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Antioxidant and prooxidant properties of flavonoids

Bachelor thesis

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„Prohlašuji, že tato práce je mým původním autorským dílem. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány.“

V Praze dne 10. května 2010 .....

Dagmar Procházková

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CONTENT

LIST OF ABBREVIATIONS

AAPH	2,2'-azobis(2-amidinopropane) dihydrochloride
ABTS	2,2-azinobis(3-ethyl-benzothiazoline-6-sulfonic) acid
CAT	catalase
DNA	deoxyribonucleic acid
$DPPH^{\bullet}$	2,2-diphenyl-picrylhydrazyl
EpRE	electrophile-responsive element
ESR	electron spin resonance
$Fl\text{-}O^{\bullet}$	flavonoid phenoxyl radical
FRAP	ferric reducing antioxidant power
GSH	glutathione
GPX	glutathione peroxidase
GR	glutathione reductase
GST	glutathione S-transferase
LDL	low-density lipoproteins
$NO^{\bullet}$	nitric oxide
NOS	nitric oxide synthase
$O_2^{\bullet -}$	superoxide anion radical
$^{\bullet}OH$	hydroxyl radical
ORAC	oxygen-radical absorbance capacity
$R^{\bullet}$	free radical
ROS	reactive oxygen species
SOD	superoxide dismutase
TEAC	trolox equivalent antioxidant capacity

1. INTRODUCTION AND AIM OF THE WORK

Flavonoids (the term is derived from the Latin word "flavus", meaning yellow) are ubiquitous plant secondary products that are best known as the characteristic red, blue, and purple anthocyanin pigments of plant tissues (Winkel-Shirley 2001). Apart from their physiological roles in the plants, flavonoids are important components of the human diet, although they are not considered as nutrients.

Use of plants for health improvement goes back to Chinese medicine 4000–6000 years ago, long before active compounds such as flavonoids were identified (Gabor 1986, taken over from van Hoorn 2002). Nowadays, interest in their possible health benefits has increased owing to their potent antioxidant and free radical scavenging activities observed *in vitro*. Most living organisms, including human beings, are aerobic organisms utilizing molecular dioxygen as a terminal electron acceptor. The reduction of molecular oxygen to water provides the energy, which allows the complexity of higher organisms. However, this reduction produces reactive oxygen species (especially superoxide, singlet oxygen, hydroxyl radical, and hydrogen peroxide), which have the potential to cause oxidative damage by reacting with various biomolecules. Flavonoids are one of the many molecules that are used by cells for the protection against the harmful effects of reactive oxygen species.

Nevertheless, the antioxidant efficacy of flavonoids *in vivo* is less documented and their prooxidant properties have been actually described *in vivo*. Due to their prooxidant properties, they are able to cause oxidative damage by reacting with various biomolecules, such as lipids, proteins and DNA.

The aim of this thesis is to discuss both the antioxidant and prooxidant effects of flavonoids. At the conclusion, a survey of the methods for antioxidant capacity determination is presented.

2. PROPERTIES OF FLAVONOIDS

2.1. Chemical structure

Flavonoids are a family of plant secondary metabolites of more than 9000 individual molecules found in all plant tissues and organs (Williams and Grayer 2004). Together with bryophytes and pteridophytes, higher plants are the only natural source of flavonoids (Rausher 2006).

Flavonoids all share the same fifteen-carbon basic skeleton, the flavan nucleus (Fig. 1), consisting of two aromatic rings with six carbon atoms (ring A and B) interconnected by a hetero cycle including three carbon atoms (ring C):

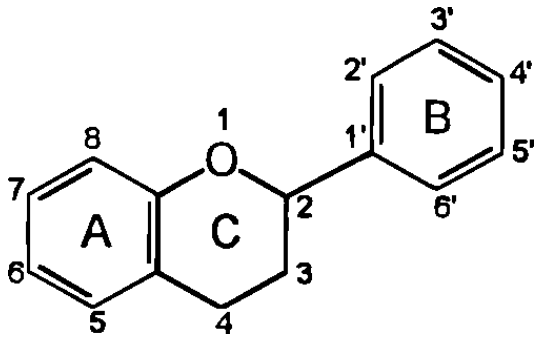


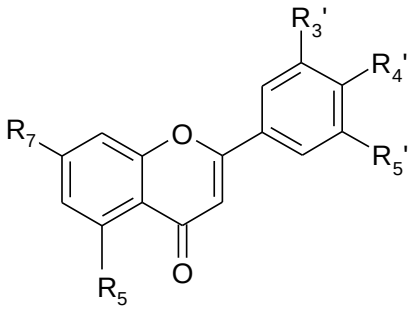
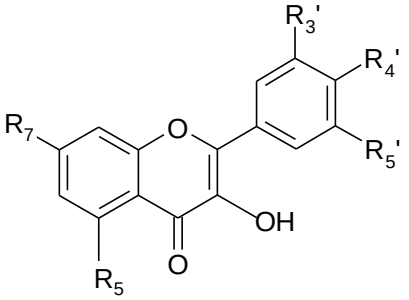
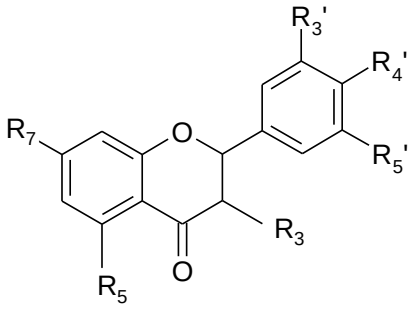
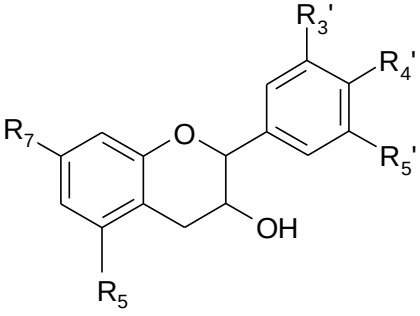
Fig. 1: Basic flavonoid structure (Pietta 2000)

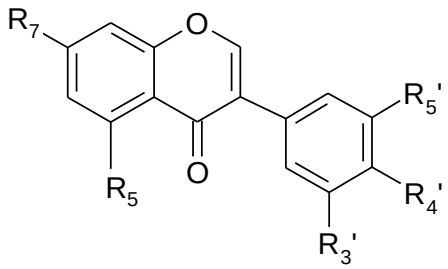
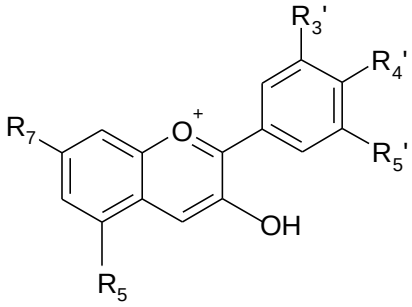
2.2. Classification of flavonoids

The various classes of flavonoids differ in the level of oxidation and pattern of substitution of the C ring, while individual compounds within a class differ in the pattern of substitution of the A and B rings (Tab. 1). The B ring is attached at the C-2 position in most of flavonoids. The relatively less common isoflavones are a notable exception to this pattern: the B-ring is attached at the C-3 position (Passamonti et al. 2009). According to the above mentioned criteria, flavonoids could be subdivided into following major subclasses:

- Flavonols, with the 3-hydroxy, 4-oxo group and 2,3-double bond in the C ring
- Flavones, lacking the 3-hydroxy group
- Flavanols, lacking the 2,3-double bond and 4-oxo group
- Flavanones, lacking the 2,3-double bond and the 3-hydroxyl group
- Isoflavones, with the B ring located in the position 3 of the C ring
- Anthocyanidins, positively charged oxonium ion in the C ring with lacking 4-oxo group

Tab. 1: Compendium of the main flavonoid groups

Subgroup	Formula	Representatives
Flavones		Apigenin, Luteolin, Chrysin, Baicalein
Flavonols		Quercetin, Kaempferol, Myricetin, Fisetin, Rutin, Quercitol, Gossypetin, Gossypin
Flavanones		Taxifolin, Naringenin, Hesperetin, Naringin
Flavanols		Catechin, Epicatechin, Catechin gallate, Gallocatechin, Epicatechin gallate, Epigallocatechin, Gallocatechin gallate, Epigallocatechin gallate

Isoflavones		Genistein, Daidzein, Formononetin, Glycitein, Biochanin A
Anthocyanidins		Malvidin, Cyanidin, Apigenidin, Delphinidin, Pelargonidin

2.3. Biosynthesis of flavonoids

The first committed step is catalyzed by chalcone synthase, which uses malonyl-CoA and 4-coumaroyl-CoA as substrates. The chain of 4-cumaroyl starter unit is extended using three molecules of malonyl-CoA. This extension initially results in a poly-β-ketochain, which, according to the nature of enzyme responsible (chalcone synthase or stilbene synthase), can be folded in two different ways giving chalcones and stilbenes, respectively. The primary flavonoid synthetic enzymes are present as multienzyme complexes on the cytosolic side of the endoplasmatic reticulum membrane (Winkel 2004). The biosynthesis of the main flavonoid subclasses is presented in Fig. 2.

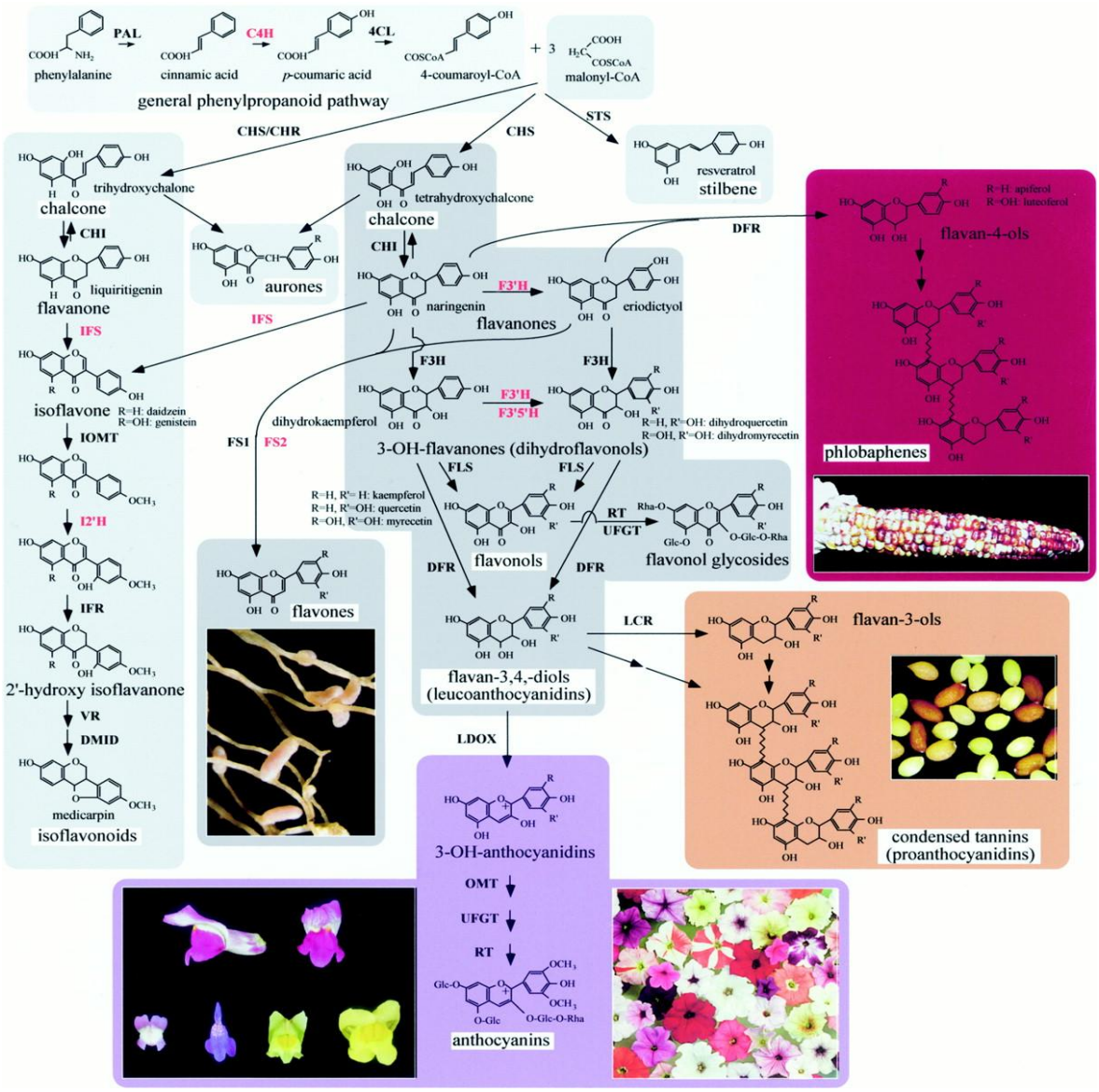


Fig. 2: Biosynthesis of major flavonoids subclasses (Winkel 2001).

Enzyme names are abbreviated as follows: cinnamate-4-hydroxylase (C4H), chalcone isomerase (CHI), chalcone reductase (CHR), chalcone synthase (CHS), 4-coumaroyl:CoA-ligase (4CL), dihydroflavonol 4-reductase (DFR), 7,2'-dihydroxy, 4'-methoxyisoflavanol dehydratase (DMID), flavanone 3-hydroxylase (F3H), flavone synthase (FSI and FSII), flavonoid 3'-hydroxylase (F3'H) or flavonoid 3',5'-hydroxylase (F3'5'H), isoflavone *O*-methyltransferase (IOMT), isoflavone reductase (IFR), isoflavone 2'-hydroxylase (I2'H), isoflavone synthase (IFS), leucoanthocyanidin dioxygenase (LDOX), leucoanthocyanidin reductase (LCR), *O*-methyltransferase (OMT), phenylalanine ammonia-lyase (PAL), rhamnosyl transferase (RT), stilbene synthase (STS), UDPG-flavonoid glucosyl transferase (UFGT), and vestitone reductase (VR).

(The figure is presented with the kind permission of Dr. Winkel.)

2.4. Occurrence and importance for plants

Flavonoids generally occur as glycosides and they are usually found attached to a sugar, tending to be more water-soluble (Ross and Kasum 2002). When pure, some plant polyphenols may not be easily soluble in water (Haslam 1996). Glucose is the most usual sugar residue. The others include galactose, rhamnose, xylose, arabinose or fructose (Rice-Evans et al. 1996).

Flavonoids are compartmented in different subcellular locations, including vesicles, vacuoles, and nuclei, or exported to the cell exterior (Mueller and Walbot 2001, Saslowsky et al. 2005, Taylor and Grotewold 2005, Yazaki 2005, taken over from Hsieh 2007). The location of flavonoids in different subcellular compartments is plant species- and cell type-specific (Hsieh and Huang 2007).

Flavonoids are widely distributed in all plant organs. The short compendium of plant occurrence of some flavonoids is given in the Table 2.

Tab. 2: Compendium of plant sources of some flavonoids

Subclass of flavonoids	Flavonoid	Source
Flavones	Apigenin	celery, parsley
	Luteolin	celery, rosemary, thyme
	Chrysin	fruit skin
Flavonols	Quercitol	wine, *Arfeuillea arborescens*
	Kaempferol	leek, broccoli, radish, grapefruit, black tea cranberry, grapes, red wine
	Myricetin	mallow, heather
	Gossypetin	soy, varnish tree
	Fisetin	*Ginkgo biloba*
	Rutin	*Hibiscus*
	Gossypin	onion, lettuce, broccoli, cranberry, apple
	Quercetin	skin, berries, olive, tea, red wine

Flavanones	Naringin	citrus peel
	Taxifolin	citrus fruits
	Naringenin	citrus fruits
	Hesperetin	citrus peel
Anthocyanidins	Malvidin	red grapes, red wine
	Cyanidin	cherry, raspberry, strawberry, grapes
	Apigenidin	coloured fruit and peels
	Delphinidin	grape, pomegranates
Isoflavones	Genistein	soybeans, chick pea, black and green beans
	Daidzein	soybeans, chick pea, black and green beans
Flavanols	Catechin	red wine
	Epicatechin	tea

Flavonoids in land plants participate in protection from UV radiation (Ryan et al. 2002) and in auxin transport (Jacobs and Rubery 1988). Many other functions such as pollinator attractants, oviposition stimulus, feeding attractants and feeding deterrents, together with allelopathy and phytoalexin effects were found by many authors as well (Iwashina 2003). Many flavonoid biosynthetic genes are induced under stress conditions and, accordingly, flavonoid levels increase during exposure to biotic and abiotic stresses, such as wounding, drought, metal toxicity and nutrient deprivation (Winkel-Shirley 2002, Dixon and Paiva 1995, taken over from Hernández 2009). A common denominator in these environmental stress conditions is the production and accumulation of reactive oxygen species (ROS). Hence, flavonoids have been suggested to act as antioxidants, protecting plants from oxidative stress (Hernández et al. 2009).

3. FLAVONOIDS AS ANTIOXIDANTS

An antioxidant can be defined as any substance which, when present in low concentrations compared to that of the oxidizable substrate, significantly delays or inhibits the oxidation of that substrate (Halliwell and Gutteridge 1995). The physiological role of antioxidants, as this definition suggests, is to prevent damage to cellular components arising as a consequence of chemical reactions involving free radicals (Young and Woodside 2001).

The antioxidant capacities of many flavonoids are much stronger that those of vitamin C and E (Prior and Cao 2000). For example, the flavonoid constituents of red wine are factors of particular interest due to so-called French paradox: the Southern French have a very low incidence of coronary heart disease despite their high fat diet and smoking habits (Renaud 1992). One of the features that has been highlighted relates to the high consumption of red wine by the French and the question as to whether the polyphenolic antioxidants from this dietary source contribute to protection from coronary heart disease along with the antioxidants in the olive oil and the high intake of antioxidant nutrients from the fresh fruit and vegetable-rich Mediterranean diet (Rice-Evans and Miller 1995). Similarly, Hertog et al. (1993) suggested an inverse correlation between the incidence of coronary heart disease and stroke and the dietary intake of flavonoids, especially quercetin.

Flavonoids can prevent injury caused by free radicals in various ways. These mechanisms are ascribed to:

(1) their capacity to scavenge free oxygen radicals directly,

(2) activating antioxidant enzymes (Nijveldt et al. 2001),

(3) chelating metal catalysts (Ferrali et al. 1997),

(4) reducing alpha-tocopherol radicals (Hirano et al. 2001, taken over from Heim et al. 2002),

(5) inhibiting oxidases (Cos et al. 1998, taken over from Heim et al. 2002),

(6) mitigating oxidative stress caused by nitric oxide (van Acker et al. 1995).

3.1. Direct scavenging of free radicals

Flavonoids are able to scavenge free radicals directly by hydrogen atom donation. Because of the high reactivity of their hydroxyl groups, radicals are made inactive according to the following equation (Korkina and Afanas'ev, 1997):

$$Fl\text{-}OH + R^{\bullet} \rightarrow Fl\text{-}O^{\bullet} + RH$$

where $R^{\bullet}$ is a free radical and $Fl\text{-}O^{\bullet}$ is a flavonoid radical. This equation is illustrated in detail in the following figure (Fig. 3):

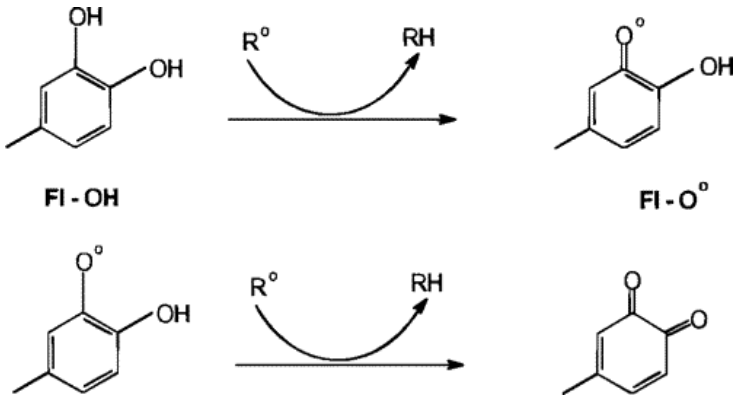
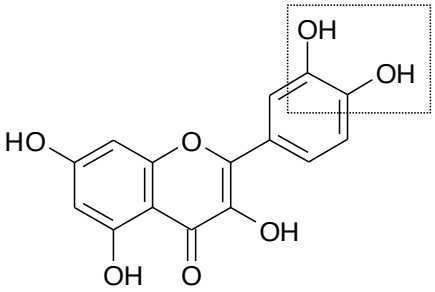


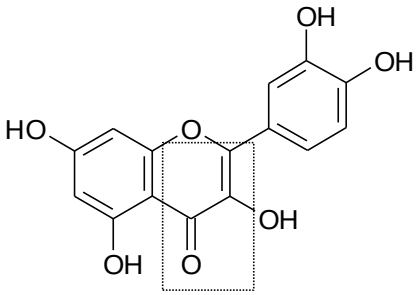
Figure 3: Scavenging of reactive oxygen species ($R^{\bullet}$) by flavonoid. The free radical $Fl\text{-}O^{\bullet}$ may react with a second radical, acquiring a stable quinine structure (Pietta 2000).

The *in vitro* flavonoid antioxidant activity depends on the arrangement of functional groups on its nuclear structure. Both the configuration and total number of hydroxyl groups substantially influence mechanism of antioxidant activity (Heim et al. 2002). The B ring hydroxyl configuration is the most significant determinant of ROS scavenging (Burda and Oleszek 2001), whereas substituent at the rings A and C has little impact on superoxide anion radical scavenging rate constants (Taubert 2003, taken over from Amić et al. 2007). The main structural features of flavonoids required for efficient radical scavenging could be summarized as follows (Bors et al. 1990, taken over from Croft 2006):

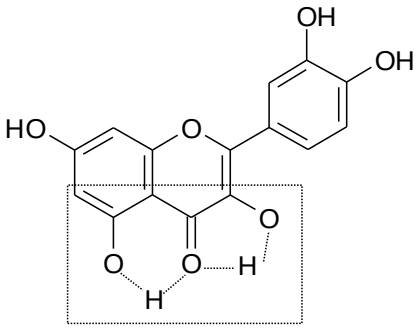
a) an *ortho*-dihydroxy (catechol) structure in the B ring, for electron delocalization:



b) 2,3-double bond in conjugation with a 4-keto function in the C-ring provides electron delocalization from the B ring:



c) hydroxyl groups at positions 3 and 5 provide hydrogen bonding to the keto group:



According to the previously stated criteria, flavonols quercetin and myricetin are the most effective radical scavengers in the aqueous phase, which has been confirmed experimentally (Rice-Evans et al. 1996).

3.2. Ability to activate antioxidant enzymes

Another possible mechanism by which flavonoids act is through interaction with various antioxidant enzymes. Furthermore, some effects may be a reset of a combination of radical scavenging and the interaction with enzyme functions (Nijveldt et al. 2001).

Flavonoids induce electrophile responsive element (EpRE)-mediated expression of enzymes, such as NAD(P)H-quinone oxidoreductase and glutathione S-transferases (GST), which are the major defense enzymes against electrophilic toxicants and oxidative stress. The ability of flavonoids to activate the EpRE-mediated response correlates with their redox properties characterized by quantum mechanical calculations. Flavonoids with a higher intrinsic potential to generate oxidative stress and redox cycling are the most potent inducers of EpRE-mediated gene expression. Modulation of the intracellular glutathione level showed that the EpRE-activation by flavonoids increases with decreasing glutathione concentration and *vice versa*, supporting an oxidative mechanism. In conclusion, the prooxidant activity of flavonoids can contribute to their health-promoting activity by inducing important detoxifying enzymes, pointing to a beneficial effect of a supposed toxic chemical reaction (Lee-Hilz et al. 2006).

It was also proved that the intracellular antioxidant function of flavonoids requires the interaction with glutathione peroxidase (GPX), at least in the cells expressing this enzyme (Nagata et al. 1999). Cocoa flavonoids increased the activities of GPX and glutathione reductase in human hepatocytes as well (Martín et al. 2009). Leung et al. (2006) provided evidence that luteolin-induced human lung carcinoma CH27 cell apoptosis was accompanied by activation of antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT), but not through the production of ROS and disruption of mitochondrial membrane potential. Therefore, the effects of luteolin on CH27 cell apoptosis were suspected to result from the antioxidant rather than the prooxidant action of this compound. Administration of the flavonoid-rich fraction along with a high fat diet caused a significant increase of SOD, CAT and GPX activities in rat erythrocytes (Kaviarasan et al. 2008).

However, some contradictory observations in this field were obtained, e.g. GST activity was significantly induced by apigenin, genistein and tangeretin in the rat heart but not in colon or liver. In red blood cells chrysin, quercetin and genistein significantly decreased the

activity of GR, CAT and GPX, whereas SOD was only significantly decreased by genistein (Breinholt 1999).

3.3. Chelating metal catalysts

Specific flavonoids are known to chelate iron and copper, thereby removing a causal factor for the development of free radicals. The proposed binding sites for trace metals to flavonoids are the catechol moiety in the ring B, the 3-hydroxyl and 4-oxo groups in the heterocyclic ring C, and the 4-oxo and 5-hydroxyl groups between the heterocyclic and the A rings (Fig. 4):

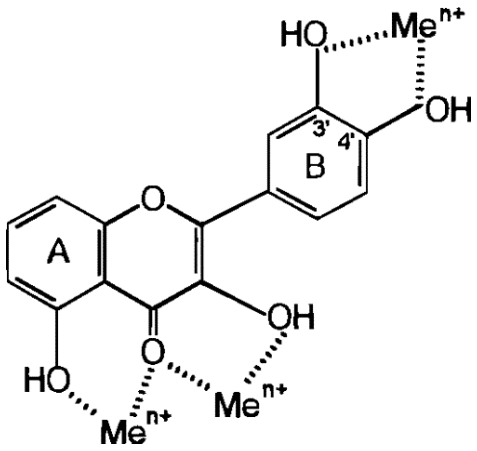


Fig. 4: Binding sites for trace metals (Pietta 2000).

The *ortho*-3´,4´-dihydroxy substitution in the B ring has been shown to be important for Cu^{2+}-chelate formation and thus being the major contributory site of the metal chelating (Brown et al. 1998). Quercetin, in particular, is known for iron-chelating and iron-stabilizing properties. *In vitro* studies have indicated that e.g. morin and quercetin form complexes with Cd(II) and exhibit strong antioxidant activity. Their sulfonic water-soluble derivatives exert low toxicity and therefore could be potential antidotes (Kopacz and Kuźniar 2003, Szeląg et al. 2003, taken over from Chlebda 2009).

3.4. Reducing α-tocopheryl radicals

The α-tocopherol functions as a major antioxidant in human low-density lipoproteins (LDL). Hirano et al. (2001) suggested that flavonoids can act as hydrogen donors to α-

tocopheryl radical. Furthermore, by interaction with α-tocopheryl, they possess a great potential to delay the oxidation of LDL. Flavonoids (kaempferol, morin, myricetin and quercetin) showed a varying protective activity against depletion of α-tocopherol in LDL, with kaempferol and morin being less effective than myricetin and quercetin (Zhu et al. 2000). Similarly, the addition of green tea catechins extracts (epigallocatechin, epigallocatechin gallate, epicatechin, epicatechin gallate) demonstrated a gradual regeneration of α-tocopherol in human LDL (Zhu et al. 1999).

3.5. Ability to inhibit oxidases

Flavonoids inhibit the enzymes responsible for $O_2^{\bullet-}$ production, such as xanthine oxidase (Hanasaki et al. 1994) and protein kinase C (Ursini et al. 1994). Quercetin and silibin inhibit xanthine oxidase activity, thereby resulting in decreased oxidative injury (Shoskes 1998, Chang et al. 1993, Iio et al. 1986, taken over from Nijveldt et al. 2001). Cos et al. (1998) carried out a study on structure-activity relationship in which luteolin (3',4',5,7-tetrahydroxyflavone) was reported to be the most potent inhibitor of xanthine oxidase. Flavonoids have been also shown to inhibit cyclooxygenase, lipoxygenase, microsomal succinoxidase and NADH oxidase (Korkina and Afanasev 1997, Brown et al. 1998).

3.6. Mitigating oxidative stress caused by nitric oxide

Nitric oxide (NO) is important in maintaining the dilation of blood vessels (Huk et al. 1998) but its high concentrations result in oxidative damage. NO is produced by the oxidation of L-arginine catalyzed by NO synthase (NOS). Nitric oxide toxicity is for the major part mediated by peroxinitrite, formed in the reaction of NO with $O_2^{\bullet-}$ (Rubbo et al. 1996).

It was reported that molecules of NO are directly scavenged by flavonoids (van Acker et al. 1995). To clarify the structure-activity relationships of flavonoids for NO production inhibitory activity, Matsuda et al. (2003) examined 73 flavonoids. Among them, apigenin, diosmetin, tetra-*O*-methylluteolin, and hexa-*O*-methylmyricetin were found to show potent inhibitory activity, and several structural requirements of flavonoids for this activity were

clarified: (1) the activities of flavones were stronger than those of corresponding flavonols; (2) the glycoside moiety reduced the activity; (3) the activities of flavones were stronger than those of corresponding flavanones; (4) methylation of the 3, 5, or 4′-hydroxyl group enhanced the activity, etc. In addition, potent NO production inhibitors were found to inhibit induction of NOS without NOS enzymatic inhibitory activity (Matsuda et al. 2003).

Flavonoids are known to directly scavenge peroxynitrite (Cao et al. 1997). The most significant determinant of their activity against peroxynitrite is the 3′,4′-catechol arrangement, followed by an unsubstituted 3-hydroxyl group. An apparent positive correlation between number of hydroxyl groups, particularly of the B ring, and antiradical activity has been described (Haenen et al. 1997, taken over from Heim et al. 2002).

Representatives of flavonols (kaempferol, quercetin, and rutin), flavones (apigenin, primuletin), flavanols (catechine) and flavanones (hesperetin, hesperidin, naringenin) were tried on NOS 2 (also called inducible NOS) induction and activity in the *in vitro* model of lipopolysaccharide-treated macrophages (cell line J774.2) (Olszanecki et al. 2002). While none of these compounds inhibited activity of NOS 2, all with unexpectedly scattered potencies inhibited induction of NOS 2 protein in lipopolysaccharide-treated J774.2 cells. So far the most potent compounds, kaempferol and apigenin, at micromolar concentrations did inhibit NOS 2 induction at the level of NOS 2 gene transcription. It was concluded that some flavonoids are potent inhibitors of NOS 2 induction. At the same time they may increase endothelial NOS 3 activity (Olszanecki et al. 2002). Several flavonoids, including quercetin, result in a reduction in ischemia-reperfusion injury by interfering with inducible NOS activity (Shoskes 1998, taken over from Nijveldt et al. 2001).

How flavonoids inhibit induction of NOS and NO production is not clearly understood yet, but several explanations are argued. The first possibility may be derived from the antioxidant property of flavonoids by which these compounds scavenge ROS. The second possibility is that flavonoids may act as a lipopolysaccharide-signaling molecule inhibitor (Kim et al. 2005).

4. FLAVONOIDS AS PROOXIDANTS

While the antioxidant properties of flavonoids support their positive role in human nutrition and disease prevention, some focus has involved the prooxidant activity of these compounds *in vitro* (Heim et al. 2002).

Flavonoids occur usually in the glycosidic form (Rice-Evans et al. 1995) and they are extensively metabolized *in vivo*, resulting in a significant alteration in their redox potentials (Williams et al. 2004). Generally, flavonoids may undergo three forms of intracellular metabolism: (1) conjugation with thiols, particularly GSH; (2) oxidative metabolism; and (3) P450-related metabolism (Williams 2004). The bioactive forms of flavonoids *in vivo* are not those forms found in plants, for example, the glycosides and, in some instances, aglycone, but instead conjugates and metabolites arising from these on intestinal absorption (Williams et al. 2004).

4.1. Direct flavonoids prooxidant function

Prooxidant activity is thought to be directly proportional to the total number of hydroxyl groups (Sofic and Prior 1997). Series of mono- and dihydroxyflavonoids demonstrated no detectable prooxidant activity, while multiple hydroxyl groups, especially in the B ring, significantly increased production of hydroxyl radicals in a Fenton system (Hanasaki et al. 1994, taken over from Heim 2002). The latter compounds included myricetin and baicalein, both of which have a pyrogallol structure in the A ring, which has also been reported to promote hydrogen peroxide production (Hodnick 1986, taken over from Heim 2002) from which Fenton reaction may generate highly reactive hydroxyl radicals (Galey 1997, taken over from Heim 2002).

4.2. Oxidation by flavonoid phenoxyl radicals

According to the "classical" definition, antioxidant is a molecule that: (1) could donate electrons or hydrogen atoms, (2) yields an antioxidant-derived radical that (3) is efficiently quenched by other electron or hydrogen sources to prevent cellular damage, and (4) whose properties are spatially and temporally correlated with oxidative stress events (Halliwell and Gutteridge 1989, Cadenas 1995, taken over from Hernández et al. 2009). Hence, the

resulting molecule formed on the flavonoid must be stable so as to prevent it from acting as a chain-propagating radical (Halliwell et al. 1995).

The end products of ROS scavenging by flavonoids are flavonoid phenoxyl radicals Fl-O$^{\bullet}$ with a lifetime of 200 µs (Bayrakçeken 2003). They are highly reactive and subjected to further oxidation, yielding, among other possible products, the more stable flavonoid quinones. Flavonoid quinones are still reactive but they can be stabilized by conjugation with nucleophiles, such as glutathione, cysteine or nucleic acids (Awad 2002, Torres et al. 2005, Kanakis 2005, taken over from Hernández 2009). This reaction is responsible for one of the prooxidant effects of flavonoids (McCord 1995, taken over from Pietta 2000). The prooxidant properties of the flavonoids apigenin, naringenin and naringin have been described by showing that their phenoxyl radicals rapidly oxidize NADH, resulting in extensive oxygen uptake and $O_2^{\bullet-}$ formation (Galati et al. 1999, Chan et al. 1999, taken over from Galati et al. 2002). Another reaction, which may be responsible for undesired prooxidant properties of flavonoids, could be the interaction of Fl-O$^{\bullet}$ with oxygen in the presence of high levels of transient metals (Fig. 5), generating quinones and $O_2^{\bullet-}$ (Pietta 2000, taken over from Amić 2007).

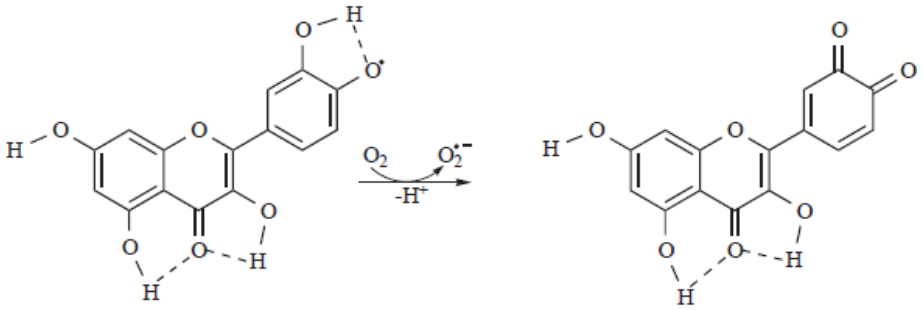


Fig. 5: Prooxidant activity of flavonoids (Amić et al. 2007)

The source of flavonoid radicals could be an autoxidation as well. Canada et al. (1990) found that the rate of autoxidation for both quercetin and myricetin was highly pH dependent with no autoxidation detected for quercetin at physiologic pH. Both the addition of iron to quercetin and myricetin and the addition of iron followed by SOD for quercetin increased the rate of autoxidation substantially. Neither kaempferol, a monohydroxylated flavonol, nor rutin, a glycosylated quercetin, showed any ability to autoxidize.

4.3. Inhibition of mitochondrial respiration

Some flavonoids cause a substrate-independent cyanide insensitive respiratory burst in isolated mitochondria and undergo autoxidation, which is associated with the production of ROS (Hodnick et al. 1989, taken over from Hodnick et al. 1994).

Hodnick et al. (1994) tested six flavonoids (robinetin, rhamnetin, eupatorin, baicalein, 7,8-dihydroxyflavon and norwogonin) for their ability to inhibit beef heart mitochondrial succinoxidase and NADH-oxidase activities. The NADH-oxidase activity was lower compared to succinoxidase activity, demonstrating a primary site of inhibition of the complex I (NADH-coenzyme Q reductase) portion of the respiratory chain. Flavonoids with adjacent tri-hydroxyl or *para*-dihydroxyl groups exhibited a substantial rate of autoxidation which was accelerated by the addition of cyanide. Flavonoids possessing a catechol configuration exhibited a slow rate of autoxidation in buffer that was stimulated by the addition of cyanide.

4.4. Oxidation by peroxidases

Alternative mechanism for flavonoid prooxidant toxicity involves the numerous peroxidases that catalyze the oxidation of polyphenols. Intracellular phenoxyl radicals (redox-cycling phenols) formed by myeloperoxidase also induce lipid peroxidation and cooxidize glutathione to form thiol radicals with concomitant oxygen activation (Goldman et al. 1999taken over from Galati et al. 2002). The prooxidant properties of the flavonoids apigenin, naringenin and naringin have been described by showing that their phenoxyl radicals rapidly cooxidize NADH, resulting in extensive oxygen uptake and $O_2^{\bullet -}$ formation (Galati et al. 1999, Chan et al. 1999, Galati et al. 2002).

4.5. Oxidation of low molecular antioxidants

The catechol ring-containing substrates (catechol, luteolin, eriodictyol and quercetin) were found to oxidize ascorbate, NADH or GSH. In general, it was found that their effectiveness of catalyzing the oxidation increased as their redox potential decreased. Thus, the more readily oxidizable flavonoids were the most effective. This is likely because the

lower redox potential catechols were more readily oxidized by peroxidase/H_2O_2 than were the higher redox potential phenols (Galati et al. 2002).

Quercetin, myricetin and kaempferol induced a concentration-dependent decrease of both the nuclear GSH content and GST activity in a model system of isolated rat liver nuclei. Myricetin, which has the maximum number of hydroxyl groups, was the most active among tested flavonoids. The impairment of the nuclear antioxidant defense GSH and GST by the polyphenolic flavonoids can lead to oxidative DNA damage, which may be responsible for their mutagenicity (Sahu and Gray 1996).

4.6. Other prooxidant properties

In the presence of reactive nitrogen species, flavonoids with A or B ring pyrogallol configurations induce DNA single-strand breakage (Ohshima et al. 1998). There is evidence that the 2,3-double bond and 4-oxo arrangement of flavones may promote the formation of ROS induced by divalent copper in the presence of oxygen (Cao et al. 1997). Yamashita et al. (1999) proposed the mechanism of site-specific DNA damage caused by quercetin. Its catechol group in the ring A or B is oxidized by a copper(II) ion bound to DNA, which can generate reactive oxygen species responsible for DNA damage (Amić et al 2007). By contrast, kaempferol and luteolin induced little DNA damage even in the presence of Cu(II). El Amrani et al. (2006) studied the oxidative DNA cleavage induced by an iron(III)-flavonoid complex (Amić et al. 2007). The proposed mechanism of iron(II)-flavonoid complex, which then binds to DNA and generates ROS, is presented in Fig. 6.

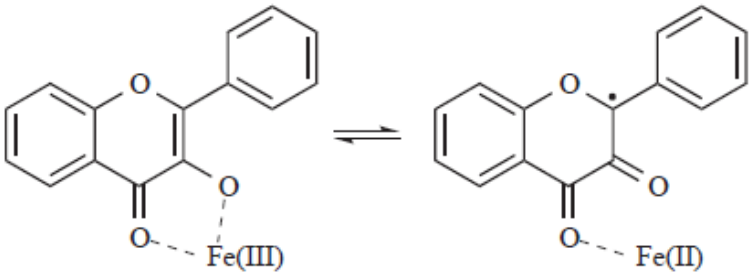


Fig. 6: Proposed route for generation of Fe(II)-flavonoid complex (Amić et al. 2007)

In addition, high ascorbate concentrations attenuate generation of ROS by flavonoids *in vitro* (Ratty and Das 1988), and it is postulated that vitamin C status modulates the prooxidant tendency of these compounds *in vivo.*

5. METHODS FOR DETERMINATION OF ANTIOXIDANT ACTIVITY *IN VITRO*

Methods used for the determination of flavonoid antioxidant activity could be divided into methods based on:

(1) the synthetic radical elimination

(2) the elimination of oxygen radicals

(3) the evaluation of redox properties of tested compound

5.1. Methods based on the synthetic radical elimination

One of the fundamental methods for evaluation of antiradical activity of studied compounds is DPPH method. It is based on the reaction of tested antioxidant with stable radical 1,1-diphenyl-2-(2,4,6-trinitrophenyl)hydrazyl ($DPPH^{\bullet}$). The reduction of $DPPH^{\bullet}$ is followed by monitoring the decrease in its absorbance at 515 nm during the reaction with an antioxidant (Brand-Williams et al. 1995):

$$DPPH^{\bullet} + AH \rightarrow DPPH\text{-}H + A^{\bullet}$$

Flavonoid antioxidant activity can be determinate also using Trolox equivalent antioxidant capacity assay (TEAC) with 2,2-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS). Synthetic stable radical cation $ABTS^{\bullet+}$ is formed by oxidation of ABTS with potassium persulfate, which is scavenged (reduced) by hydrogen-donating antioxidant present in the sample (see equation below). The $ABTS^{\bullet+}$ typically has a bluish-green color with maximum absorbance values at 645 nm, 734 nm and 815 nm. This absorbance is reduced upon the capture of $ABTS^{\bullet+}$ by antioxidant in concentration-dependent manner (Re et al. 1999).

$$ROOH + ABTS^{\bullet+} \leftrightarrow ROO^{\bullet} + ABTS + H^{+}$$

5.2. Methods based on the elimination of oxygen radicals

The electron spin resonance (ESR), spectroscopic technique, is one of the few methods that can directly detect free radical species in the sample. However, several other methods based on elimination of oxygen radicals by antioxidants exist.

The oxygen-radical absorbance capacity (ORAC) assay is based on the ability of studied compound to slow down or stop the radical reaction. Detection is based on the decrease in fluorescence of protein β-phycoerythrin upon peroxyl radical attack. These radicals are generated by AAPH [2,2'-azobis(2-amidinopropane) dihydrochloride]. The decrease in fluorescence of ß-phycoerythrin in the presence of AAPH is linear with time and a period of complete protection of ß-phycoerythrin against AAPH is related to antioxidant concentrations (Glazer 1990). The obtained results are compared to the activity of standard Trolox and expressed as ORAC units, where 1 ORAC unit equals the net protection produced by 1 μM Trolox. The uniqueness of this assay is that total antioxidant capacity of a sample is estimated by taking the oxidation reaction to completion (Cao et al. 1993).

The methods evaluating elimination of $O_2^{\bullet-}$ are based on the formation of blue colored formazane either from nitroblue tetrazolium chloride (Chaitanya and Naithani 1994) or from 3-bis(2-methoxy-4-nitro-5-sulphophenyl-2H-tetrazolium-5-carboxanilide) sodium salt (Able et al. 1998). This formazan is formed upon reaction of before mentioned dyes with $O_2^{\bullet-}$. Various $O_2^{\bullet-}$generating systems are used, e.g. 5-methylphenazinium methosulfate and NADH or system xanthine/xanthine oxidase (Paulova et al. 2004).

The other approach to evaluation of antioxidant activity of selected compounds is determination of H_2O_2 scavenging ability. The hydrogen peroxide assay is based on monitoring of absorbance of yellow colored titanium-peroxide complex at 415 nm (Rao *et al.* 1996).

$$H_2O_2 + Ti^{4+} \rightarrow \text{Titanium-peroxide complex (yellow color)}$$

5.3. Methods based on the evaluation of redox properties of tested compound

Antioxidant capacity could be determined chemically by ferric reducing antioxidant power (FRAP) assay. FRAP assay measures the change in absorbance at 593 nm due to the formation of a blue colored Fe^{II}-tripyridyltriazine compound from colorless oxidized Fe^{III} form by the action of electron donating antioxidants (Benzie and Strain 1996). FRAP assay

has several limitations (e.g. non-physiological pH) and reflects only the ability of tested compound to reduce Fe^{III} ions (Paulova et al. 2004).

The other methods that belong to this group are cyclic voltametry and HPLC with electrochemical detection (Paulova et al. 2004).

6. CONCLUSIONS

Flavonoids are of great interest for human health due to their anti-inflammatory, antiplatelet, antiviral, vasodilatory, anti-ischemic and anticancer activities (Subarnas and Wagner 2000, Lou et al. 1992, Formica and Regelson 1995, Duarte et al. 1993, Rump et al. 1995, Duthie et al. 2000, taken over from Hernández et al. 2006). The greatest interest in these substances has been stimulated by the potential health benefits arising from their antioxidant capacity. However, flavonoid prooxidant properties have been described as well.

Contradictory observations are sometimes obtained, e.g. Husain et al. (1987) reported that myricetin and quercetin scavenge $^{\bullet}OH$ generated by UV photolysis of H_2O_2 whereas Laughton et al. (1989) reported that these flavonoids accelerate the production of $^{\bullet}OH$ from H_2O_2 in the presence of Fe^3-EDTA. Similarly, although green tea belongs between beverages with antioxidant properties (e.g. Rice-Evans et al. 1996), Frankel et al. (1997) showed that high concentrations of green tea extract resulted in prooxidation. Joubert et al. (2005) also showed that a critical balance seems to exist between the prooxidant and antioxidant behaviors of rooibos flavonoids. In addition, the prooxidant activity of flavonoids can contribute to their health-promoting activity by inducing important detoxifying enzymes, pointing to a beneficial effect of a supposed toxic chemical reaction (Lee-Hilz 2006).

Concentrated extracts of flavonoid-rich plants are widely marketed as nutraceuticals, targeting the aging population and individuals with cardiovascular disease, cancer and chronic inflammatory conditions (Heim 2002). Although the health benefit of flavonoids from additives is undoubted, they hardly could have the same health benefit as flavonoids present in whole varied food. Plants contain a huge range of agents that might protect human beings against development of various diseases (Halliwell 2006). Thus it could be anything in the dietary plants that protects against disease, and high body antioxidant levels could be a 'biomarker' of a good diet. If so, reproducing these levels with supplements may not give the same benefit (Halliwell 1999, taken over from Halliwell 2007).

Flavonoids are typical xenobiotics, metabolized as such and rapidly removed from the circulation. High levels may even be toxic, but low levels of toxins can sometimes be good for the organism by raising levels of xenobiotic-metabolizing and antioxidant defense enzymes (Halliwell 2007).

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