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Plant Polyphenols as Antioxidants Influencing the Human Health

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1. Introduction

Widely distributed in plant kingdom and abundant in our diet plant polyphenols are today among the most talked about concerning the classes of phytochemicals. There are several thousand plant-derived compounds of biogical interest that have more than one phenolic hydroxyl group attached to one or more benzene rings, thus qualifying as polyphenols. In recent years, polyphenols have gained a lot of importance because of their potential use as prophylactic and therapeutic agents in many diseases, and much work has been presented by the scientific community which focuses on their antioxidant effects. Traditionally, herbal medicines with antioxidant properties have been used for various purposes and epidemiological data also point at widespread acceptance and use of these agents. Plant polyphenols have been studied with intention to find compounds protecting against a number of diseases related to oxidative stress and free radical-induced damage, such as cardiovascular and neurodegenerative diseases, cancer, diabetes, autoimmune disorders and some inflammatory diseases. In order to evaluate the efficacy of polyphenols as antioxidants as well as to elucidate the mode of their action, researchers today are using a wide range of experimental models, from the simplest chemical antioxidant assays through the biologically more relevant cellular-based assays to the most accurate animal models, and ultimately clinical studies in humans. The latest scientific knowledge offers a more detailed understanding of the biological effects of polyphenols and their role in human health promotion and disease prevention.

This chapter is focused on plant polyphenols, taking into consideration aspects relative to their biosynthesis, structure, botanical sources, chemical analysis, evaluation of antioxidant action, bioavailability as well as their potential health benefits.

2. Biosynthesis, classification and distribution of plant polyphenols

Phenolic compounds are ubiquitous in the plant species but their distribution at the plant tissue, cellular and subcellular levels is not uniform. Insoluble phenolics are found in cell walls, while the soluble phenolics are present within the vacuoles. They are essential to the plant's physiology being involved in diverse functions such as structure, pigmentation, pollination, pathogen and herbivore resistance, as well as growth and development. Insoluble

phenolics, linked to various cell components, contribute to the mechanical strength of cell walls and play a regulatory role in the plant growth and morphogenesis. Phenolics from cell inside take a part in response to stress and pathogens. An enhancement of phenylpropanoid metabolism and the amount of phenolic compounds can be observed under different environmental factors and stress conditions (Dewick, 2001; Korkina, 2007). The most plant phenolics are derived from *trans*-cinnamic acid, which is formed from L-phenylalanine by the action of L-phenylalanine ammonia-lyase (PAL), the branch point enzyme between primary (shikimate pathway) and secondary (phenylpropanoid pathway) metabolism. The shikimate pathway provides an alternative route to the formation of aromatic compounds particularly the aromatic amino acids. L-Phenylalanine, as C_6C_3 building block, is precursor for a wide range of natural products. In plants, frequently the first step is the elimination of ammonia from the side chain to generate cinnamic acid which is later modified to hydroxycinnamic acid (p-coumaric acid). Other related derivatives are obtained by further hydroxylation and methylation reactions sequentially building up substitution patterns typical of shikimate pathway metabolites. Loss of two carbons from the side chain of hydroxycinnamic derivatives leads to formation of hydroxybenzoic acids. Flavonoids are biosynthesized via a combination of the shikimic acid and acylpolymalonate pathways. The crucial biosynthetic reaction is the condensation of one molecule p-coumaroyl-CoA with three molecules malonyl-CoA to a calcone intermediate that consists of two phenolic groups which are connected by an open three carbon bridge. Plants collectively synthesize several thousand known different phenolic compounds and the number of these fully characterised is continually increasing. They can be considered as the most abundant plant secondary metabolites with highly diversified structures, ranging from simple molecules such as phenolic acids to highly polymerized substances such as tannins. The common feature of plant phenolic compounds is the presence of a hydroxy-substituted benzene ring within their structure. They may be classified into different groups as a function of the number of phenol rings contained and the structural elements that bind these rings to one another. Distinctions are thus made between the flavonoids, phenolic acids, stilbenes and lignans. These compounds occur primarily in conjugated form, with one or more sugar residues linked to hydroxyl groups, although direct linkages of the sugar unit to an aromatic carbon atom also exist. Associations with other compounds, such as organic acids, amines and lipids, and linkages with other phenols are also common (Dewick, 2001; Boudet, 2007; Martens & Mithöfer, 2005).

Flavonoids comprise the most abundant class of plant polyphenols with more than 6000 structures which have been identified. They share a carbon skeleton of diphenyl propanes, two benzene rings (A and B) joined by a linear three carbon chain ($C_6C_3C_6$). This central chain usually forms a closed pyran ring (C) with one of the benzene rings. Based on the variation in the type of heterocycle involved, flavonoids may be divided into six subclasses: flavones, flavonols, flavanones, flavanols, anthocyanidins and isoflavonoids. This subdivision is primarily based on the presence (or absence) of a double bond on position 4 of the C (middle) ring, then a double bond between carbon atoms 2 and 3 of the C ring, and hydroxyl groups in the B ring (Table 1). Flavones are characterized by the presence of a double bond between C2 and C3 in the heterocycle of the flavan skeleton. The B ring is attached to C2 and usually no substituent is present at C3. This exactly represents the difference to the flavonols where a hydroxyl group can be found at that C3 position while flavanones have a saturated three-carbon chain. Flavanols contain a saturated three-carbon chain with a hydroxyl group in the C3. Anthocyanidins are positively charged at acidic pH

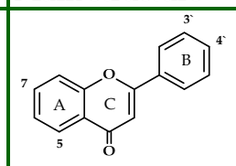
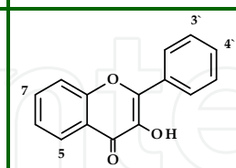
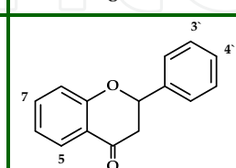
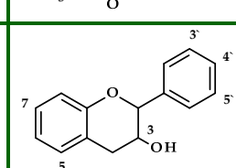
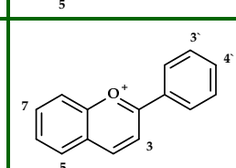
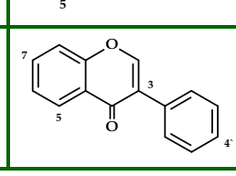
Class	Main structure	Compound	Plant source	Reference
Flavone		apigenin 4',5,7-OH luteolin 3',4',5,7-OH	*Matricaria recutita* *Achillea millefolium* *Cynara scolymus* *Thymus vulgaris*	(Švehlíková & Repčák, 2006; Trumbeckaite et al., 2011) (Mulinacci et al., 2004; Bazylko & Strzelecka, 2007)
Flavonol		quercetin 3',4',3,5,7-OH kaempferol 4',3,5,7-OH	*Sambucus nigra* *Betula pendula* *Ginkgo biloba* *Moringa oleifera*	(Keinänen & Julkunen-Tiitto, 1998; Verberic et al., 2009) (Beek & Montoro, 2009; Verma et al., 2009)
Flavanone		naringenin 4',5,7-OH hesperetin 3',5,7-OH 4'-OCH_3	*Citrus paradisi* *Humulus lupulus* *Citrus limon* *Citrus sinensis*	(Kanaze et al., 2003; Helmja et al., 2007) (González-Molina et al., 2010; Khan et al., 2010)
Flavanol		catechin 4',5',3,5,7-OH gallocatechin 3',4',5',3,5,7-OH	*Quercus petraea* *Potentilla erecta* *Hamamelis virginiana* *Camellia sinensis*	(Vivas et al., 2006; Tomczyk & Latté, 2009) (Dauer et al., 2003; Ashihara et al., 2010)
Anthocyanidin		cyanidin 3',4',3,5,7-OH pelargonidin 4',3,5,7-OH	*Vaccinium myrtillus* *Cenaturea cyanus* *Pelargonium sp.* *Fragaria ananassa*	(Du et al., 2004; Takeda et al., 2005) (Mitchell et al., 1998; Fossen et al., 2004)
Isoflavonoid		daidzein 4',7-OH genistein 4',5,7-OH	*Glycine max* *Trifolium pratense* *Glycine max* *Genista tinctoria*	(Beck et al., 2005; Peng et al., 2004) (Rimbach et al., 2008; Rigano et al., 2009)

Table 1. Classification of some common flavonoids indicating their major plant sources.

and this equilibrium form is called flavylium cation (2-phenylbenzopyrylium). In the flavonoid structure, a phenyl group is usually substituted at the 2-position of the pyran ring. In isoflavonoids the substitution is at the 3-position (Harborne & Williams, 2000; Dewick, 2001; Middleton et al., 2000). Individual differences within each group arise from variation in number and arrangement of the hydroxyl groups and their extent of alkylation and/or glycosylation. Flavonoids occur both in the free form and as glycosides, most are O-glycosides but a considerable number of flavonoid C-glycosides are also known. The O-glycosides have sugar substituents bound to a hydroxyl group of the aglycone, usually located at position 3 or 7, whereas the C-glycosides have sugar groups bound to a carbon of the aglycone, usually C6 or C8. The most common carbohydrates are rhamnose, glucose, galactose and arabinose. Flavonoid-diglycosides are also frequently found. Two very common disaccharides contain glucose and rhamnose, 1→6 linked in neohesperidose and 1→2 linked in rutinose. An interesting combination of flavonoid and lignan structure is found in a group of compounds called flavonolignans. They arise by oxidative coupling process between flavonoid and a phenylpropanoid, usually coniferyl alcohol. Additionally,

the flavanols exist as oligomers and polymers referred to as condensed tannins or proanthocyanidins (Harborne & Williams, 2000; de Rijke et al., 2006). Table 1 summarizes the chemical structures of the most common flavonoids and their botanical sources.

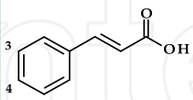
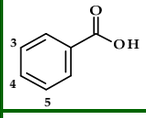
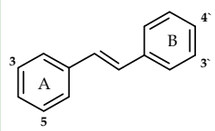
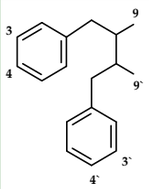
Class	Main structure	Compound	Plant source	Reference
Phenolic acid	Hydroxycinnamic acid (HCA) 	ferulic acid 4–OH; 3–OCH_3	*Citrus sinensis* *Pinus maritima*	(Swatsitang et al., 2000; Virgili et al., 2000)
		caffeic acid 3,4–OH	*Ocimum basilicum* *Helianthus annuus*	(Kwee & Niemeyer, 2011; Weisz et al., 2009)
		chlorogenic acid (5-O-caffeoylquinic acid)	*Coffea arabica* *Ilex paraguariensis*	(Koshiro et al., 2007; Marques & Farah, 2009)
		rosmarinic acid (α-O-caffeoyl-3,4-dihydroxyphenyl-lactic acid)	*Rosmarinus officinalis* *Melissa officinalis*	(Petersen & Simmonds, 2003; Weitzel & Petersen, 2011)
	Hydroxybenzoic acid (HBA) 	p-HBA 4–OH	*Daucus carota* *Vitex negundo*	(Sircar & Mitra, 2009; Guha et al., 2010)
		gallic acid 3, 4, 5–OH	*Quercus robur* *Hamamelis virginiana*	(Mämmelä et al., 2000; Wang et al., 2003)
Stilbene		resveratrol 3,5,4'-OH	*Vitis vinifera* *Polygonum cuspidatum*	(Pascual-Martí et al., 2001; Chen et al., 2001)
		piceatannol 3,5,3',4'-OH	*Vitis vinifera* *Euphorbia lagascae*	(Kim et al., 2009; Duarte et al., 2008)
Lignan		secoisolariciresinol 4,9,4',9'-OH 3,3'–OCH_3	*Linum usitatissimum* *Secale cereale*	(Li et al., 2008; Smeds et al., 2009)
		isotaxiresinol 4,3',4'–OH 3–OCH_3	*Taxus yunnanensis* *Taxus wallichiana*	(Banskota et al., 2004; Chattopadhyay et al., 2003)

Table 2. Some typical non-flavonoid phenolic compounds of various structures and their plant source.

Phenolic acids are the most common non-flavonoid naturally occurring phenolics which contain two distinguishing constitutive carbon frameworks: the hxydroxycinnamic (C_6C_3) and hydroxybenzoic (C_6C_1) structure. Although the basic skeleton remains the same, the numbers and position of hydroxyl and methoxyl groups on the benzene rings create the variety (Table 2). Only a minor fraction of phenolic acids exists in the free form. Instead, the majority are linked through ester, ether or acetal bonds either to structural components of the plant, larger polyphenols or smaller organic molecules (e.g., glucose, quinic acid). These linkages give rise to a vast array of derivatives (Robbins, 2003). Hydroxybenzoic acids are found in the free form as well as combined into esters of glycosides. Some of them are constituents of hydrolysable tannins which are compounds containing a central core of glucose or another polyol esterified with gallic acid or its dimer hexahydrohydiphenic acid, also called gallotannins and ellagitannins (Dai & Mumper, 2010).

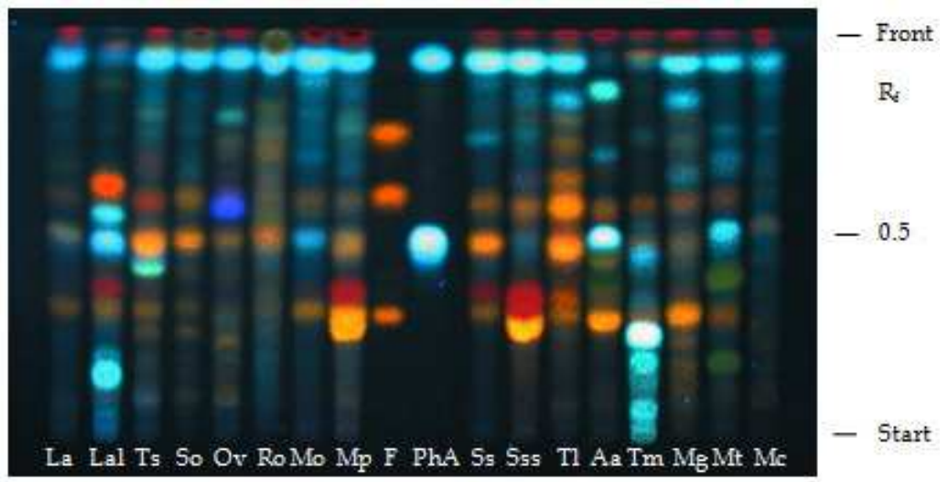
Stilbenes (1,2-diarylethenes) belong to a relatively small group of non-flavonoid class of phenolic compounds found in a wide range of plant sources. Ring A usually carries two hydroxyl groups in the m-position, while ring B may be substituted by hydroxyl and methoxyl groups in various position (Table 2). Stilbenes exist as stereoisomers and naturally occurring stilbenes are overwhelmingly present in the *trans* form. They occur in free and glycosylated forms and as dimeric, trimeric and polymeric stilbenes, the so-called viniferins. One of the most relevant and extensively studied stilbene is *trans*-resveratrol found largely in grapes (Cassidy et al., 2000).

Lignans also constitute a group of non-flavonoid phenolics that are structurally characterized by the coupling of two phenylpropanoid units by a bond between the β-positions in the propane side chains (Table 2). According to recent nomenclature recommendations the units are treated as propylbenzene units, giving the positions 8 and 8' to these linked carbon atoms. When the phenylpropane units are linked by another carbon–carbon bond, the compound class is named as neolignan. Lignans comprise a whole class of compounds with a similar basic skeleton, but with large variations in substitution patterns. They are mostly present in the free form, while their glycoside derivatives are only a minor form (Willför et al., 2006).

3. Phytochemical characterization of polyphenols

The biological properties of polyphenols and their health benefits have intensified research efforts to discover and utilise methods for the extraction, separation and identification of these compounds from natural sources. Despite a great number of investigations, the separation and quantification of various polyphenolics remain difficult, especially the simultaneous determination of their different structural groups. Quantification of phenolic compounds in plant materials is influenced by their chemical nature, the extraction method employed, sample particle size, storage time and conditions, as well as assay method, selection of standards and presence of interfering substances such as waxes, fats, terpenes and chlorophylls. A number of spectrophotometric methods have been developed for quantification of plant phenolics. These assays are based on different principles and can be classified as either those which determine total phenolic content or those quantifying a specific group of class of phenolic compounds (Ignat et al., 2011; Naczk & Shahidi, 2006). The Folin–Ciocalteu assay is widely used for determination of total phenolics. This assay relies on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid reagent to form blue coloured complexes that are determined spectrophotometrically at 760 nm (Dai & Mumper, 2010; Singleton et al., 1999). Tannins are capable of binding proteins to form water-insoluble substances. The absorbance corresponding to non-tannins is measured after precipitation of tannins with hide powder or casein, and the tannin content is than calculated by the difference (EDQM, 2006; Jurišić Grubešić et al., 2005). Vanillin and proanthocyanidin assays are usually used to estimate total procyanidinidins (condensed tannin), while hydrolysable tannins can be quantified by the potassium iodate method (Dai & Mumper, 2010; Hartzfeld et al., 2002). Determination of phenolic acids can be performed by the method described in European Pharmacopoeia using nitrite-molybdate reagent of Arnow which forms complexes with ortho-diphenols (Blažeković et al., 2010; EDQM, 2006). On the other hand, complexation of the phenolics with Al(III) is the principle of spectrophotometric assay used for quantification of total

flavonoids (Jurišić Grubešić et al., 2007; Vladimir-Knežević et al., 2011). Determination of anthocyanins is based on their characteristic behaviour under acidic conditions causing the transformation of anthocyanins to red-coloured flavylium cation (Ignat et al., 2011). The above mentioned spectrophotometric assays give an estimation of the total phenolic contents, while various chromatographic techniques are employed for separation, identification and quantification of individual phenolic compounds.



Experimental conditions: HPTLC silica gel 60 F_{254} plate; ethyl acetate - formic acid - water 8:1:1 (V/V/V) as mobile phase; NST/PEG for detection, UV 365 nm. Tracks: F (Flavonoids) - rutin (R_f=0.30), isoquercitrin (R_f=0.59), quercitrin (R_f=0.74) and PhA (Phenolic acids) - chlorogenic acid (R_f=0.48), rosmarinic acid (R_f=0.92) as references; La - *Lavandula angustifolia* Mill., Lal - *Lamium album* L., Ts - *Thymus serpyllum* L., So - *Salvia officinalis* L., Ov - *Origanum vulgare* L., Ro - *Rosmarinus officinalis* L., Mo - *Melissa officinalis* L., Mp - *Mentha* x *piperita* L., Ss - *Salvia sclarea* L., Sss - *Satureja subspicata* Bartl. ex Vis., Tl - *Thymus longicaulis* C. Presl, Aa - *Acinos arvensis* (Lam.) Dandy, Tm - *Teucrium montanum* L., Mg - *Micromeria graeca* (L.) Benth. ex Reich., Mt - *Micromeria thymifolia* (Scop.) Fritsch, Mc - *Micromeria croatica* (Pers.) Schott.

Fig. 1. HPTLC chromatogram of flavonoids and phenolic acids in metanolic extracts of various Lamiaceae species originating from Croatia.

Thin layer chromatography (TLC) is useful for a rapid screening of plant extracts for phenolic substances prior to detailed analysis by instrumental techniques especially because many samples can be analysed simultaneously (Fig. 1). The separation of phenolic compounds is mainly achieved by using silica gel as stationary phase and various mobile phases with UV monitoring or following specific spray reagents (de Rijke et al., 2006). High performance liquid chromatographic technique (HPLC) is now most widely used for both separation and quantification of phenolic compounds. The chromatographic conditions of the HPLC methods include the use of, almost exclusively, a reversed-phase C18 column, UV–Vis diode array detector, and a binary solvent system containing acidified water (solvent A) and a polar organic solvent (solvent B). Reversed phase (RP) HPLC has become a dominating analytical tool for the separation and determination of polyphenols with different detection systems, such as diode array detector (DAD), mass or tandem mass spectrometry. Liquid chromatography–mass spectrometry (LC–MS) techniques are nowadays the best analytical approach to study polyphenols from different biological sources, and are the most effective

tool in the study of the structure of phenolic compounds. Tandem-MS detection has largely replaced single-stage MS operation because of much better selectivity and the wider-ranging information that can be obtained. Capillary electrophoresis (CE), which is an alternative separation technique to HPLC, is especially suitable for the separation and quantification of low to medium molecular weight polar and charged compounds, the resultant separations being often faster and more efficient than the corresponding HPLC separations. Gas chromatography (GC) is another technique that has been employed for separation and identification of different phenolic compounds. GC methods developed for the analysis of polyphenols require the derivatisation to volatile compounds by methylation, trifluoroacetylation, conversion to trimethylsilyl derivatives and mass-spectrometric detection (Ignat et al., 2011; Naczk & Shahidi, 2006; de Rijke et al., 2006).

4. Antioxidant effectiveness of plant polyphenols

Oxidative stress is defined as an imbalance between production of free radicals and reactive metabolites, so-called oxidants or reactive oxygen species (ROS), and their elimination by protective mechanisms, referred to as antioxidants. This imbalance leads to damage of important biomolecules and cells, with potential impact on the whole organism. Oxidative stress can damage lipids, proteins, carbohydrates and DNA in cells and tissues, resulting in membrane damage, fragmentation or random cross linking of molecules like DNA, enzymes and structural proteins and even lead to cell death induced by DNA fragmentation and lipid peroxidation. These consequences of oxidative stress construct the molecular basis in the development of cardiovascular diseases, cancer, neurodegenerative disorders, diabetes and autoimmune disorders. ROS are products of a normal cellular metabolism and play vital roles in the stimulation of signalling pathways in cells in response to changes in intra- and extracellular environmental conditions. Most ROS are generated in cells by the mitochondrial respiratory chain. During endogenous metabolic reactions, aerobic cells produce ROS such as superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), hydrogen peroxide (H_2O_2) and organic peroxides as normal products of the biological reduction of molecular oxygen. The electron transfer to molecular oxygen occurs at the level of the respiratory chain, and the electron transport chains are located in the membranes of the mitochondria. Under hypoxic conditions, the mitochondrial respiratory chain also produces nitric oxide (NO), which can generate reactive nitrogen species (RNS). ROS/RNS can further generate other reactive species by inducing excessive lipid peroxidation. In order to combat and neutralize the deleterious effects of ROS/RNS, various antioxidant strategies have involved either the increase of endogenous antioxidant enzyme defences (e.g., superoxide dismutase, glutathione peroxidase, glutathione reductase and catalase) or the enhancement of non-enzymatic defences (e.g., glutathione, vitamins) through dietary or pharmacological means. Antioxidants can delay, inhibit or prevent the oxidation of oxidizable substrate by scavenging free radicals and diminishing oxidative stress. However, in disease conditions, the defence against ROS is weakened or damaged and the oxidant load increases. In such conditions, external supply of antioxidants is essential to countervail the deleterious consequences of oxidative stress (Ratnam et al., 2006; Reuter et al., 2010). It has been proposed that polyphenols can act as antioxidants by a number of potential mechanisms. The free radical scavenging, in which the polyphenols can break the free radical chain reaction, as well as suppression of the free radical formation by regulation of enzyme activity or chelating metal ions involved in free radical production are reported to be

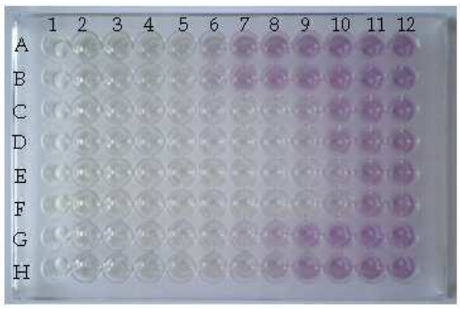
the most important mechanisms of their antioxidant activity. The interaction between polyphenolic compounds with other physiological antioxidants is another possible antioxidant pathway for these compounds (Fraga et al., 2010; Perron & Brumaghim, 2009).

In order to evaluate the efficacy of polyphenols as antioxidants as well as to elucidate the mode of their action, researchers today are using a wide range of experimental models, from the simplest chemical antioxidant assays through the biologically more relevant cellular-based assay to the most accurate animal models, and ultimately clinical studies in humans. Taking into account all known advantages and limitations, each currently employed method provides useful information at certain level of biological organization.

4.1 Chemical-based antioxidant assays

Widely used for initial antioxidant screening, chemical-based assays are the most popular methods for an evaluation of antioxidant properties of plant extracts as well as their bioactive constituents. Due to the implication of redox mechanisms in the pathogenesis of numerous human diseases, the vast majority of the available *in vitro* approaches to polyphenolic antioxidant research are based on the redox-linked colorimetric assays. Although it has been established that polyphenols exert antioxidant effects via different modes of action, free radical scavenging abilities seems to be the most important. Namely, polyphenolic compounds possess ideal structure chemistry for free radical neutralization because they have: (i) phenolic hydroxyl groups that are prone to donate a hydrogen atom or an electron to a free radical; (ii) extended conjugated aromatic system to delocalize an unpaired electron (Dai & Mumper, 2010). Among widely employed antioxidant assays, scavenging capacity assays against stable, non-biological radicals (e.g., $DPPH^{\bullet}$ and $ABTS^{\bullet+}$) and assays against specific ROS/RNS can be distinguished. Especially popular DPPH assay is a convenient and accurate method which provides a simple and rapid way to evaluate free radical-scavenging ability. This assay uses a stable and commercially available organic radical 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$). Interaction of this purple-coloured radical with an antioxidant agent, which is able to neutralize its free radical character, leads to the formation of yellow-coloured diphenylpicrylhydrazine (Fig. 2) and resulting colour change, proportional to the effect, can be quantified spectrophotometrically. Based on the DPPH free radical scavenging effectiveness, it has been established that numerous polyphenolic compounds of different classes are strong antioxidants (Okawa et al., 2001; Villaño et al., 2007; Vladimir-Knežević et al., 2011). Also frequently used colorimetric method is Trolox equivalent antioxidant capacity (TEAC) assay, which is based on scavenging of blue-green ABTS radical cation by antioxidants present in sample (Nenadis et al., 2004).

Oxygen radical absorbance capacity (ORAC), deoxyribose assays as well as superoxide anion scavenging capacity are usually used to predict scavenging abilities of plant polyphenols against biologically relevant peroxyl, hydroxyl and superoxide radicals, respectively (Cano et al., 2002; Kang et al., 2010). Reducing properties of polyphenols are also very important in terminating free radical-induced chain reactions and are mainly studied in terms of iron(III)-iron(II) transformation abilities. For that purpose ferric reducing antioxidant power (FRAP) assay or potassium ferricyanide reducing method are used (Firuzi et al., 2005; Vladimir-Knežević et al., 2011). In the context of the methods appropriate for quantification of polyphenol total antioxidant capacity, the phosphomolybdenum assay based on the ability to reduce Mo(VI) to Mo(V) is also worth of mention (Blažeković et al.,



Experimental: The DPPH scavenging activities of ethanolic extract of *Micromeria croatica* (Pers.) Schott (A, B), polyphenolic constituents rosmarinic acid (C, D), and quercetin (E, F), as well as referent antioxidant butylated hydroxyanisole (G, H) were evaluated by mixing seral dilution of samples (concentration range 200-0.20 μg/mL; 1-11) with ethanolic solution of DPPH (100 μM), with regard to DPPH control (12). After incubation for 30 min, absorbance was measured at 490 nm using microplate reader.

Fig. 2. Application of DPPH assay on *Micromeria croatica* extract and polyphenolic constituents.

2010). Another important antioxidative mechanism of polyphenols arises from their capability to chelate transition metals, such as iron and copper, through multiple hydroxyl groups and the carbonyl moiety, when present (Leopoldini et al., 2011). Iron(II) is a primary cause of the ROS generation *in vivo* and plays a pivotal role in contributing to oxidative stress, DNA damage, and cell death, so it has been the target of many antioxidant therapies. By removing and neutralising prooxidative metals, polyphenols may prevent oxidative damage of important biomolecules due to highly reactive hydroxyl radicals generated by the Fenton reaction which is catalysed by Fe^{2+} or Cu^{+} ions (Mladenka et al., 2011; Perron & Brumaghim, 2009). Moreover, polyphenols may also act as preventive antioxidants through the inhibition of prooxidative enzymes, like xanthine oxidase (Selloum et al., 2001) which is a physiological source of superoxide anions in eukaryotic cells. Among all biologically important macromolecules, unsaturated membrane lipids are particularly prone to oxidative damage. Therefore, the *in vitro* models of lipid peroxidation, as a well-established mechanism of cellular injury, can be used as an indicator of oxidative stress in cells and tissues. Polyphenols were proved to inhibit chain reaction of oxidative degradation of polyunsaturated fatty acids integrated in micelles, emulsions, liposomes, low-density lipoproteins (LDL) and animal tissues, which was previously induced by different prooxidans (hydroxyl radicals, iron(II) ions, UV radiation). The extent of lipid peroxidation was mostly evaluated spectrophotometrically, in terms of quantification of thiobarbituric acid-reactive substances as end products - TBARS assay (Blažeković et al., 2010; T. Miura et al., 2000).

4.2 Cell-based antioxidant assays

Since the biological systems are much more complex than the simple chemical mixtures and antioxidant compounds may operate via multiple mechanisms, the studies employing complex cellular testing systems provide a more useful approach towards understanding the *in vitro* antioxidant capacities of polyphenols and their behaviours in living cells to reduce oxidative damage (Niki, 2010). The cellular antioxidant activity assay (CAA), developed by Wolfe & Liu (2007), centres on dichlorofluorescin, a probe molecule trapped within cells that can be easily oxidised to produce fluorescence. The test uses 2,2′-azobis(2-amidinopropane) dihydrochloride-generated peroxyl radicals to oxidise dichlorofluorescin,

and the ability of antioxidant compounds to inhibit this process in HepG2 human hepatocarcinoma cells. The study has suggested that polyphenols can act at the cell membrane and break peroxyl radical chain reactions at the cell surface, or they can be taken up by the cell and react with ROS intracellularly. Therefore, the efficiency of cellular uptake and/or membrane binding combined with the radical scavenging activity likely dictates the antioxidative effectiveness of different polyphenols. Blasa et al. (2011) consider that red blood cells represent the cheapest and easily available cell type and their ability to cope with ROS/RNS is relevant in the body.

4.3 *In vivo* animal studies

To overcome differences between *in vitro* test systems and the whole organism, as well as the obvious constraints of human clinical studies, animal studies are widely applied in antioxidant research (Mortensen et al., 2008). The capacity and efficacy of antioxidants *in vivo* may be assessed most accurately due to their effect on the level of oxidation in biological fluids and tissues, thus animal studies of polyphenols are mainly focused on serum total antioxidant capacity, lipid peroxidation and antioxidant enzymes activity measurements. Hepatoprotective studies with animal models use exogenously administered hepatotoxin such as carbon tetrachloride (CCl_4), a free radical generating compound that induce an oxidative stress, and related damage of biomolecules and cell death. Polyphenols were found to be able to protect the liver against cellular oxidative damage and maintenance of intracellular level of antioxidant enzymes (Amat et al., 2010; Fernandez-Martinez et al., 2007). Neuroprotective properties of polyphenolic antioxidant compound curcumin were reported based on its ability to inhibit homocysteine (Hcy) neurotoxicity and related Hcy-induced lipid peroxidation in animals' hippocampi (Ataie et al., 2010). Studies performed in apolipoprotein E-deficient mice proved that polyphenols from wine and tea can prevent the development and/or reduce progression of atherosclerosis, probably due to their potent antioxidative activity and ability to protect LDL against oxidation (Hayek et al., 1997; Y. Miura et al., 2001).

4.4 Human studies

At present there is a big gap between the knowledge of *in vitro* and *in vivo* effectiveness of polyphenols as antioxidants, and human studies are still scarcer than those on animals. Oxidative damage and antioxidant protection by polyphenols have been studied in healthy people (preventive effect) or those suffering from certain disease (therapeutic effect). The available human studies of polyphenols can be divided into intervention studies, which are under defined circumstances (such as clinical trials) applying nutritional intervention and measuring the biological outcome (effect), and observational epidemiological studies. Various epidemiological studies have shown an inverse association between the consumption of polyphenols or polyphenol-rich foods and the risk of cardiovascular diseases. In this context, strong epidemiological evidences indicate that moderate consumption of wine is associated with a significant reduction of cardiovascular events. However, the respective contribution of wine polyphenols and alcohol to these effects was not well clarified (Hansen et al., 2005; Manach et al., 2005). Additionally, recent clinical trials on resveratrol, as the main antioxidant in wine, have been largely focused on characterizing its pharmacokinetics and metabolism as well as the potential role in the management of

diabetes, obesity, Alzheimer's disease and cancer (Patel et al., 2011). Considering the lack of relevant data, the future clinical trials should be aimed at identifying the cardiovascular benefits of resveratrol. In order to verify the polyphenols as antioxidants in *in vivo* conditions there is still a lot of scientific research to be done. The emerging findings suggest that continued research through well-designed and adequately powered human studies that undoubtedly verify health-promoting antioxidant activity of polyphenols in *in vivo* conditions are more than welcome.

5. Bioavailability of polyphenols

Bioavailability is usually defined as the fraction of an ingested nutrient or compound that reaches the systemic circulation and the specific sites where it can exert its biological activity (Porrini & Riso, 2008). To establish conclusive evidence for the effectiveness of dietary polyphenols in disease prevention and human health improvement, it is useful to better define the bioavailability of polyphenols. The health effects of polyphenols in human and in animal models depend on their absorption, distribution, metabolism and elimination. The chemical structure of polyphenols determines their rate and extent of absorption as well as the nature of the metabolites present in the plasma and tissues. The most common polyphenols in human diet are not necessarily the most active within the body, either because they have a lower intrinsic activity or because they are poorly absorbed from the intestine, highly metabolized, or rapidly eliminated (Manach et al., 2004). Evidence of their absorption trough the gut barrier is given by the increase in the antioxidant capacity of plasma after intake of plant polyphenols. More direct evidence on the bioavailability of phenolic compounds has been obtained by measuring the concentration in plasma and urine after the ingestion of either pure compounds or plant extracts with the known content of the compounds of interest (Baba et al., 2005; Koutelidakis et al., 2009).

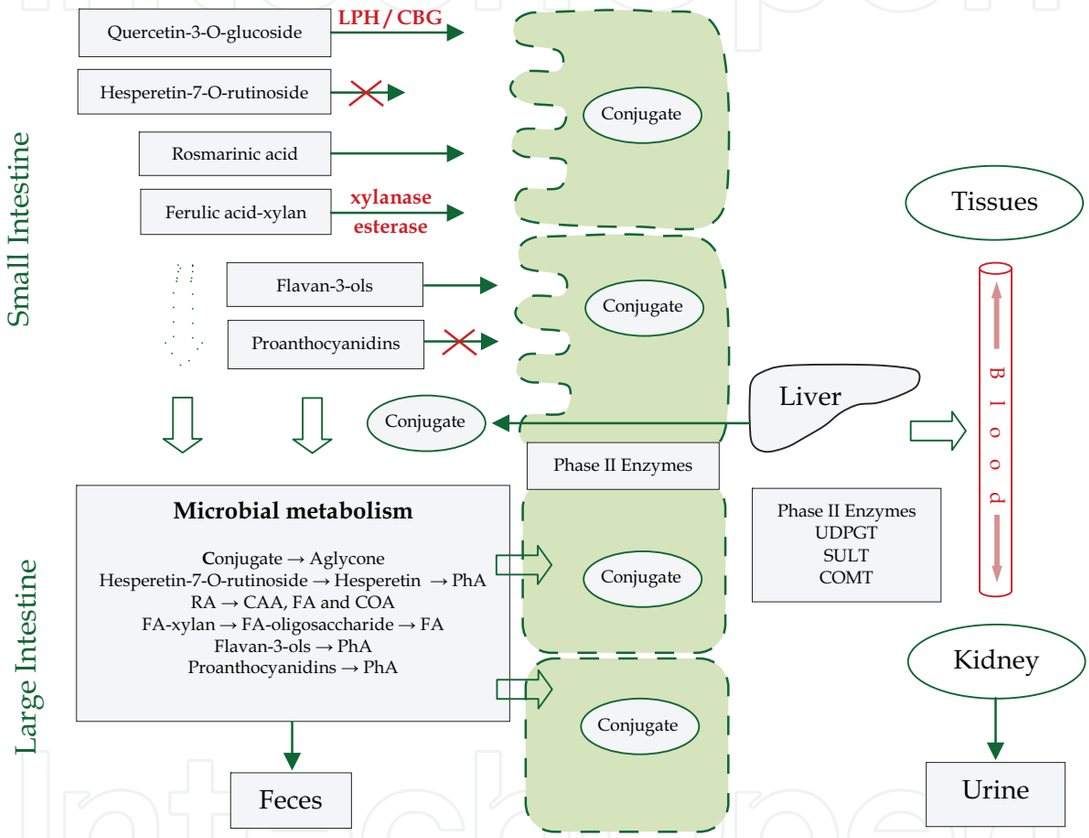
Polyphenols are present in plants as aglycones, glycosides, esters or polymers. Aglycones can be absorbed from the small intestine, while glycosides, esters and polymers must be hydrolyzed by intestinal enzymes, or by the colonic microflora, before they can be absorbed (Fig. 3). Although this can be considered as a general rule, some exceptions occur like unchanged anthocyanin glycosides detected in human plasma and urine (Kay et al., 2004). The absorption of plant flavonoids which are predominantly bound to different sugars mostly occurs in the small intestine. There are two possible routes by which the glycosides could be hydrolysed and the first one involves the action of lactase phlorizin hydrolase (LPH) in the brush-border of the small intestine epithelial cells. The released aglycone may then enter epithelial cells by passive diffusion due to increased lipophilicity. The second mechanism includes cytosolic β-glucosidase (CBG) within the epithelial cells where the polar glucosides are transported through the active sodium-dependent glucose transporter 1 (SGLT1). On the other hand, SLGT1 was reported not to transport flavonoids and, moreover, those glycosylated flavonoids, and some aglycones, have the capability to inhibit the glucose transporter. It has been observed that polyphenols differ in site of their absorption. Data on absorption of quercetin-4'-glucoside and quercetin-3-rutinoside illustrate the major impact of structural differences on the site of absorption. Quercetin-4'-glucoside is absorbed in the small intestine after hydrolysis, while the quercetin-3-rutinoside is absorbed in the colon as free and metabolised forms produced by the microflora (Crozier et al., 2010). Hydroxicinnamic acids, when ingested in the free form, are rapidly absorbed

from the stomach or the small intestine. However, in plants these compounds mainly occur as esters and therefore a release of free form is required before absorption. For example, ferulic acid, the main phenolic compound present in cereals, is esterified to arabinoxylans of the grain cell walls. Although it has been shown that esterases are present throughout the entire gastrointestinal tract, the release of ferulic acid mainly occurs in the colon after the hydrolysis by microbial xylanase and esterase (Poquet et al., 2010). Proanthocyanidins as the most widespread polyphenols in plants are very poorly absorbed due to their polymeric nature and high molecular weight. Since their concentrations can be much higher in the lumen of gastrointestinal tract then in plasma or other body tissues, proanthocyanidins may have direct effects on the intestinal mucosa, which are important because the intestine is particularly exposed to oxidizing agents and may be affected by inflammation and numerous diseases such as cancer. Polyphenols that are not absorbed in the stomach or small intestine will be carried to the colon where they undergo substantial structural modifications. Those absorbed in the upper part of the gastrointestinal tract, metabolized in the liver and excreted in the bile or directly from the enterocyte back to the small intestine will also reach the colon but in a different form (e.g. glucuronide conjugates). The colonic microflora hydrolyzes glycosides into aglycones and extensively metabolizes the aglycones into simpler compounds, such as phenolic acids (Fig. 3). Conjugated metabolites secreted in bile can also be hydrolised by β-glucuronidases of microbial origin. The released aglycones can be reabsorbed and undergo enterohepatic cycling, as indicated by a second plasma peak. Some microbial metabolites exert biological activity, such as equol produced from daidzein having better phytoestrogenic properties than the original isoflavone. Inter-individual variations in formation of active metabolites caused by different composition of colonic microflora are also observed (Del Rio et al., 2010; Manach et al., 2004).

The absorption of polyphenols is followed by extensive conjugation and metabolism. Phenolic compounds are conjugated in the intestinal cells and later in the liver that restricts their potential toxic effects and facilitates their biliary and urinary excretion by increasing their hydrophilicity. This process mainly includes glucuronidation, sulfation and methylation catalyzed by following enzymes: UDP glucuronosyl transferase (UDPGT), phenol sulfotransferase (SULT) and cathel-O-methyltransferaze (COMT), respectively (Scalbert & Williamson, 2000). The conjugation mechanisms of polyphenols are highly efficient, and free aglycones are generally either absent or present in low concentrations in plasma after the consumption of nutritional doses. Green tea catechins, whose aglycones can constitute a significant proportion of the total amount in plasma, present an exception (Lee et al., 2002). Rosmarinic acid, partly absorbed in unchanged form and partly metabolized by microflora in the colon, is found to be present in plasma as conjugated and/or methylated forms accompanied by the conjugated forms of its metabolites (caffeic, ferulic and m-coumarinic acid). In the same study, looking at the metabolites formed from rosmarinic acid between rats and humans large differences are observed and attributed to the differences in the enzyme location, affinity of substances and the characteristics of the enzymatic reactions, which corresponds to data for epicatechin and quercetin (Baba et al., 2005).

Generally, it is important to identify the circulating metabolites of polyphenols because the nature and position of the conjugating groups can affect their biological properties (Day et al., 2000). The accumulation of polyphenol metabolites in the target tissues where they exert

biological activities is the most important phase of polyphenol metabolic pathway, however, a few studies reported on their concentrations in human tissues. Isoflavones were found to accumulate in breast and prostate tissues (Hong et al., 2002; Maubach et al., 2003). Tea polyphenols and theaflavins were detected in the prostate, and their tissue bioavailability was greater in humans than in animals (Henning et al., 2006). Since, polyphenols are extensively modified and the forms appearing in the blood and tissues are usually different from the forms found in plants, greater attention should be focused on exploring the potential biological activity of polyphenol metabolites. In understanding bioavailability of plant polyphenols, the differences between animal models and human should be taken into account.



CAA – Caffeic acid, CBG - Cytosolic β-glucosidase, COA - m-Coumarinic acid, COMT - Cathel-O-methyltransferaze, FA – Ferulic acid, LPH - Lactase phlorizin hydrolase, PhA – Phenolic acids, RA – Rosmarinic acid, SULT - Phenol sulfotransferase, UDPGT - UDP glucuronosyl transferase.

Fig. 3. Pathway of absorption and metabolism of selected polyphenols.

6. Polyphenols in aging and oxidative stress-related diseases

Aging is a natural process that is defined as a progressive deterioration of biological functions after the organism has attained its maximal reproductive competence; it leads to the disabilities and diseases that limit normal body functions. Major hypotheses of aging include altered proteins; DNA damage and less efficient DNA repair; inappropriate cross-

linking of proteins, DNA and other structural molecules; a failure of neuroendocrine secretion; cellular senescence in the cell culture system; an increase in free radical-mediated oxidative stress and changes in the order of gene expression (T. Farooqui & A.A. Farooqui, 2009). It has been pointed out that oxidative stress experienced during normal metabolism has primary contribution to aging. Today much attention is given to the therapeutic effects of polyphenols, which have been shown to mitigate age-associated phenomena such as oxidative stress, chronic inflammation, and toxin accumulation (Queen & Tollefsbol, 2010). Plant polyphenols are serious candidates in explanations of the protective effects of vegetables and fruits against various diseases. Epidemiologic studies are useful for evaluation of the human health effects of long-term exposure to physiologic concentrations of polyphenols, but reliable data on polyphenol contents of food are still scarce (Arts & Holman, 2005). With respect to cardiovascular health, polyphenols may alter lipid metabolism, inhibit low-density lipoprotein (LDL) oxidation, reduce atherosclerotic lesion formation, inhibit platelet aggregation, decrease vascular cell adhesion molecule expression, improve endothelial function and reduce blood pressure. Polyphenols have also been shown to exert beneficial cognitive effects, to reverse specific age-related neurodegeneration and to exert a variety of anti-carcinogenic effects including an ability to induce apoptosis in tumour cells (Vauzour et al., 2010), inhibit cancer cell proliferation (Walle et al., 2007), prevent angiogenesis (Mojzis et al., 2008) and tumour cells invasion (Weng & Yen, 2011).

Cardiovascular disease (CVD) is one of the leading causes of death in many economically developed nations. Some of the major risk factors for CVD can not be changed (age, sex, genetic predisposition), but diet and lifestyle are modifiable risk factors (Leifert & Abeywardena, 2008). Accordingly, current knowledge favours the notion that risk factors other than raised plasma cholesterol play an important role in the development of CVD, such as oxidative stress, vascular inflammation and endothelial dysfunction (Zalba et al., 2007). Many human studies have demonstrated that altered oxygen utilization and/or increased formation of reactive oxygen species (ROS) contribute to atherogenesis, hypertension and progression of other CVD. For example, olive oil polyphenols increased the level of oxidized LDL autoantibodies (OLAB) which have protective role in atherosclerosis. In a crossover, controlled trial 200 healthy men were randomly assigned to 3-week sequences of 25 mL/day of three olive oils with high (366 mg/kg), medium (164 mg/kg) and low (2.7 mg/kg) phenolic contents. OLAB concentrations increased in a dose-dependent manner with the polyphenol content of the administered olive oil (Castañer et al., 2011). Phytoestrogen genistein was tested on sixty healthy postmenopausal women in a double blind, placebo controlled, randomized study where participants received either genistein (54 mg/day) or placebo. After six months of genistein therapy, the ratio between nitric oxide and endothelin-1 was increased and flow-mediated endothelium-dependent vasomotion in postmenopausal women improved (Sqadrito et al., 2002). Medina-Remón et al. (2011) confirmed that intake of polyphenols is negatively correlated with hypertension (Table 3). Human studies have also showed an association of moderate intake of alcoholic drinks containing polyphenols with a reduced risk of cardiovascular disease. The intake of these drinks decreased Nuclear factor-kappa B (redox sensitive transcription factor implicated in the pathogenesis of atherosclerosis) activation (Blanco-Colio et al., 2007). These observations have been proposed to explain the French paradox: the coexistence of relative high fat intake and the lower rate of cardiovascular disease in France, due to regular intake of drinks containing polyphenols.

Sample	Participants/Study	Effect	Reference
Diet rich in polyphenols	589 participants; blood pressure measured; total polyphenol excretion determined in urine	Polyphenol intake negatively associated with blood pressure levels and prevalence of hypertension	Medina-Remón et al., 2011
Grape antioxidant dietary fibre – 5.25 g dietary fibre, 1.4 g polyphenols per day	34 participants; 16 weeks supplementation with grape antioxidant dietary fibere; blood pressure and LDL levels measured	Significant reduction of total cholesterol, LDL level, systolic and diastolic blood pressures	Pérez-Jiménez et al., 2008
Alcoholic drinks with polyphenols; 2660 mg/L red wine 357 mg/L rum, and 89 mg/L brandy	16 volunteers; 16 g/m^2 of ethanol during 5 days; fat-enriched diet; examined NF-kappa B activation and circulating MCP-1 levels	Decreased NF-kappa B activation and MCP- 1 levels	Blanco-Colio et al., 2007
Green and black tea (EGCG)	21 postmenopausal healthy woman; flow-mediated dilation and nitro-mediated dilation measured before and 2 h after consumption of tea	Significant increase of endothelium-dependent flow-mediated dilation	Jochmann et al., 2008
Flavonoids from food	10054 participants; flavonoid intake estimated on the basis of the flavonoid concentrations in food; the incident cases of the diseases identified from national public health registers	Total cancer incidence lower at higher quercetin (mainly lung cancer); prostate cancer risk lower at higher myricetin; breast cancer risk lower at higher quercetin intake	Knekt et al., 2002
Regular diet	Patients afflicted with cancer of the oral cavity, pharynx, larynx and esophagus	Favonoid intake associated with a reduction in risk of incidence of cancer	De Stefani et al., 1999
Quercetin	51 patients with confirmed cancer no longer amenable to the standard therapy	Tyrosine kinase inhibited; CA 125 level decreased; α-fetoprotein level decreased	Ferry et al., 1996
Fruit and vegetable juices containing a high concentration of polyphenols	Population-based prospective study of 1836 Japanese Americans	Hazard ratio for Alzheimer's disease 0.24 for subjects who drank juices at least 3 times per week; 0.84 for subjects who drank juices 1 to 2 times per week	Dai et al., 2006
Functional drink rich in polyphenols	100 participants; plasmatic level of tHcy determined	Decrease of tHcy plasmatic level in Alzheimer patients	Morillas-Ruiz et al., 2010
Coffee and tea	200 participants, pure ethnic Chinese	Dose-dependent protective effect of Parkinson's disease in coffee and tea drinkers	Tan et al., 2003

CA 125 - Tumour marker present in greater concentration in ovarian cancer, EGCG - Epigallocatechin-3-gallate, LDL – Low-density lipoprotein, MCP-1 - Monocyte chemoattractant protein-1, NF-kappa B - Nuclear factor-kappa B, tHcy – total Homocysteine.

Table 3. Overview of the selected human studies.

A multi-stage process such as cancer development is characterised by the cumulative action of multiple events occurring in a single cell and can be described by three stages (initiation, promotion and progression) and ROS can act in all these stages of carcinogenesis (Valko et al., 2006). The reported data imply that growth factor-stimulated ROS generation can mediate intracellular signalling pathways by activating protein tyrosine kinases, inhibiting protein tyrosine phosphatase, and regulating redox-sensitive gene expression (Aslan & Özben, 2003). Different clinical and epidemiological studies presented in Table 3 have confirmed negative correlation between intake of polyphenols and incidence of cancer (Knekt et al., 2002; De Stefani et al., 1999; Ferry et al., 1996).

Among the most common neurologic diseases are neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS). The risk factors that have been identified for these diseases are mitochondrial dysfunction and oxidative damage which play important roles in the slowly progressive neuronal death (Esposito et al., 2002). The growing evidence suggests that the oxidative damage caused by the β-amyloid peptide in the pathogenesis of AD may be hydrogen peroxide mediated. The polyphenols from apple and citrus juices, such as quercetin, are able to cross the blood-brain barrier and show neuroprotection against hydrogen peroxide (Dai et al., 2006). The increased serum total homocysteine (tHcy) is identified as a risk factor for the development of AD. As the thiol group of homocysteine undergoes auto-oxidation in the plasma and generates ROS, polyphenols have preferences for the preventive-therapeutic use in AD (Morillas-Ruiz et al., 2010). The characteristic of PD is the selective degeneration of dopamine neurons in the nigrostriatal system where increased total iron has been found (Esposito et al., 2002). The protective effect of tea catehins against neuronal diseases may involve its radical scavenging and iron chelating activity and/or regulation of antioxidant protective enzymes (Weinreb et al., 2004) which was confirmed by Tan et al. (2003). Human studies considering neurodegenerative diseases are presented in Table 3.

Human studies suggest that plant polyphenols may reduce the risk of various chronic diseases, but there is a need for more studies to provide definitive proofs of their protective role. Further epidemiological studies should identify proper biomarkers of disease risk and demonstrate they are influenced by the consumption of plant polyphenols (Schalbert et al., 2005). There is also lack of validated biomarkers of polyphenol intake since polyphenol family encompasses very diverse compounds with highly different bioavailabilities, so the results obtained for one polyphenol compound cannot be generalized to others (Manach et al., 2005). Nevertheless, epidemiological studies are useful tool to study the health effects of polyphenols, providing us with information about significant differences among groups of people who have different lifestyles and have been exposed to different environmental factors.

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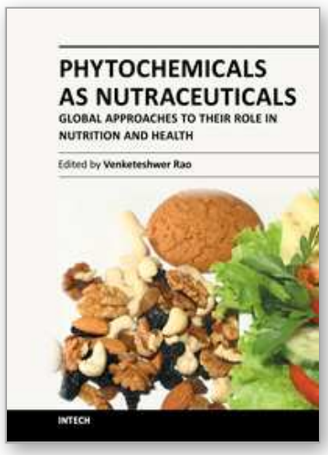

Phytochemicals as Nutraceuticals - Global Approaches to Their Role in Nutrition and Health

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Phytochemicals are biologically active compounds present in plants used for food and medicine. A great deal of interest has been generated recently in the isolation, characterization and biological activity of these phytochemicals. This book is in response to the need for more current and global scope of phytochemicals. It contains chapters written by internationally recognized authors. The topics covered in the book range from their occurrence, chemical and physical characteristics, analytical procedures, biological activity, safety and industrial applications. The book has been planned to meet the needs of the researchers, health professionals, government regulatory agencies and industries. This book will serve as a standard reference book in this important and fast growing area of phytochemicals, human nutrition and health.

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