver, the main organ involved in flavanone metabolism, where further glucuronidation, sulfation, and in some cases methylation occur, thus converting the rest of aglycones and phenolic acids into their respective metabolites. Due to lack of catechol groups in hesperetin and naringenin, no methylation by catechol-o-methyltransferase (COMT) was observed which is in contrast to catechin and quercetin (Felgines *et al.*, 2000). Two metabolic pathways are possible with eriocitrin (eriodictyol rutinoside): one is the formation of phenolic acids (3,4-dihydroxycinnamic acid) by the microflora and the second is the formation of eriodictyol, homoeriodictyol (3′-methoxy-4′,5,7-trihydroxyflavanone) and hesperetin (4′-methoxy-3′,5,7-trihydroxyflavanone) conjugates due to methylation of the catechol group of the aglycone. The conversion of eriodictyol to homoeriodictyol and hesperetin through methylation in liver was also reported (Miyake *et al.*, 2000).

Recently, a study was conducted to determine the effect of tumor on flavanone metabolism. The similar naringenin concentrations in liver and kidney of healthy and tumor-bearing rats suggested that there was no effect of tumor on intestinal and hepatic metabolism of flavanones (Silberberg *et al.*, 2006).

Moreover, the impact of full-fat yogurt on the bioavailability and metabolism of orange flavanones in human was investigated by analysing the human plasma and urine over different intervals of time. Addition of yogurt into orange juice significantly reduced the quantity of flavanone metabolites excreted up to 5 h after ingestion. However, a statistical analysis over a longer time span (0-24 h) did not show any significant effect of yogurt addition (Mullen *et al.*, 2008).

Glucuronidation and sulfation are the major conjugation pathways of flavanone aglycones. Structural studies on the plasma and urinary metabolites showed that the major metabolites of naringenin are naringenin-7-glucuronide, naringenin-4′-glucuronide, naringenin-7-sulfate-4′-glucuronide, naringenin-7-glucuronide-4′-sulfate and naringenin-7,4′-disulfate (Tripoli *et al.*, 2007; Brett *et al.*, 2009). Similarly, the main hesperetin conjugates are hesperetin-7-glucuronide, hesperetin-3′-glucuronide, hesperetin diglucuronide and hesperetin sulfoglucuronide (Matsumoto *et al.*, 2004; Mullen *et al.*, 2008). Among all these metabolites, glucuronides largely prevail (87%) but the importance of the other metabolites should not be underestimated (Manach *et al.*, 2003). The position at which glucuronidation occurs might influence the resulting bioactivity including the antioxidant activity (Tripoli *et al.*, 2007). Up to now, no data have been reported about the antioxidant activity of flavanone glucuronides. However, since the common flavanones hesperetin and naringenin are devoid of catechol group, which is the critical structural determinant of the antioxidant (reducing) activity for polyphenols, both are weak antioxidants and their glucuronides (with one less free phenolic OH group) are expected to be even less potent. It is thus quite likely that the bioactivity expressed by flavanone glucuronides is largely unrelated to their redox properties and rather reflects their interactions with specific proteins.

1.6.3. Pharmacokinetics

After the oral administration of 500 mg of naringin, urine analysis of healthy volunteers was optimised for the determination of naringenin and its metabolites (Ishii *et al.*, 1997). Moreover, the pharmacokinetics of naringenin and its glucuronides in rat plasma and brain tissue was successfully performed by HPLC (Peng *et al.*, 1998). The study was extended to determine the naringenin levels in rat blood, brain, liver and bile using microdialysis coupled with a HPLC system (Tsai, 2002). Most probably, the first report on the pharmacokinetics of flavanones in human subjects was published by Erlund and co-authors in 2001. After ingestion of orange or grapefruit juice (8 mL/kg of body weight), the plasma

concentration of hesperetin and naringenin aglycones (after deconjugation) was found in the range 0.6 – 6 μmol/L. Moreover, elimination half-lives ($t_{1/2}$) in the range 1.3 – 2.2 h showed a relatively fast clearance. The percentage of flavanones excreted in urine was lower than that of their absorption, which indicated a substantial distribution to tissues for these phenolic compounds (Erlund *et al.*, 2001). In another study, ingestion of hesperetin and naringenin (135 mg of each) under fasting conditions resulted in their appearance as metabolites in blood plasma 20 min later. The peak plasma concentration (C_{max}) of 2.7 μmol/L and 7.4 μmol/L was reached 4.0 and 3.5 h afer ingestion, respectively (Kanaze *et al.*, 2007). Plasma and urine analyses pointed to the higher naringenin bioavailability in comparison to hesperetin (Gardana *et al.*, 2007; Kanaze *et al.*, 2007). An in vitro hydrolysis showed a faster hydrolysis rate for hesperidin and narirutin (flavanone rutinosides) than for naringin and neohesperidin (flavanone neohesperiosides) (Wang *et al.*, 2008). More recently, the same group demonstrated the bioavailability of hesperetin and naringenin after the consumption of *Citrus aurantium* L. and *Citrus sinensis* Osbeck (Cao *et al.*, 2010).

The permeability of epithelial cells to flavanones is a good determinant of their intestinal absorption. Flavanones are transported from apical side (gut lumen) to basolateral side (blood). In in vitro models, hesperetin (aglycone) was found to be efficiently absorbed across Caco-2 cell monolayers in comparison to hesperidin (hesperetin glycoside). The absorption mechanisms involved transcellular passive diffusion along with a newly proposed mechanism of proton-coupled active transport (Kobayashi *et al.*, 2008a). The study was further elaborated to explain the H^+-driven polarised absorption and similar mechanisms were found for naringenin and eriodictyol aglycones (Kobayashi *et al.*, 2008b).

The faster absorption of flavanone aglycones compared to flavanone glycosides was also shown for eriodictyol and eriocitrin in humans (Miyake *et al.*, 2006).

Concentration of flavanone conjugates in plasma and urine is an important criterion to determine the site of absorption and estimate the bioavailability in human. It varies according to glycoside concentration and flavanone structure. After ingestion of 1 L of orange juice containing 444 mg of hesperidin and 96 mg of narirutin, the highest plasma concentration of hesperetin and naringenin (after deconjugation) were 1.28 ± 0.13 μmol/L and 0.20 ± 0.04μmol/L, respectively. The levels of flavanones in urine were expressed as percentage of their intake and amounted to 7.87 ± 1.69% for naringenin and 6.41 ± 1.32 % for hesperetin. The relative urinary excretion of flavanones was not significantly affected by the dose

ingested. The relative urinary excretion of naringenin from grapefruit was found higher (8.9%) (Manach *et al.*, 2003). In another study, high naringenin concentrations of 128 ± 2 μM, 144 ± 8 μM and 139 ± 15 μM were detected at time 10h in the plasma of rats fed with naringenin (0.25 % of total diet), naringenin-7-glucoside (0.38 %) and naringenin-7-rhamnoglucoside (0.5 %), respectively. The urinary excretion of naringenin was two times higher in naringenin-fed rats than in naringenin-7-rhamnoglucoside-fed ones (Felgines *et al.*, 2000). Still the missing part in most bioavailability studies is the availability of authentic conjugates for use as standards. The present study is aimed at bridging this gap by developing chemical syntheses of flavanone glucuronides. Those conjugates are also very much needed for investigating the mechanisms of their bioactivity in cell models.

1.7. Interaction of flavanones with Human Serum Albumin (HSA)

In the last decade, the biological studies performed to explore the possible health effects of flavanones were devoted to assess either their metabolism and bioavailability or their possible therapeutic value as potential drugs. But unfortunately much less attention was given to study the delivery of flavanones to specific biological sites. Flavonoids are transported to their biological sites by the blood plasma. Serum albumin is the major component of blood plasma, occurring there at a concentration of 0.6 mM. Beside the maintenance of colloidal osmotic blood pressure and bodily detoxification, serum albumin transports fatty acids, vast types of drugs and dietary polyphenols (Dangles & Dufour, 2006). The literature on the structural aspects and binding locations of HSA is well described by a number of comprehensive reviews. The determination of the amino acid sequences of HSA (585 amino acids) was the first important step for the determination of the binding properties of albumin (Behrens *et al.*, 1975; Meloun *et al.*, 1975). Then, X-ray crystallography made it possible to elucidate the three-dimensional structure of HSA and precisely characterise the binding domains (He & Carter, 1992). Mainly, HSA consists of three helical domains I (1-195), II (196-383) & III (384-585) and each domain is further subdivided into two subdomains A and B (Fig 7). The protein has an overall shape of heart and its structure is stabilised by 17 disulfide bonds. Subdomains IIA (site I) and IIIA (site II) are most studied because of their involvement in the binding of drugs and other xenobiotics. Both subdomains bind through hydrophobic cavities lined by some positively charged amino acid residues (*Lys*) at the entrance of the pockets (Sugio *et al.*, 1999).

A significant amount of the literature available on albumin-flavonoid interactions reports not only quantitative thermodynamic data (binding constants), but also qualitative analyses aimed at locating the possible binding sites (Dufour & Dangles, 2005; Banerjee *et al.*, 2008; Lu *et al.*, 2007; Rawel *et al.*, 2005). In particular, quercetin and its metabolites were studied for their affinity with HSA (Murota *et al.*, 2007; Zsila *et al.*, 2003). In addition to other conventional techniques, fluorescence spectroscopy is the analytical tool that is most widely used to investigate binding to HSA (Oravcovà *et al.*, 1996). Indeed, there is a fluorescent tryptophan residue (*Trp214*) in site I, which can be excited at 295 nm and emits fluorescence at 340 nm. From the quenching of this fluorescence by a given ligand, the binding constant can be estimated (Sulkowska, 2002).

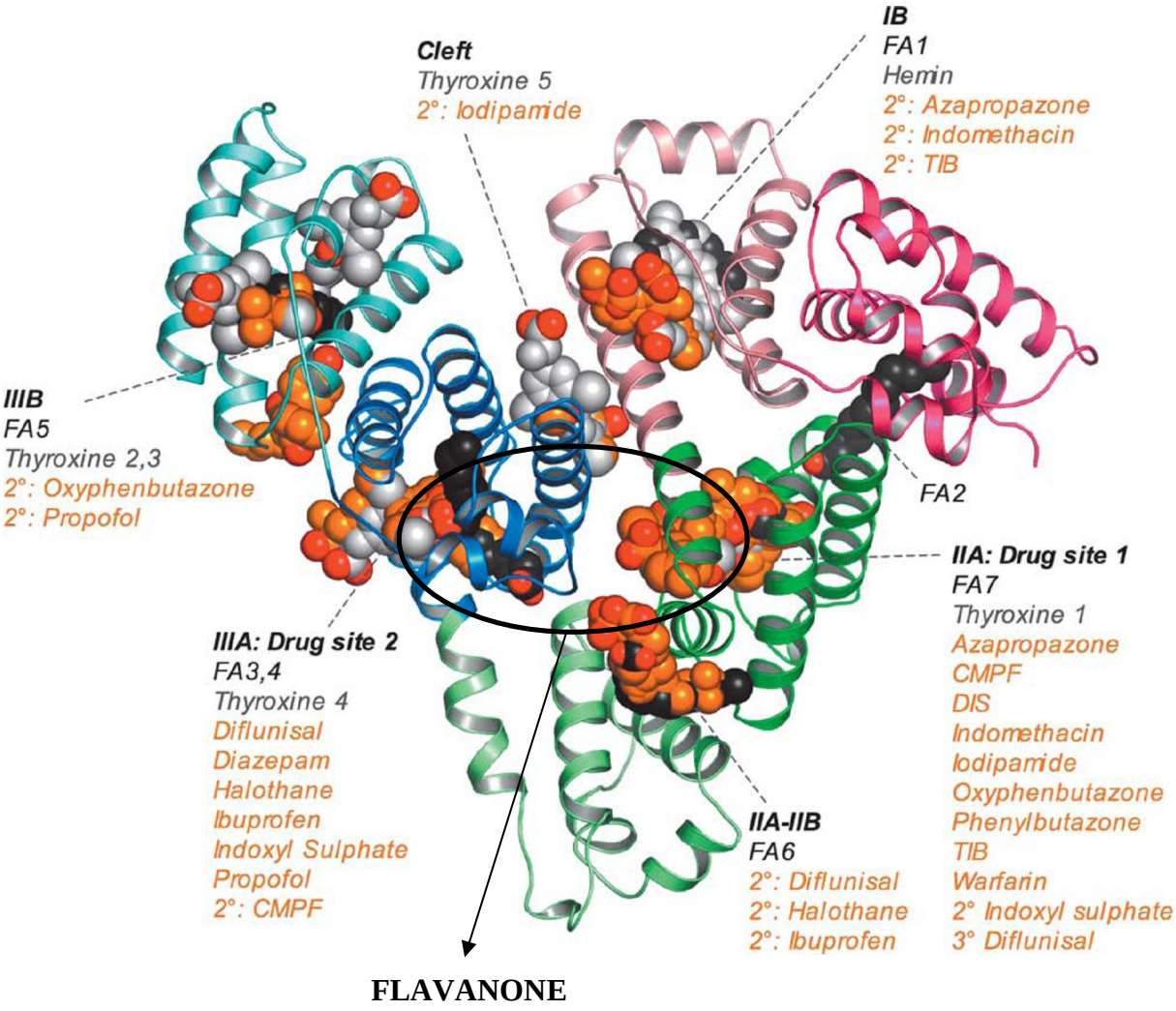


Figure 7: Three dimensional structure of HSA with common ligands bound at different binding sites. (adapted from Ghuman *et al.*, 2005)

The affinity of flavanones to HSA has been more recently investigated. In an original study, piezoelectric quartz crystal impedance (PQCI) analysis was performed to measure the affinity of hesperidin for immobilised HSA. The association constant calculated was 2×10^3 M^{-1} using Scatchard analysis (Liu *et al.*, 2004). The interaction of hesperidin with bovine serum albumin (BSA) was also investigated by fluorescence spectroscopy (Wang *et al.*, 2007). Xie and co-authors used fluorescence spectroscopy with support of Fourier-transformed infrared (FT-IR) and UV-visible spectroscopies to determine the binding constant, binding site and binding mechanism of hesperetin to HSA. From the Stern-Volmer equation, a binding constant of about 81×10^3 M^{-1} was estimated at pH 7.4. The *K* value decreased with increasing the pH from 6.4 to 8.4 due to a) conformational changes of HSA which affect the shape of the hydrophobic binding cavities and b) the increased dissociation of the phenolic hydroxyl groups of hesperetin. Moreover, FT-IR spectroscopy suggested that hesperetin binds to subdomain IIA. The main mechanisms involved in the interaction include the hydrophobic effect (van der Waals interactions between the ligand and hydrophobic amino acid residues with concomitant desolvation), electrostatic interactions between Lys residues and the flavanone phenolate ion, formed after deprotonation of the most acidic OH group at position C7, and hydrogen bonding between the phenolic OH and keto groups of hesperetin and the polypeptide chain or other polar amino acid residues (Xie *et al.*, 2005a). The study was further extended to investigate the association of naringenin ($K = 127\times10^3$ M^{-1}) with HSA (Xie *et al.*, 2005b). Conjugation of flavanone aglycones may affect their affinity for has. For instance, the binding constant of naringin, a naringenin diglycoside, was found lower ($K = 18\times10^3$ M^{-1}) than that of naringenin (Zhang *et al.*, 2008). So far, the affinity for HSA of true circulating flavonoid metabolites has not been investigated.

1.8. Bioactivity of Flavanones

1.8.1. Introduction

Over the last few decades, extensive research has been conducted on dietary compounds that could be protective against lethal diseases, in particular cardiovascular diseases and some types of cancers. These potentially bioactive compounds include phytoestrogens, carotenoids, ascorbic acid, citrus limonoids, organosulfur compounds and a good number of polyphenols. The basic mechanisms implicated in the potential health effects of polyphenols are mainly the inhibition of lipid and DNA oxidation (antioxidant activity) and the regulation of gene expression (Kris-Etherton *et al.*, 2002; Patil *et al.*, 2009). Like other

polyphenols, flavanones are also studied for their effects on specific cells. However, the missing part remains the investigation of true flavanone metabolites. Examples of in vitro / in vivo studies conducted to explore the beneficial effects of flavanones and the mechanisms involved are discussed below.

1.8.2. Radical-Scavenging Effect

Reactive oxygen species (ROS) / reactive nitrogen species (RNS) in biological systems are typically unstable and oxidizing species that are produced in low concentration for physiological signalling pathways and in larger concentration (oxidative stress) to destroy viruses and bacteria in leucocytes during infection (inflammatory response). The chronic exposure to oxidative stress is considered an initiating event in the development of degenerative diseases (Brown & Borutaite, 2006; Forman *et al.*, 2008). ROS/RNS include the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), the hydroxyl radical ($HO^{\bullet}$), the hypochlorite ion (ClO^-), nitrogen dioxide (NO_2) and peroxynitrite ($ONOO^-$), lipid oxyl and peroxyl radicals ($RO^{\bullet}$, $ROO^{\bullet}$) produced during the autoxidation of polyunsaturated fatty acids... Phenolic compounds are extensively studied for their ability to reduce ROS/RNS (antioxidant activity, AA), thereby preventing the oxidative damage they cause to the host's biomolecules.

The antioxidant activity of flavanones depends upon the number and spatial arrangement of phenolic OH groups (Cai *et al.*, 2006; Sadeghipur *et al.*, 2005). Up to now, in vitro and in vivo investigations have been performed to determine the antioxidant potential of flavanone aglycones, chalcones, and glycosides. No literature is available about the antioxidant capacity of flavanone glucuronides.

A comparative study on the antioxidant properties of nine different flavanones (naringin, neohesperidin, neoeriocitrin, hesperidin, narirutin, naringenin, hesperetin, heridictyol and isosakuraternin) using the crocin bleaching inhibition assay has shown that the presence of a catechol nucleus (3′,4′-dihydroxy substitution on the B-ring) and its *O*-methylation have no significant effect on the AA of aglycones, which is surprising. By contrast, an increase in AA was observed with the glycosides having a catechol nucleus while *O*-methylation of the catechol has an opposite effect (Di Majo *et al.*, 2005). *O*-glycosylation often reduces the AA, which points to the participation in the radical-scavenging reaction of the OH group involved in the glycosidic bond (Acker *et al.*, 1996). The different glycosydic

moieties may also have a small effect on AA. For instance, the glycosylation of hesperetin on the C7-OH group by neohesperidose affects the AA while the glycosylation by rutinose has no effect (Di Majo *et al.*, 2005). While substitution on a hydroxyl group typically decreases the AA, addition of a hydroxyl group can strongly increase it. For example, 3′,5′-dihydroxynaringin (pyrogallol B-ring) is ca. 70 times as potent as naringenin (Ye *et al.*, 2009). Flavanones present a higher AA in a hydrophilic environment. In a lipophilic environment, some flavanones (neohesperidin, hesperetin, isosakuranetin) show a reduced antioxidant potential while others (naringin, narirutin, naringenin, neoeriocitrin, heridictyol) even become prooxidant (Finotti and Di Majo, 2003). Overall, common dietary flavanones being devoid of a catechol nucleus are only poor antioxidants and their metabolites are expected to be even less potent. Hence, the most significant mechanisms involved in their health effects must be unrelated to their antioxidant activity.

1.8.3. Anti-Inflammatory Effect

The phenomena of inflammation have been well described in literature through many reviews. Inflammation is the most obvious manifestation of immune defence. It is manifested by swelling, pain, heat, and redness in the affected tissue and helps eliminate the sources of damage (viruses, bacteria…) and initiate healing. Inflammation is produced by immune cells within the tissue, releasing specific mediators which control local circulation and cell activities (Silverstein, 2009). Inflammation response to external stimuli may arise from the action of amines (histamine and 5-hydroxytryptamine), short peptides (bradykinin), long peptides (interleukin-1 (IL-1)), lipids (prostaglandins (PGs) and leukotrienes (LTs)), and many regulatory enzymes (protein kinase C, phosphodiesterase, lipoxygenase, and cyclooxygenase) (Vane & Botting, 1987). Many of the chronic and uncured diseases which plague our civilization are due to a dysfunctioning of the immune response.

Hesperidin (hesperetin 7-rutinoside) was found to inhibit kinases and phosphodiesterases responsible for cellular signal transduction and activation during an inflammation response (Manthey *et al.*, 2001). An inhibitory effect of hesperidin on pleurisy (chronic inflammation of lungs) induced by carrageenan was investigated in rats. The results showed a reduction in the volume of exudates and the number of migrating leucocytes by 48% and 34%, respectively, which makes hesperidin a mildly anti-inflammatory agent. Furthermore, this research group observed that hesperidin can reduced yeast-induced hyperthermia in rats (Emim *et al.*, 1994). In another study, hesperidin showed an inhibitory

effect on lipopolysaccharide (LPS)-induced overexpression of cyclooxygenase-2, inducible nitric oxide synthase (iNOS), overproduction of prostaglandin E2 and nitric oxide (NO) (Sakata *et al.*, 2003). Similar anti-inflammatory effects were also found for poncirin in RAW 264.7 macrophage cells (Kim *et al.*, 2007). A study also showed the anti-inflammatory activity of hesperidin by inhibiting arachidonic acid and histamine release (Galati et al, 1994).

Examples of naringenin and its glycosides as strong anti-inflammatory agents are:

a) the inhibition of pro-inflammatory cytokine induced by lipopolysaccharide in macrophages and *ex vivo* human whole-blood models to prevent Periodontitis (Bodet *et al.*, 2008); b) the attenuation of LPS / IFN (interferon)-γ-induced TNF (tumour necrosis factor)-α production in glial cells by inhibiting iNOS (inducible nitric oxide synthase) expression and nitric oxide production, p38 mitogen-activated protein kinase (MAPK) phosphorylation, and downstream signal transducer and activator of transcription-1 (STAT-1) to protect neuroinflammatory injury (Vafeiadou *et al.*, 2009); c) the reduced production of nitrate and nitrite (indicators of inflammatory process) in DSS (dextran sodium sulphate)-induced ulcerative colitis mice models to control the formation of intestine edema (Amaro *et al.*, 2009).

1.8.4. Anti-Cancer Effect

Advances in cancer research have been spectacular during the past decade. However, it is very unfortunate that the rate of cancer incidence is increasing at an alarming rate. The more recent estimation on cancer in France has given the figure of 320 000 cases diagnosed in 2005 in which 180 000 were in man and 140 000 in woman (INC report). While it cannot be concluded that technological progress is promoting cancer rate, it is clear that serious research in combating cancer is still essential.

Cancer is a complex family of diseases. In terms of molecular and cell biology, cancer is a disease of abnormal gene expression. This altered gene expression occurs through a number of mechanisms, including direct insults to DNA (such as gene mutations, translocations, or amplifications) and abnormal gene transcription or translation. When the cellular DNA of one or more normal cells has been exposed to carcinogens and substances or agents that can damage genetic material, the cell undergoes genetic alterations that result in malignant transformations and the process is known as carcinogenesis. Carcinogens include

chemical agents (from industrial pollutants, tobacco etc…), viruses (Human papilloma virus, hepatitis B & C virus), ionising (X-rays) and ultraviolet radiations, physical substances (asbestos, wood dust) and many others. ROS (superoxide, hydrogen peroxide, and hydroxyl radical) were also found major causes of not only DNA damage but also protein and lipid damages which lead to aging (Ames & Gold, 1998).

Unlike other flavonoids, flavanones have not been extensively studied for their bioactivities. Moreover, the studies have remained limited to aglycones and glycosides. Recently, hesperetin 7-glucuronide (Hp7G) was demonstrated to affect osteoblast differentiation (Trzeciakiewicz *et al.*, 2010). The major citrus flavanones can be effective in fighting carcinogenesis by minimizing DNA damage, tumor development and proliferation.

1.8.4.1. Antimutagenic effect

Flavanones can protect DNA damage by their capacity to absorb UV light. The results from a UV irradiated model of plasmidic DNA showed a considerable protecting effect of naringenin against UV-induced damage of DNA (Kootstra, 1994). The moderate antioxidant capacity of flavanones can also be useful in protecting against mutation by free radicals generated near DNA. Furthermore, naringin also inhibits H_2O_2-induced cytotoxicity and apoptosis, possibly via its effect on H_2O_2-induced expression of an apoptosis-associated gene (Kanno *et al.*, 2003). Naringenin may exhibit anti-mutagenic changes by stimulating DNA repair, following oxidative damage in human prostate cancer cells (Gao *et al.*, 2006).

1.8.4.2. Inhibition of tumor development

The pharmacological importance of flavanones can also be evaluated by their action against tumor development. So *et al.* (1996) studied the effect of hesperetin and naringenin on the development of breast cancer induced by 7,12-dimethylbenz[a]anthracene in female rats. The results showed that tumor development was delayed in rats fed with orange juice / naringin-supplemented diet (So *et al.*, 1996). Later on, concerning the anti-angiogenic effect of flavanones, an enzyme-linked immunosorbent assay (ELISA) was used to measure the vascular endothelial growth factor (VEGF) release from mammary adenocarcinoma human breast cancer cells. Naringin appeared more potent than rutin, apigenin, kaempferol and chrysin (Schindler *et al.*, 2006). 8-Prenylnaringenin, a derivative of naringenin, inhibits angiogenesis induced by basic fibroblast growth factor, VEGF, or the synergistic effect of two

cytokines in combination, in an in vitro and in vivo study (Pepper *et al.*, 2004). Eight flavanones, including flavanone, 2′-hydroxyflavanone, 4′-hydroxyflavanone, 6-hydroxyflavanone, 7-hydroxyflavanone, naringenin, naringin, and taxifolin, were investigated for their antitumor effects in colorectal carcinoma cells (HT29, COLO205, and COLO320HSR). 2′-Hydroxyflavanone came up as the most potent chemopreventive agent and thus showed a significant inhibitory effect on tumor formation. A recent study on hesperetin supplementation in male albino Wistar rats showed its inhibition of 1,2-demithylhydrazine (DMH)-induced colon carcinogenesis. The investigation suggested that hesperetin may inhibit phase I enzymes (involved in carcinogen activation), induce phase II xenobiotic metabolising enzymes and scavenge the electrophilic carcinogenic species (Aranganathan *et al.*, 2009).

1.8.4.3. Anti-proliferation

Naringenin was successfully investigated for its cell antiproliferation effect on an HT-29 colon cancer cell line. Cell proliferation measured by a colorimetric assay was significantly inhibited especially when HT-29 cells are exposed to naringenin at doses greater than 0.7 mM. The results suggested a potential role for citrus fruits as a source of chemoprotective agents against colon cancer (Frydoonfar *et al.*, 2003). In a comparative study, flavanones showed a significant anti-proliferative activity against lung, colon, breast, prostate and melanoma cancerous cell lines, although less efficiently than flavones. Moreover, glycosylation reduced the anti-proliferative activity in both flavonoid classes (Manthey & Guthrie, 2002). A C2-C3 double bond seems important for the anti-proliferative activity as results from different studies clearly showed the greater anti-proliferative activity of flavones compared to flavanones (Agullo *et al.*, 1996; Kawaii *et al.*, 1999; Manthey & Guthrie, 2002; Rodriguez *et al.*, 2002). Another structural element that may influence anti-proliferative activity is the substitution of the flavonoid nucleus. For instance, methylation of hesperetin and eriodictyol at C7-OH increased the anti-proliferative capacity (Benavente-Garcia and Castillo, 2008). Several mechanisms have been put forward to explain the antiproliferative activity of flavonoids. The most accepted hypothesis is the inhibition of several kinases involved in signal transduction such as protein kinases C, tyrosine kinases, PI 3-kinases or S6 kinase (Casagrande & Darbon, 2001).

The effects of 17β-estradiol (E2) hormone cover a wide range of physiological processes in mammals such as reproduction, cardiovascular health, bone integrity, cognition

and behaviour etc. Besides these roles in human physiology, E2 is also involved in the development of many diseases, including various types of cancers. The mechanism proposed to explain the chemoprotective activity of naringenin suggests that the flavanone binds to ERα (estrogen receptor α) as an antagonist, thereby limiting the effect of E2 in promoting cellular proliferation (Bulzomi *et al.*, 2010).

1.8.5. Cardiovascular Effects

Cardiovascular diseases (CVD) affect the heart and surrounding blood vessels and can take many forms, such as high blood pressure, coronary artery disease, heart disease and stroke. CVD are the largest cause of death in the EU and account for approximately 40% of deaths (2 million deaths per year). Increased oxidative stress was found one of the major factors causing CVD and its control by antioxidants including polyphenols is of great biological significance.

1.8.5.1. Vasorelaxant & Vasoprotective effects

The vascular endothelial cells are very important in normal coronary functions. The regulation of vascular tone and blood flow to organs is controlled by endothelial cells, which synthesize and release a number of factors such as prostacyclin, nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF) and endothelin. Among these factors, NO is critical in the preservation of normal vascular functions and there is a clear relationship between coronary artery disease and NO dysfunctioning (Benavent-Garcia, 2008).

Flavonoids promote endothelial NO synthase (eNOS) and at the same time inhibit the inducible NOS (iNOS) (Olszanecki *et al.*, 2002). In vitro, inhibition of iNOS in LPS-activated RAW 264.7 cells is not significant with flavanones (naringenin) in comparison to flavones and flavonols (apigenin, luteolin, quercetin), which may demonstrate the significance of a C2-C3 double bond (Kim *et al.*, 1999). Recently, the vasorelaxant potential of hesperetin and hesperidin (Orallo *et al.*, 2004) and naringenin and naringin (Orallo *et al.*, 2005) was demonstrated in rats. These vasorelaxant effects are probably due to the inhibition of different phosphodiesterase isoenzymes.

1.8.5.2. Effect on Coronary Heart Disease (CHD)

There is compelling evidence that CHD is principally related with an elevation of LDL cholesterol. Normally, cholesterol, cholesterol esters and triglycerides are transported by LDL (especially Apolipoprotein B (ApoB)) from their sites of absorption or synthesis to sites of bioactivity. So any malfunctioning of these lipoproteins causes the accumulation of cholesterol in circulatory system and ultimately generates CHD.

The anti-atherosclerosis potential of citrus flavanones, hesperetin and naringenin, was attributed to their ability to regulate apoB secretion and cellular cholesterol homeostasis in human hepatoma cells line HepG2. A decrease in apoB accumulation was observed in the media following 24h incubation with hesperetin and naringenin. This reduced apoB secretion was related to a reduced cellular concentration of cholesteryl ester (Wilcox *et al.*, 2001). The mechanism involved in naringenin anti-atherosclerosis activity was a reduced apoB secretion primarily due to the inhibition of microsomal triglyceride transfer protein and the enhancement of LDL receptor (LDLr)-mediated apoB-containing lipoprotein uptake (Borradaile *et al.*, 2003).

1.8.5.3. Anti-atherosclerotic effects

Atherosclerosis consists in the hardening and narrowing of arteries, which progressively takes place and blocks arteries, putting blood flow at risk. Atherosclerosis is the usual cause of heart attacks, strokes, and peripheral vascular disease. Hesperetin was shown to limit the rise of hepatic lipid contents and enzymes activities involved in triacylglycerol synthesis in rats fed with 1% orotic acid (Cha *et al.*, 2001). Moreover, a hypolipidemic effect of hesperetin was also reported even during the high lipid concentrations (Kim *et al.*, 2003).

In rats fed a high-cholesterol diet, 0.1% naringenin reduced the levels of plasma cholesterol and hepatic triacylglycerols. This effect was accompanied by a decrease in the activity of 3-hydroxy-3-methylglutaryl-coenzyme A reductase and acyl-CoA cholesterol acyltransferase (Lee *et al.*, 1999; Lee *et al.*, 2003). In another study on rabbits fed with cholesterol-rich diet, naringin showed an increase in superoxide dismutase and catalase activities to cooperate in the detoxification of free radicals produced. Moreover, naringin was also shown to regulate gene expression of superoxide dismutase, catalase and glutathione peroxidase (Jeon *et al.*, 2001).

1.8.6. Other biological effects

Anti-microbial Effects: the antiviral activity of hesperidin was demonstrated against herpes simples, polio, parainfluenza, and syncytial viral infections while naringin, was inactive (Kaul et al, 1985). In a recent study, hesperetin showed a moderate antimicrobial activity against Salmonella typhi and S. typhimurium (Kawaguchi *et al.*, 2004).

Anti-allergic activity: A study by Matsuda *et al.* (1991) suggests that hesperidin has an antiallergic activity via the inhibition of histamine release from pertinent mast cells in rats.

Miscellaneous: Knekt *et al.* (2002) found an association between a high intake of hesperetin and naringenin and a lower incidence of cerebrovascular disease and asthma.