Condensed and Hydrolysable Tannins as Antioxidants Influencing the Health

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Abstract: Natural polyphenols are a wide class of secondary plant metabolites and represent an abundant antioxidant component of human diet. An important, but often neglected group of natural polyphenols, are tannins. This review offers a general description of chemistry of both hydrolysable and condensed tannins (proanthocyanidins), the mechanisms of their antioxidation action, like free radical scavenging activity, chelation of transition metals, inhibition of prooxidative enzymes and lipid peroxidation. The mechanisms of action of antibacterial, antiviral, anticarcinogenic, cardiovascular system preventing, and antiinflammatory effects as well as the absorption, metabolic fate and positive *in vivo* effects of tannins are enclosed.

Key Words: Tannins, antioxidant, diseases, proanthocyanidins, free radicals, biological activity, phlorotannins.

INTRODUCTION

There are many natural compounds with a wide scale of biological activities. Especially, there is a great interest in polyphenols, which have been studied for many years and are still very active domain of research because of their prospective use in medicine. Polyphenols are a large class of substances, which contain over 8000 compounds, from those with simple structure (e.g. phenolic acids) to the polymeric substances like tannins [1,2]. Flavonoids are very important and well known group of compounds with various pharmacological effects [3-7]. An important, but often neglected group of polyphenols, are also tannins.

In 1957, Bate-Smith and Swain defined plant tannins as water-soluble phenolic compounds having a molecular weight between 500 and 3000 Dalton [8]. Their characteristic properties include forming of insoluble complexes with proteins, polysaccharides, nucleic acids, or alkaloids. Within this general character, tannins exhibit number of various bioactivities, which are often related to their antioxidant activity. Tannins are classified into two major groups on the basis of their structure: the hydrolysable and the condensed tannins [9]. This article is focused on both groups of tannins, nevertheless more attention is given to condensed tannins, because they are represented in nature more widely and are an important components of human food.

HYDROLYSABLE TANNINS

Hydrolysable tannins are compounds containing a central core of glucose or another polyol esterified with gallic acid, also called gallotannins, or with hexahydroxydiphenic acid, also called ellagitannins. Pentagalloylglucose (PGG) is a basic unit of the metabolism of hydrolysable tannins, from which other molecules are derived. Gallotannins consist of a central molecule, such as glucose, surrounded by gallic acid units. Ellagitannins contain hexahydroxydiphenic acid, or its dilactone form, ellagic acid (Fig. **1**). The great variety in the structure of these compounds is due to the many possibilities in formation of oxidative linkages. Intermolecular oxidation reactions give rise to many oligomeric compounds having a molecular weight between 2000 and 5000 Dalton [9].

Plants are able to biosynthesize gallotannins, ellagitannins, or form the mixture of both types of hydrolysable tannins. While condensed tannins are presented in many species of higher plants, presence of hydrolysable tannins is limited to Angiospermae, Dicotyledons. Gallic acid derivatives are presented in several families, e.g. *Ericaceae, Geraniaceae,* or *Fagaceae*. Ellagitannins are presented in subclasses of *Hamamelidae, Dilleniidae* and *Rosidae* species [10-12].

CONDENSED TANNINS

Condensed tannins are oligomers or polymers composed of flavan-3-ol nuclei. They are also called proanthocyanidins, because they are decomposed to anthocyanidins in heated ethanol solutions. The most frequent basic units of condensed tannins are derivatives of flavan-3-ols: (+)-catechin, (-)-epicatechin, (+)-gallocatechin and major polyphenols of green tea: (-)-epigallocatechin (EGC) and (-)-epigallocatehin gallate (EGCG) (Fig. **2**). The structural diversity is caused by variation in hydroxylation pattern, stereochemistry at the three chiral centers, and the location and type of interflavan linkage. Furthermore, derivatisations as *O*-methylation, *C*- and *O*-glycosylation, and *O*-galloylation are frequently reported. Proanthocyanidins are classified according to their

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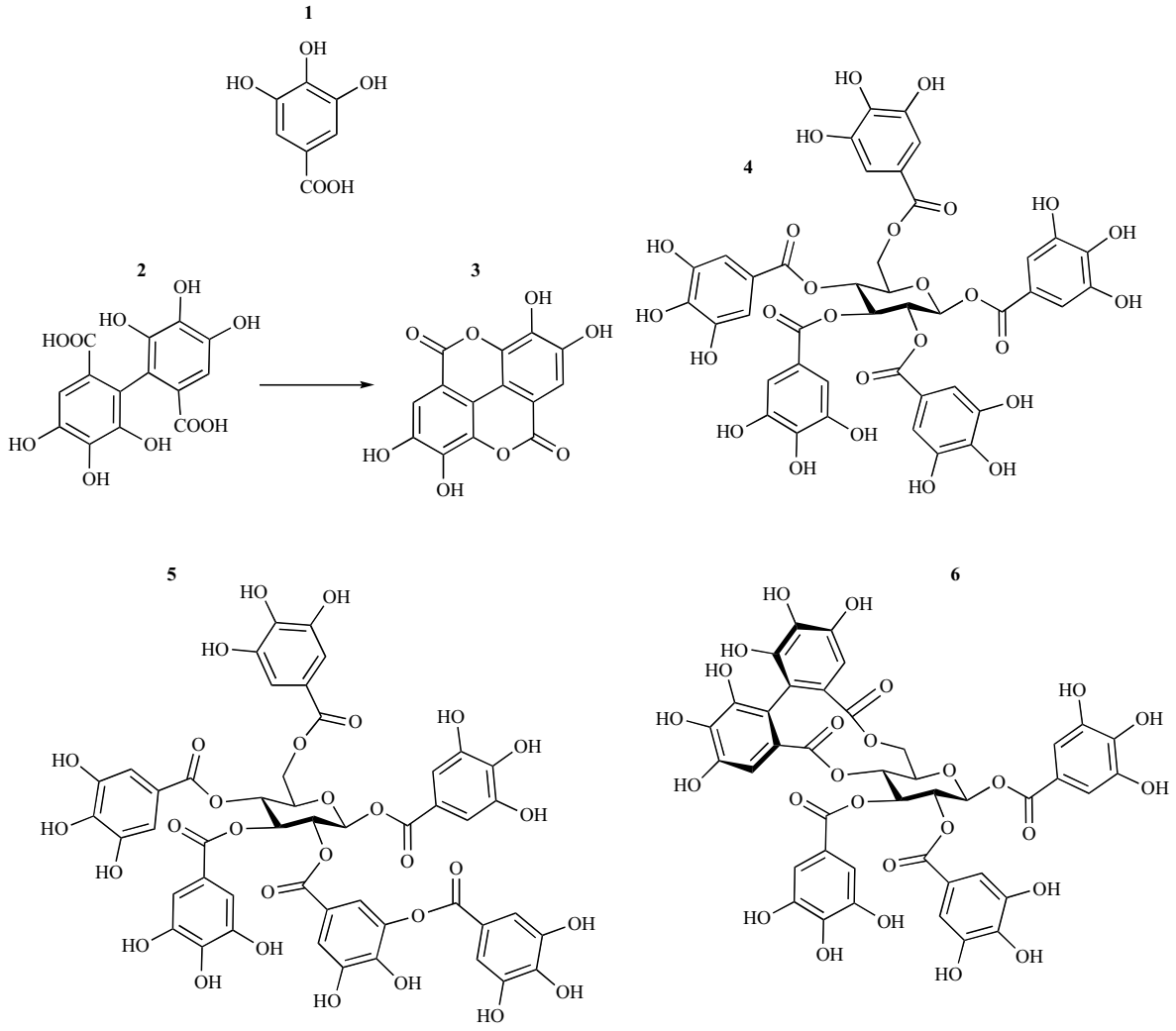

Fig. (1). Characteristic structures of hydrolysable tannins: (1) gallic acid; (2) hexahydroxydiphenic acid; (3) ellagic acid; (4) pentagalloylglucose, the basic unit of hydrolysable tannins; (5) 2-*O*-digalloyl-1,3,4,6-tetra-*O*-galoyl-β-D-glucopyranose, the example of gallotannin; (6) tellimagradin II., the typical ellagitannin.

hydroxylation pattern into several subgroups, e.g. procyanidins (3,5,7,′,4′-OH) or prodelphinidins (3,5,7,3′,4′,5′-OH) [13,14].

Procyanidins of the B-type (dimeric) and C-type (trimeric) are characterized by single linked flavanyl units, usually between C-4 of the flavan-3-ol of the upper unit and C-6 or

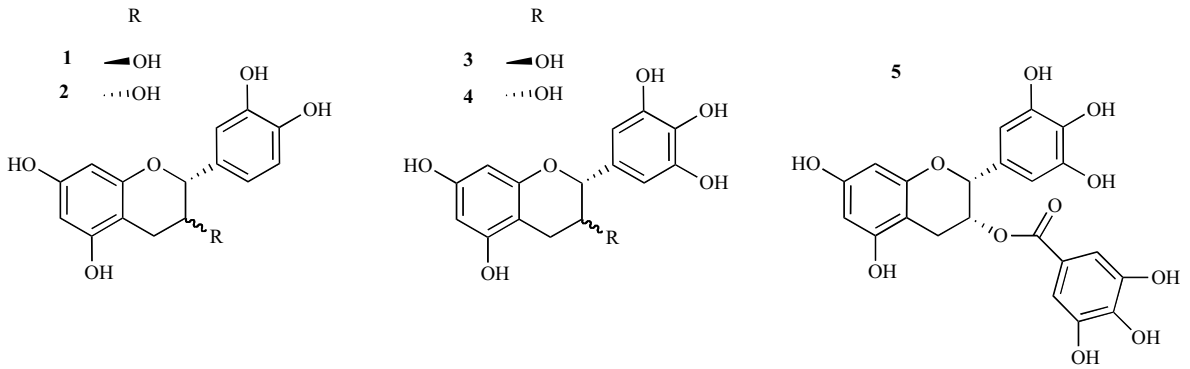

Fig. (2). The most frequent structure units of condensed tannins: (1) (+)-catechin; (2) (-)-epicatechin; (3) (+)-gallocatechin; (4) (-)-epigallocatechin; (5) (-)-epigallocatechin gallate.

C-8 of the lower unit. Proanthocyanidins of the A-type possess an additional ether linkage between C-2 of the upper unit and a 7 or 5-OH of the lower unit (Fig. **3**) [13,14]. Polymers, composed of up to fifty monomers, are formed by the addition of more flavans. Especially polyepicatechins and copolymers of procyanidins and prodelphinidins are common.

Proanthocyanidins have been isolated from many species of plants, and they are also important components of human

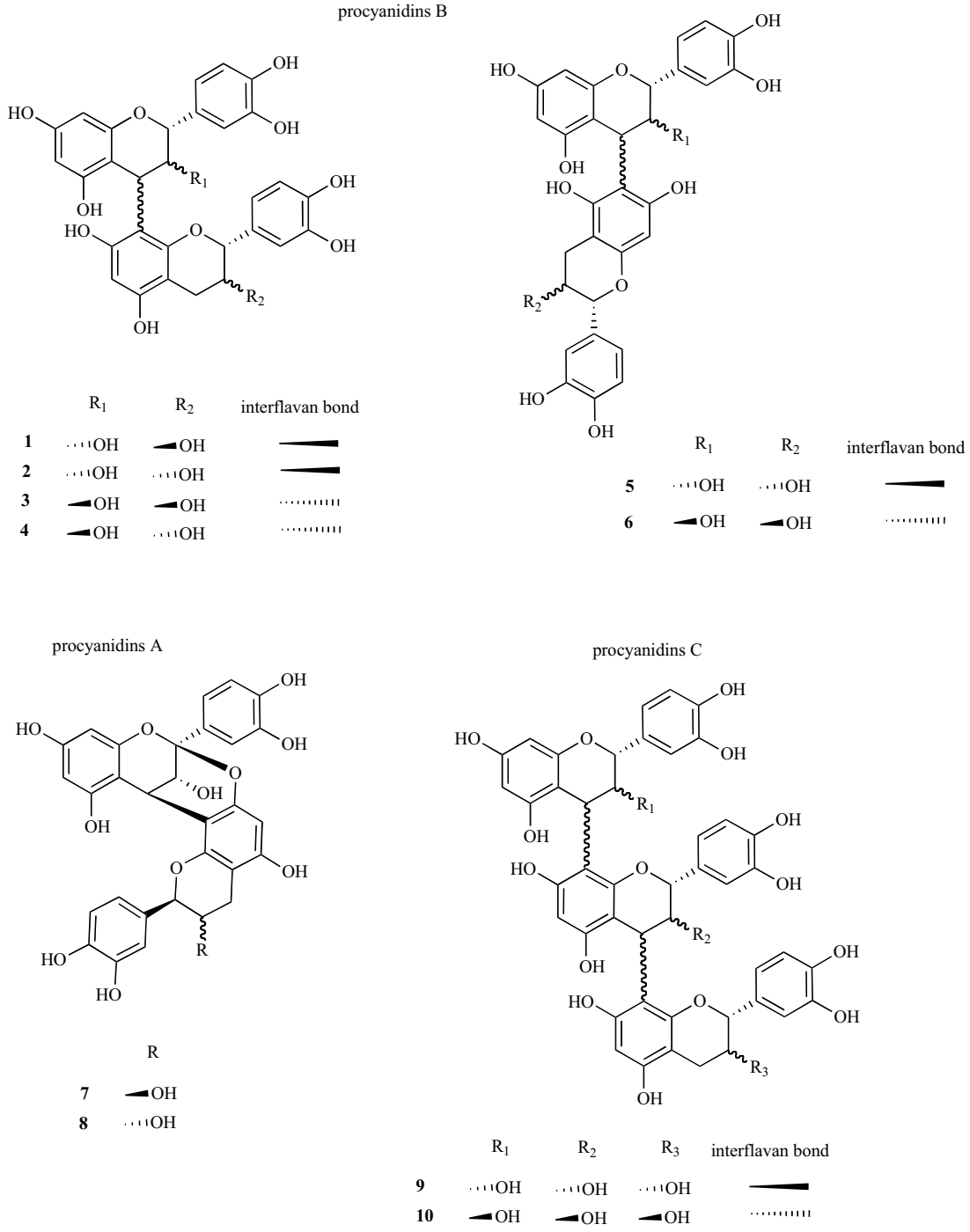

Fig. (3). Oligomeric procyanidins: (1) procyanidin B-1 (epicatechin-(4β→8)-catechin); (2) procyanidin B-2 (epicatechin-(4β→8)-epicatechin); (3) procyanidin B-3 (catechin-(4α →8)-catechin); (4) procyanidin B-4 (catechin-(4α →8)- epicatechin); (5) procyanidin B-5 (epicatechin-(4β→6)-epicatechin); (6) procyanidin B-6 (catechin-(4α→6)-catechin) (7) procyanidin A-1 (epicatechin-(4β→8,2β→7)-catechin); (8) procyanidin A-2 (epicatechin-(4β→8,2β→7)-epicatechin); (9) procyanidin C-1 (epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin); (10) procyanidin C-2 (epicatechin-(4α →8)-catechin-(4α →8)-catechin).

food. The largest group of proanthocyanidins is formed by procyanidins. Procyanidin B-1 is presented in grapefruit, sorghum, and cranberries, B-2 in apples, cocoa beans, and cherries, B-3 in strawberries and hops and B-4 in raspberries and blackberries [15,16]. Well-known source of protoanthocyanidins is also red wine, green tea, cocoa and chocolate [17].

ABSORPTION AND METABOLISM OF TANNINS

It has been assumed for a long time that tannins are not absorbed due to their high molecular weight and their ability to form insoluble complexes with components of food, such as proteins. Though there are some studies which confirm that the absorbtion of tannins is higher than it was assumed there are still many questions about their biological availability [18]. Generally, absorbtion of tannins decreases with the increasing polymerisation degree.

It was found that dimeric and trimeric procyanidins are absorbed by intestinal epithelium without any considerable limitations. Caco-2 cell line was used as an *in vitro* model of intestinal epithelium. The absorbtion decrease with further polymerisation, and the transport of hexamers did not proceed [19].

The *in vitro* study which dealt with the stability of procyanidins in acidic environment, as found in the gastric milieu, stated that high-molecular substances are fragmented to absorbable monomers and dimers [20]. Another study claims that higher procyanidins are not degraded in stomach environment of six healthy volunteers, and only original monomers and dimers are absorbed [21]. This discrepance could be interpreted in view of various character of tested proanthocyanidins: some compounds can be more disposed to degradation than others.

According to one study epicatechin is the primary bioavailable form of procyanidin dimers B-2 and B-5 after perfusion of isolated small intestine, while other study in rats arrived at a conclusion that procyanidin B-2 is absorbed and excreted predominantly unmodified and is metabolised only partly [22,23]. These results show that quantitative composition of a tested mixture and also the type of used biological model affect the process of absorption.

Human intestinal microflora play an important role in the metabolisation of tannins. It is known that the part of non-absorbed tannins is degraded *in vitro* by human colonic microflora to various phenolic products, which can be absorbed and participate in various pharmacological effects [24]. Non-absorbed high-molecular tannins and tannin-protein complexes have also an important role for protection of intestinal tract because they keep their antioxidant activity [25].

TANNINS AS ANTIOXIDANTS

Introduction

Antioxidants are defined as substances that, when present at low concentrations compared to those of an oxidizable substrate, significantly delay or prevent oxidation of that substrate. Recently, great attention has been given to antioxidant agents by reason of their medical use. It is given by association of many human diseases with oxidative stress. Free radicals play an important role in pathogenesis of ageing, various cardiovascular diseases, type 2 diabetes, or cancer. For example, in the radiation-induced carcinogenesis, highly reactive hydroxyl radicals are recognized as primary cause of the disease, whereas in diseases like atherosclerosis or rheumatoid arthritis, the oxidative stress is not the inciting agent, but supports their pathology [13, 26-28].

Primarily, free radicals play an important physiological role, for example, they are mediators of energy transfer, immunity defence factors, or signal molecules of cell regulation. In special conditions they may become hurmful and injure the organism. For the maintenance of the redox balance and prevention of increased formation of free radicals, organism uses some mechanisms, such as scavenging of free radicals, prevention of new formation through regulation of the enzymes which form them, support of antioxidant enzymes, or inactivation of transition metals which support formation of free radicals [29].

Secondary plant metabolites, such as flavonoids or tannins, can be also involved in complex system of antioxidant defense. The basic mechanisms of antioxidant activity of tannins are free radical scavenging activity, chelation of transition metals and inhibition of prooxidative enzymes.

Free Radical Scavenging Activity

The basic concept of free radical scavenging activity of polyphenols, including tannins, is the ability of the antioxidant to donate electron to a free radical and produce a more stable and therefore less harmful radical structure. DPPH (1,1-diphenyl-2-(2,4,6-trinitrophenyl)hydrazyl) or ABTS ((2, 2-azinobis)3-ethyl-2,3-dihydrobenzothiazol-6-sulphonic acid) radicals are often used for *in vitro* determination of free radical activity [30,31]. A number of tested condensed and hydrolysable tannins scavenged these radicals [32-36]. Out of other radicals, free radical scavenging activity against superoxide radical [37], hydroxyl radical [38,39], and peroxyl or nitric oxide (NO)[40,41] was determined.

Generally, it applies to proanthocyanidins that scavenging activity increases with the number of hydroxyls, especially if they are in ortho position on benzene nucleus and if gallic acid is introduced. The activity is also influenced by the size of molecule; it increases from monomers to trimers, afterwards it decreases [33,34].

The comparison of antioxidant activities of B-type procyanidins showed ambiguous results. One study found that procyanidin B-2 is more active than B-3 and B-5 in DPPH assay, while other study did not prove any differences in ABTS scavenging activity of six different B-type procyanidins [32,34]. In superoxide radical scavenging assay, procyanidins B-1 and B-3 showed similar scavenging activity [37]. These differences are not surprising because one compound can act differently against different radicals.

Tannins, due to their higher molecular weight and high degree of hydroxylation of aromatic rings, show high antioxidant potential. In comparison to *in vitro* antioxidant activity of various types of polyphenols, dimeric procyanidins are the most active on the basis of scavenging ABTS radical, hypochlorous acid, or in FRAP test for the evaluation of re-

ducing power. Dimeric procyanidins were followed by flavanols, flavonols, hydroxycinnamic acids, and simple phenolic acids [42].

It was found that, unlike other phenolic antioxidants, procyanidins might not show prooxidative activity. Quercetin and other flavon(ol)s form o-quinone structures in quenching reactions that may act as prooxidants in further redox reactions. While the study, which used electron spin resonance technique (ESR) which dealt with mechanism of antioxidant activity of hydrolysable and condensed tannins, showed the contrary. The *O*-quinones of proanthocyanidins formed after reaction with a radical are subject to subsequent nucleophilic addition reactions and as a result more complicated structures are formed, which keep great number of hydroxyl groups and thereby also their antioxidant activity (Fig. **4**). From this point of view proanthocyanidins are better antioxidants than monomeric flavon(ol)s. The similar experiments with hydrolysable tannins were not so unambiguous [43]. Another study which evaluated anti- and prooxidation properties of gallic and ellagic acid and tannin (tannic acid) according to the DNA injury in mussels *Unio tumidus* cells found that high concentrations of these substances injure DNA and showed prooxidant activity [44].

Oligomeric procyanidins (B-1, B-3, and others) and phenolic monomers (catechin, epicatechin, and flavonoid taxifolin) are the main bioactive compounds of standardized *Pinus maritima* bark extract (Pycnogenol®). This pine originated from south part of France, and in many countries is used as the cardioprotective preparation (Pycnogenol®) which causes vasodilation effect, inhibition of angiotensin-converting-enzyme (ACE), or increase in capillary permeability. Pine bark extract also contains phenolic acids (such as caffeic, ferulic, and p-hydroxybenzoic acids) as minor constituents and glycosylation products, i.e., glucopyranosyl derivatives of either flavanols or phenolic acids as minute constituents. Recently, the great scientific attention regard the medicinal use of this extract. Studies indicate that Pycnogenol® components are highly bioavailable. The complex extract exhibit higher biological activity than isolated substances, which means that the constituents act synergistically. The extract has strong free radical scavenging activity against reactive oxygen and nitrogen species. The procyanidins contribute significantly to the ESR free radical signal. Pycnogenol® modulates NO metabolism in activated macrophages by quenching the NO radical and inhibiting both iNOS (inducible nitric oxide synthase) mRNA expression and iNOS activity. The ability to regenerate ascorbyl radical and protect endogenous vitamin E and glutathion (GSH) against oxidative damage also support the complex antioxidant properties of Pycnogenol® [45].

Chelation of Transition Metals

The transition metals like iron and copper perform many functions in human organism. For example, they play an important physiological role like cofactors of antioxidant enzymes such as superoxide dismutase, catalase or glutathione peroxidase. They are usually bound to proteins such as ferritin or caeruloplasmin [46]. When they occur separately, they can catalyze the radical reactions. The typical example of this reactions is well known Fenton reaction [47]:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO\bullet$$

There are many studies that examinated chelation activity of polyphenols, especially flavonoids [48-50]. In the study which dealt with antioxidant activity of tannin, it was found that tannic acid was more efficient in protecting against 2-deoxyribose degradation than classical hydroxyl radical scavengers. The *in vitro* study concluded that chelation of iron ions by this substance may be more important for its antioxidant activity than the mechanism of quenching free radicals itself [51]. Other study determined that chelation of iron and resulting inhibition or Fenton reaction participates in high antioxidant activity of *Vitis vinifera* procyanidins. Antioxidant potency of procyanidins was studied in phosphatidylcholine liposomes, using iron-promoted lipid peroxidation [52].

The ability to chelate metals was also proved by the study which dealt with stability of aluminium-proanthocyanidin complexes. This study concludes that existence of phenolic groups, especially in B-ring o-position, is very important for chelating activity. The stability of complexes increased with increasing polymerisation degree [53].

The recent study which dealt with beneficial influence of oligomeric proanthocyanidins on lead-induced neurotoxicity

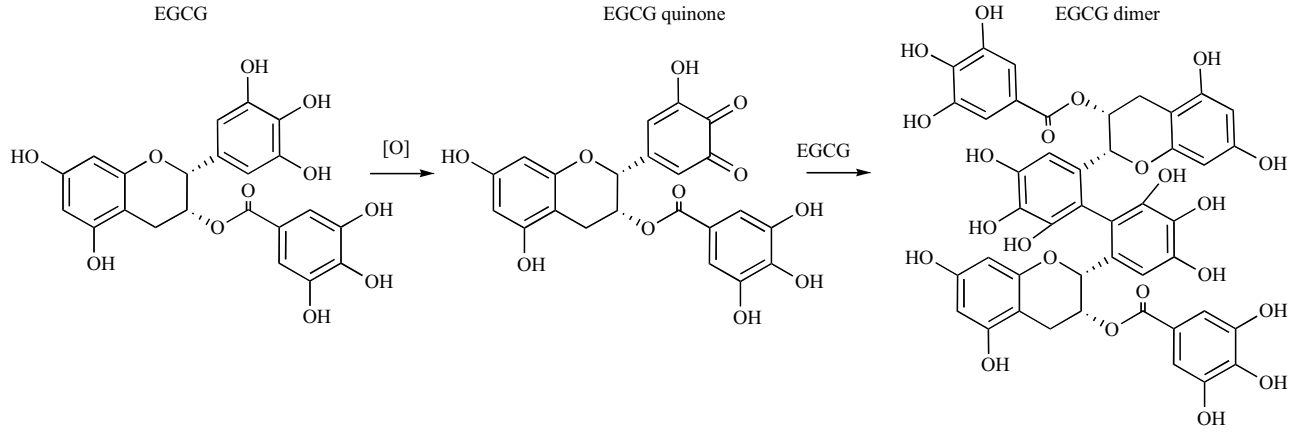

Fig. (4). Polyphenolic reaction of epigallocatechin gallate (EGCG).

in rats showed ambiguous results. It was confirmed that proanthocyanidins exhibit antioxidant and chelating activity in *in vitro* studies, but the lead-induced toxicity did not decrease in *in vivo* experiment and Pb^{2+} was cumulated even in some organs [54].

Inhibition of Enzymes

Antioxidant activity can also be exhibited through inhibition of prooxidative enzymes.

Tannins decrease formation of NO through inhibition of nitric oxide synthases (NOS). For example, hydrolysable tannins isolated from East Asian plant *Melastoma dodecandrum* inhibited the induction of the iNOS in the course of macrophage activation with lipopolysaccharide and recombinant mouse interferon-gamma [55]. Next *in vitro* study which tested monomeric catechins up to octameric procyanidins isolated from hops (*Humulus lupulus*) showed that procyanidin B-2 was the most active against brain NOS, while procyanidin B-3, catechin, and epicatechin did not inhibit this enzyme [56].

Xanthin oxidase is also ranked among enzymes with prooxidative activity. For example, ellagitannins isolated from New Caledonian plant *Cunonie macrophylla* inhibited *in vitro* this enzyme. The enzyme activity was measured spectophotometrically following the conversion of xanthine to uric acid. Ellagic acid-4-*O*-β-D-xylopyranoside (Fig. **5**) was the most active from tested compounds [57].

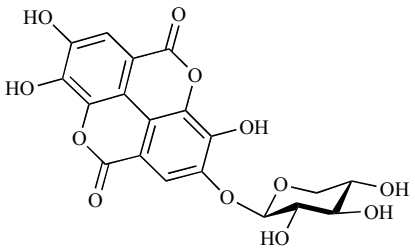

Fig. (5). Ellagic acid-4-*O*-β-D-xylopyranoside; ellagitannin from New Zealand plant *Cunonie macrophylla.*

Several studies dealt with effect of tannins on lipoxygenases (LOX) which can damage membrane lipids. It was found that phlorotannins, isolated from brown seaweed *Eisenia bicyclis,* exhibited high *in vitro* inhibition activity of soybean LOX and 5-LOX. The phlorotannins, polyphenols of marine algae, are structurally different from hydrolysable and condensed tannins, which are produced by terrestrial plants. Phlorotannins are oligomers up to polymers of phloroglucinol. Eckol (trimer), phlorofucofuroeckol A (pentamer), dieckol (hexamer) and 8,8′-bieckol (hexamer) (Fig. **6**) were even more active than known inhibitor of LOX epigallocatechin gallate. These compounds had also pronounced *in vitro* inhibitory effects on secretory phospholipase $sPLA_2$ [58].

The influence of polymerization on inhibition of 5-LOX was showed in cocoa procyanidins (*Theobroma cacao*). Recombinant human 5-LOX was significantly inhibited by (-)-epicatechin in a dose-dependent manner. Among the procyanidin fractions, only the dimer fraction and, to a lesser extent, the trimer through pentamer fractions exhibited comparable effects, whereas the larger procyanidins (hexamer through nonamer) were almost inactive [59].

The study with tannins rich Pycnogenol® showed the *in vitro* inhibition of horseradish peroxidase, LOX, NOS, and xanthine oxidase. Authors concluded that inhibition of these enzymes is probably non-specific, and it is given by high affinity of polyphenols to proteins [45,60]. Non-competitive enzyme inhibition was also described with other plant extracts. For example, *Vitis vinifera* procyanidins inhibited xanthine oxidase, proteolytic enzymes elastase and collagenase, as well as *β*-glucuronidase and hyalouronidase [52].

Lipid Peroxidation

Lipid peroxidation belongs to important pathological processes. This process is involved in oxidative modification of low-density lipoproteins (LDL) which ultimately leads to the formation of atherosclerotic lesions [61]. It was found that proanthocyanidins can protect LDL against oxidation. For example, catechins and their oligomers of coffee-beans inhibited *in vitro* human LDL oxidation in the following order: procyanidin C-1 > procyanidin B-2 > (+)-catechin > (-)-epicatechin. It was also confirmed that the number of hydroxyl groups is related to the antioxidant activity [62].

Generally, it is possible to say that the substances which inhibit lipid peroxidation act through the mechanism of quenching initiatory radicals (hydroxyls), or already formed oxidative products (peroxyl, alcoxyl). The mechanism of chelation of transition metals can be also involved. Many studies proved *in vitro* inhibition of lipid peroxidation by hydrolysable as well as condensed tannins [35,37,63-65]. For example, hydrolysable tannin punicalagin (Fig. **7**) is contained in pomegranate (*Punica granatum*) and participates in high antioxidant potential of prepared extracts and drinks. Punicalagin inhibit lipid peroxidation induced by Fe^{2+} in a liposomal model [63]. Also tannins isolated from cranberry (*Rhodococcum vitis-idaea*) exhibited high *in vitro* inhibition of lipid peroxidation. Out of six tested substances, cinnamtannin B-1 was the most active (Fig. **7**) [37].

Above mentioned studies used various methods for evaluation of inhibition of lipid peroxidation and they generally showed that the ability to inhibit lipid peroxidation is high, comparable with vitamin E.

ROLE OF TANNINS IN HUMAN DISEASES

Anti-Cancer Activity

Though many studies indicated an interesting anti-tumor activity of hydrolysable as well as condensed tannins, mechanisms of action have not been clearly determined yet. The antioxidant activity can play a positive role. Tannins suppress the oxidative stress, which is important for pathogenesis of cancer and influence apoptosis of cells. The study, which investigated whether the anti-cancer effects of oligomeric proanthocyanidins are induced by apoptosis on human colorectal cancer cell line (SNU-C4) pronounced that cytotoxic effect of proanthocyanidins on SNU-C4 cells appeared in a dose-dependent manner. Proanthocyanidin treatment revealed typical morphological apoptotic features, increased level of Bax and caspase-3, and decreased level of Bcl-2 mRNA expression. Bax and Bcl-2 belong to the

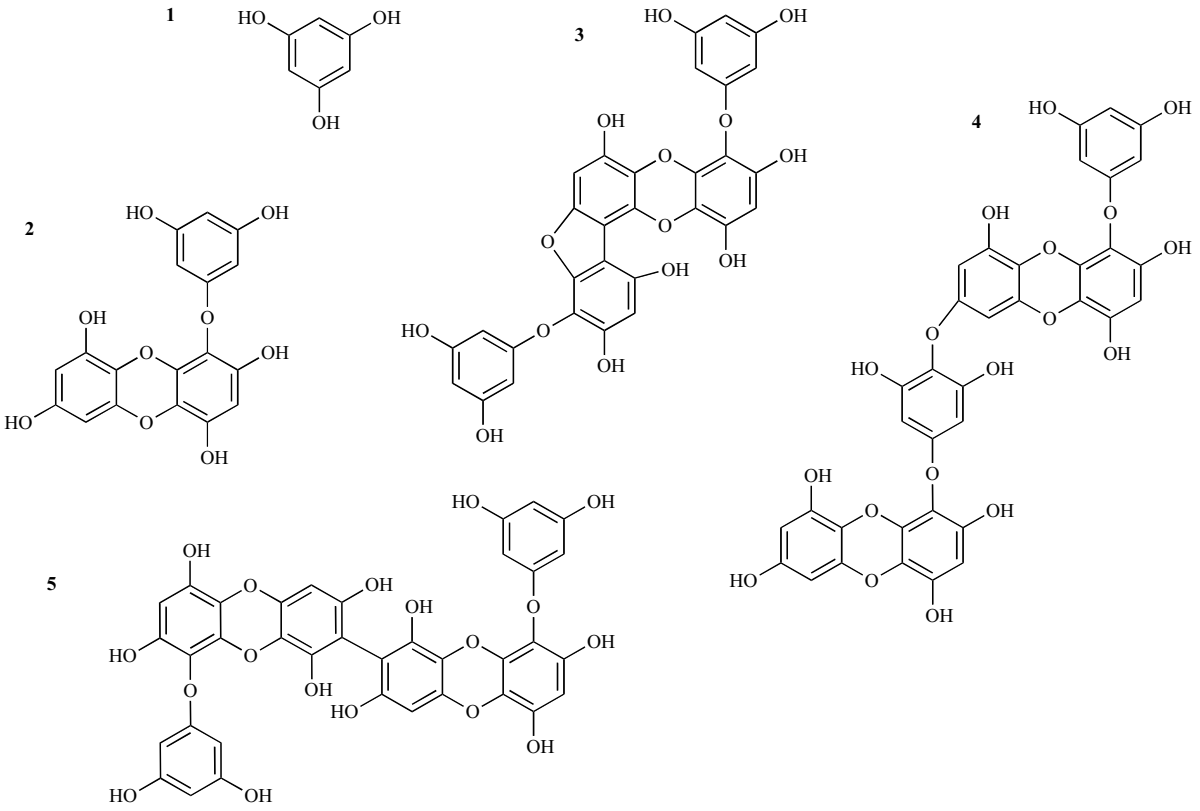

Fig. (6). Phlorotannins: (1) phloroglucinol; (2) eckol; (3) phlorofucofuroeckol A; (4) dieckol; (5) 8,8′-bieckol.

groups of genes which pomote (Bax) or inhibit (Bcl-2) apoptosis. Caspases are proteases which play essential roles in apoptosis. Caspase-3 enzyme activity was also significantly increased by treatment of proanthocyanidins. The study concludet that proanthocyanidins caused cell death by apoptosis through caspase pathways [66].

Several studies examinated anti-tumor activity of punicalagin, tannin extract and juice from pomegranate [63,67]. Tested samples exhibit antiproliferative activity on human oral (KB, CAL27), colon (HT-29, HCT116, SW480, SW620) and prostate (RWPE-1, 22Rv1) tumor cells and induced apoptosis in HT-29 and HCT116 cancer colon cells [63].

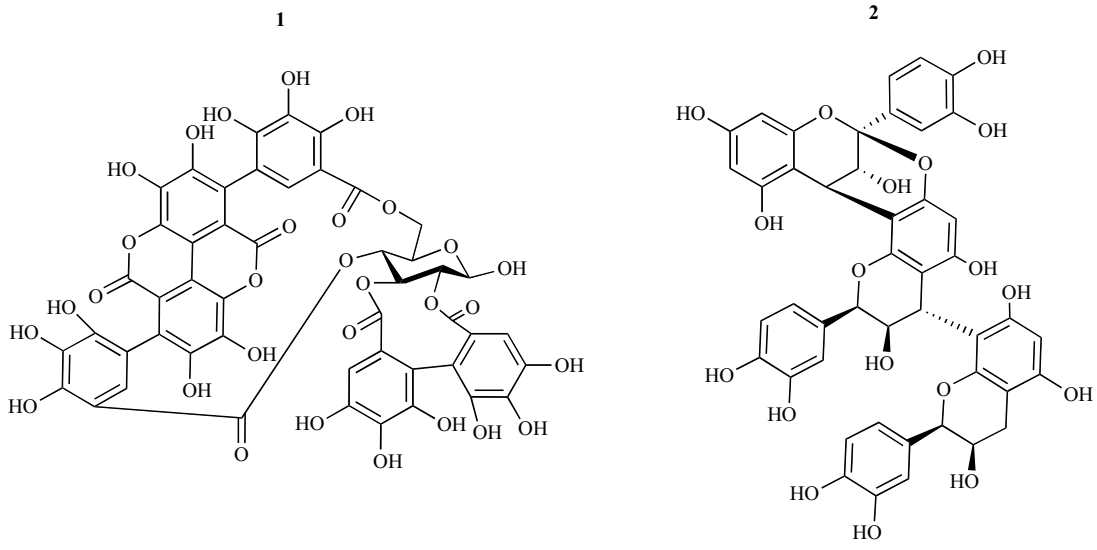

Fig. (7). (1) **Punicalagin**, pomegranate ellagitannin (*Punica granatum*); (2) **cinnamtannin B-1**, cranberry trimeric proanthocyanidin (*Rhodococcum vitis-idaea*).

Continuos study examined the effects of pomegranate on inflammatory cell signals (TNF-α, NF-κB, Akt) in the HT-29 human colon cancer cell line. TNF-α (tumor necrosis factor), cytokine mainly secreted by macrophages, is involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation. The most active suppressor of TNF-α induced cyclooxygenase-2 protein expression was pomegranate juice followed by tannin extract and punicalagin. Additionally, pomegranate juice, tannin extract and punicalagin but no ellagic acid reduced phosphorylation of the p65 subunit and binding to the NF-κB response element, which is an important regulator of gene expression and promotes transcription of many inflammatory mediators. Pomegranate juice also abolished TNF-α-induced Akt activation. Akt, genes implicated in cellular signaling, are needed for NF-κB activity. The study concluded that polyphenolic phytochemicals in the pomegranate play an important role in the modulation of inflammatory cell signaling in colon cancer cells [67].

Tannins are considered to be non-mutagenic substances; some of them even exhibit anti-mutagenic activity. The initial step in the formation of cancer is damage to the genome of a somatic cell producing a mutation in an oncogene or a tumor-suppressor gene. For example, inhibition of mutagen methyl methansulphonate and metabolically activated carcinogen benzo[a]pyrene was described in juices and organic solvent extracts from strawberries, raspberries, and blueberries. Of prepared solvent extracts, the hydrolyzable tannin containing fraction from strawberries (Sweet Charlie cultivar) was the most effective at inhibiting mutations [68]. Another study described the effect of cocoa liquor proanthocyanidins on pyridine derivate induced mutagenesis *in vitro* and on *in vivo* carcinogenesis in female rats were investigated. Study concluded that cocoa proanthocyanidins inhibit *in vitro* mutagenicity of pyridine derivate, as well as rat pancreatic carcinogenesis in the initiation stage, but not mammary carcinogenesis induced by pyridine derivate [69].

The protective effect of tannins can be also linked to inhibition of ornithine decarboxylase (ODC), an enzyme which participates in biosynthesis of polyamines; increased polyamines expression is a marker of tumor development. Inhibition of ODC by plant metabolites was determined in several studies [70]. Proanthocyanidin-rich fraction of American cranberry (Vaccinium macrocarpon) exhibited significant *in-vitro* chemopreventive activity indicated by an ornithine decarboxylase assay [70].

Antiinflammatory Activity

Antiinflamatory activity is one of the important effects of tannins. The mechanisms of action have not been definetely solved yet due to the complex character of inflammatory processes and thereby many possibilities of their interference. Most of the studies, which dealt with anti-inflammatory activity of tannins, targeted their antioxidant activity and interference with nuclear NF-κB, which promotes transcription of many inflammatory cytokinins including e.g. IL-8, TNF-α or RANKL. The effects on the activation of extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (p38MAPK), which are upstream enzymes known to regulate COX-2 expression in many cell types, were also examined [67,71,72-74].

Topical aplication of pomegranate fruit acetone extract, contaning anthocyanins and hydrolysable tannins, to mice inhibited TPA (12-O-tetradecanoylphorbol-13-acetate) - mediated increase in skin edema and hyperplasia, epideral ODC activity and protein expression of ODC, and COX-2. The study also found that topical application of extract resulted in inhibition of TPA-induced phosphorylation of ERK and JNK, as well as activation of NF-κB. The study provided clear evidence that pomegranate extract possesses antiskin-tumor-promoting effects in CD-1 mouse [71].

Gallotannin pentagalloylglucose (PGG) inhibited *in-vitro* NF-κB activation and IL-8 production in human monocytic cells (U937) stimulated with phorbol myristate acetate or TNF-α. Furthermore, PGG prevented degradation of the NF-κB inhibitory protein I-κBα. The paper concluded that PGG can inhibit IL-8 gene expression by a mechanism involving its inhibition of NF-κB activation, which is dependent on I-κBα degradation [72].

Ellagitannin furosin, isolated from *Euphorbia helioscopia*, was examined for the effects of bone metabolism. Furosin *in-vitro* decreased the differentiation of both murine bone marrow mononuclear cells and Raw 264.7 cells into osteoclasts. Furosin targeted at the early stage of osteoclastic differentiation and had no cytotoxic effect on osteoclast precursors. The mechanism of effect was due to the inhibition of the receptor activator of NF-κB ligand (RANKL)-induced activation of p38 mitogen-activated protein kinase (p38MAPK) and c-Jun N-terminal kinase (JNK)/activating protein-1 (AP-1). Furthermore, furosin reduced resorption pit formation in osteoclasts, which was accompanied by disruption of the actin rings. The study concluded that the furosin would be the potential candidate for treatment of bone diseases [73].

The study which compared the the effect of flavonoid monomers, dimers, trimer and Pycnogenol on NO production, tumor necrosis TNF-α secretion and NF-κB activity demonstrated that procyanidins act as modulators of the immune response in macrophages. Monomers and dimers repressed NO production, TNF-α secretion and NF-κB-dependent gene expression induced by interferon γ, whereas the trimeric procyanidin C-2 and Pycnogenol enhanced these parameters. In addition, in unstimulated Raw 264.7 macrophages, both procyanidin C-2 and Pycnogenol increased TNF-α secretion in a concentration- and time-dependent manner [74]. This results show the importance of the influence of the structure and polymerization on the antiinflammatory activity of these substances.

Cardiovascular Protection

Proanthocyanidins have cardiovascular protective effect due to their antioxidant activity, inhibition of LDL oxidation, ability of vasodilation, antiplattelet activity, and protection against ischemia-repefusion injury. Antioxidant activity of proanthocyanidins and the ability to protect lipid peroxidation was described above.

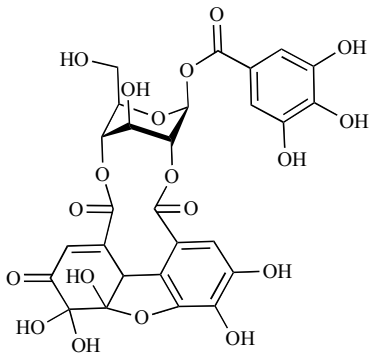

Fig. (8). Furosin, ellagitannin from *Euphorbia helioscopia.*

The ability to relax blood vessels is due to NO released from endothelial cells by the action of proanthocyanidins and subsequent increased in cyclic GMP levels in the vascular smooth muscle cells. The NO/cyclic GMP pathway is known to be involved in many cardiovascular-protective roles. *In vitro* vasodilating activity of isolated grape seeds procyanidins tended to increase with degree of polymerization, epicatechin content, and with galloylation [75].

Proanthocyanidins can inhibit the rennin-angiotensin-aldosteron system through affecting angiotensin I converting enzyme (ACE) [76], or through antagonism on angiotensin receptor [77]. Rennin-angiotensin-aldosteron system is an endocrine system which influence vascular tone, fluid and electrolyte balance and the sympathetic nervous system. The *in vitro* analysis of inhibition of ACE activity and consequently assessment of ACE activity using angiotensin-I as substrate, in both cultured HUVEC (human umbilical vein endothelial cells) and purified enzyme indicated the close relevance in structure/activity releationship of tested epicatechin, procyanidin dimer, tetramer and hexamer. Procyanidin tetramer significantly inhibit the ACE activity by cultured HUVEC, whereas dimer and hexamer caused a non-significant inhibition. When ACE activity was assayed using the isolated rabit lung enzyme, maximal ACE inhibition was exerted by tetramer and hexamer. The influence of the presence of plasma protein albumin on the activity of tested compounds was also *in vitro* determinated. The presence of albumin did not reverse the ACE inhibition by dimer and tetramer, but decreased hexamer inhibition by 65%. The study concluded that although the number of epicatechin units in procyanidin is one determinant of the specificity and extent of inhibition, the way that epicatechin units are bound should also be considered [76]. It was demonstrated *in vitro* that proanthocyanidins could inhibit angiotensin II binding to the angiotensin I receptor. Inhibitory activity increased with the degree of polymerization to a maximal activity for pentamers and hexamers [77].

The increased plattelet aggregation contributes to pathology of cardiovascular diseases. Several *in-vivo* studies described inhibition of plattelet aggregation by procyanidin rich cocoa. Consumption of flavanol-rich cocoa inhibited several measures of platelet activity including, epinephrine- and ADP-induced glycoprotein IIb/IIIa and P-Selectin expression, platelet microparticle formation, and epinephrine-collagen and ADP-collagen induced primary hemostasis [78,79]. High inhibition of thrombin-induced plattelet aggregation was exhibited by tannins isolated from leaves of *Arbutus unedo*, so called "Strawberry tree", growing in Mediterranean region, which is traditionaly used as a remedy for decrease of high blood pressure [80].

Other positive effects of tannins on cardiovascular system include their ability to decrease tissue injury induced by ischemia and reperfusion. For example, the antiarrhythmic and cytoprotective effect of an oral 3-week-pretreatment with oligomer procyanidins derived from Vitis vinifera was investigated on the isolated perfused heart after global no-flow ischemia [81].

Antimicrobial Protection

The ability of tannins to antagonise various pathogens has been recognised for a long time. Mechanisms, which facilitate inhibition of bacteria or fungi growth include: e. g. non-specific ability of tannins to bound bacterial enzymes, direct action on metabolism of pathogens through inhibition of oxidative phosphorylation, or ability to complex transitional metals ions, which are important for pathogens growth [13]. *In vitro* studies which dealt with this topic proved inhibition of many strains of bacteria including genus *Aeromanas, Bacillus, Clostridium, Enterobacter, Helicobacter, Klebsiella, Proteus, Pseudomonas, Shigella, Escherichia, Staphylococcus,* or *Streptococcus*. Out of fungi, it is possible to mention *Aspergillus, Coniophora*, or *Penicillium* [82,83].

Cranberries (*Vaccinium macrocarpon*) have been used for treatment of urinary infections caused by bacteria *Escherichia coli* for a long time. It was found that just proanthocyanidins adhere the urinary tract epithelium and protect *E. coli* adhesion. The activity of various tannins isolated from cranberries was compared, and it was found that the A-type proanthocyanidin trimers are the most active. A-type proanthocyanidin dimers possessed lower activity than trimers and substances with B-type connection and monomers didn't show any antibacterial activity [84,85].

Polymeric proanthocyanidin purified from the fruit of Zanthoxylum piperitum (spice used in Japan) decreased the minimum inhibitory concentrations of beta-lactam antibiotics for methicillin-resistant Staphylococcus aureus. *In vitro* study of the effects of the compound indicated that it suppressed the activity of beta-lactamase and largely decreased the stability of the bacterial cell membrane [86].

The mechanism of antiviral activity can be due to the linkage of tannins on protein surface of viruses or on cell membrane of host cells. Through this adsorption, penetration and eventually virus uncoating is restricted. Inhibition of enzymes, e.g. reverse transcriptase, can be also included. There are several studies, which determined inhibition of Herpes simplex virus (HSV), or human immunodeficiency virus (HIV) by various condensed and hydrolysable tannins [87,88]. For example, several chemically defined plant extracts were investigated for their antiviral action on herpes simplex virus (HSV-1, HSV-2)-infected African green monkey kidney cells and human adenocarcinoma cells. Among them, the monomeric hydrolyzable tannins, oligomeric ellagitannins and condensed tannins, having galloyl groups or hexahydroxydiphenoyl groups, had the most potent anti-

HSV activity. On the other hand, gallic acid, neutral polysaccharides, chemically modified (N,N-dimethylaminoethyl-, carboxymethyl-, and sulfated-) glucans, sialic acid-rich glycoproteins, and uronic acid-rich pine cone polysaccharide showed little or no activity. The study concluded that effect of the tannins was due to inhibition of virus adsorption to the cells [87].

A series of dimeric procyanidins and some related polyphenols were tested for *in-vitro* anti-HSV and HIV activities, radical-scavenging effects and complement-modulating properties. In general, more pronounced activities were seen with epicatechin-containing dimers for anti-HSV, anti-HIV, and radical-scavenging effects, while the presence of ortho-trihydroxyl groups in the B-ring was important in compounds exhibiting anti-HSV and radical-scavenging effects and complement classical pathway inhibition. Double interflavan linkages gave rise to interesting antiviral effects (HSV and HIV) and complement inhibition [88].

Tannins are also examined as antiprotozoal agents. For example, recent study which dealt with antileishmanial activity of 67 tannins examined macrophage activation for release of NO, TNF and interferon (IFN)-like activities. The effect of tannins on macrophage functions were further assessed by expression analysis (iNOS, IFN-alpha, IFN-gamma, TNF-alpha, IL-1, IL-10, IL-12, IL-18). The most of tannins revealed little direct toxicity for extracellular promastigote Leishmania strains. In contrast, many polyphenols appreciably reduced the survival of the intracellular, amastigote parasite form *in vitro*. Data from functional bioassays suggested that the effects of polyphenols on intracellular Leishmania parasites were due to macrophage activation rather than direct antiparasitic activity. Gene expression analyses confirmed functional data, however, *in vivo* experiments are essential to prove the therapeutic benefits of polyphenolic immunomodulators [89].

Human Intervention Studies

The most of above mentioned studies described only *in vitro* activity of tannins. These studies dealt with biological activity of tannins, but do not consider absorbtion, bioavailability and metabolism, and that is why the results of *in vitro* activities do not always correspond with *in vivo* efficiency. The studies on enzymes, tissue cultures or animals and especially human long-lasting intervention studies which evaluate modifications of many biomarkers can bring confirmation of gained results. This part of review examines the effect of proanthocyanidins demonstrated in some of human intervention studies.

Recently, review from Williamson and Manach on clinical data of polyphenols including proanthocyanidins was published [90]. Williamson and Manach mentioned nearly forty human interventions studies in which the health effects of proanthocyanidins rich food were demonstrated. It's necessary to point out that the human intervention studies unfortunately did not use pure compounds, but only proanthocyanidins rich food (extracts and juices from apples, grape seed, pomegranates, cranberries, blueberries, black currant, furthermore various types of chocolate, cocoa, red wine, or Pycnogenol®), which is due to high difficulty of preparing sufficient amount of pure compounds. Subsequently, also other active substances, except tested proanthocyanidins and their metabolic products, can be included in the activity; e.g. catechins, which occur with proanthocyanidins very often. The Williamson and Manach stated that predominant health effects of proanthocyanidins rich food are on cardiovascular system. Biomarkers affected are: increases of general plasmatic antioxidant activity, decreased plattelet aggregation, decreased lipid peroxides plasmatic concentration, decreased LDL concentration and increased HDL concentration, decreased disposition of LDL to oxidation, endothelium induced vasodilatation and decreased blood pressure, positive effect on capillary permeability and fragility, increased ascorbic acid plasmatic concentration, decreased expression of P-selectin, decreased thromboxane serum concentration, increased microvessels diameter, increased homocysteine and vitamin B6 plasmatic concentration, endothelium functions support, increased platelet subjected production of NO, inhibition of superoxide, increased α-tocopherol concentration, and decreased concentration of self antibody against oxidized LDL [90].

Increased general immunity, decreased UV sensitivity, menstrual and abdominal pain control, and decreased urinary infections relapse number are other effects caused by proanthocyanidins rich food and mentioned by the clinical review of Williamson and Manach [90].

The number of health effects determined in *in vivo* studies is often superior to results obtained with pure substances in *in vitro* tests. It can be due to the effect of potentiation of activities of individual compounds, due to the action of various metabolites formed by colon microflora, or due to the effects of previously mentioned ballast substances (e.g. catechins).

ADVERSE EFFECTS

Tannins form nonabsorbable complexes with proteins, sugars, digestive enzymes, or metal ions and decrease nutritive value of food. Therefore, it is not advisable to ingest high extent of tannins. It was proved that animals, which were fed with tannin free feed, had higher weight gain compared with those fed with tannin rich feed. Tannins influenced also vitamins utilization; tannin included in rats' diet decrease vitamin A content in liver and utilization of vitamin B_{12}. Also the absorption of iron was decreased by formation of insoluble complexes in people, who ate tannin rich sorghum [91].

Gallotannin has been shown to produce hepatic necrosis in humans and grazing animals. The breakdown of polyribosomes in mouse liver and inhibition of the incorporation of aminoacids into hepatic protein was found after subcutaneous injections of gallotannin to mice [92].

In the past, several studies have informed about possible mutagenic and carcinogenic activities of tannins [94]. For example, in Caribbean region great consumption of tannin rich food was associated with higher occurrence of esophageal cancer [93]. Nevertheless, direct association between the incidence of cancer and tannins has never been documented, and many other studies present that tannins are non-mutagenic and noncarcinogenic [12,35,92].

As it was mentioned above, one study, which evaluated anti- and prooxidation properties of gallic and ellagic acid and galottannin according to the DNA injury in mussels *Unio tumidus* cells, found that high concentrations of these substances injure DNA and showed prooxidant activity. The study cocluded that the bioactivity of gallic acid, ellagic acid and galottannin could be more complicated [44].

CONCLUSION

Tannins show various health benefit activities, especially antioxidant, antitumor, cardioprotective, antiinflammatory and antimicrobial activity. Mechanisms of activity, as well as their bioavailability have not been satisfactorily clear yet. It is not advisable to ingest large amount of tannins, until other studies aimed at finding average daily intake and possible adverse effects in higher doses of proanthocyanidins are done. However, small doses of tannin reach food can be beneficial to human health.

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ABREAVIATIONS

ABTS	=	(2,2-azinobis)3-ethyl-2,3-dihydrobenzothiazol-6-sulphonic acid) radical
ACE	=	angiotensin converting enzyme
DPPH	=	1,1-diphenyl-2-(2,4,6-trinitrophenyl)hydrazyl) radical
EGC	=	(-)-epigallocatechin
EGCG	=	(-)-epigallocatechin gallate
GSH	=	glutathion
HSV	=	herpes simplex virus
HIV	=	human immunodeficiency virus
HUVEC	=	human umbilical vein endothelial cells
IFN	=	interferon
LOX	=	lipoxygenase
LPL	=	low-density lipoprotein
NF-κB	=	nuclear factor-κB
NO	=	nitric oxide
NOS	=	nitric oxide synthase
iNOS	=	inducible nitric oxide synthase
ODC	=	ornithine decarboxylase
PGG	=	pentagalloylglucose
TNF-α	=	tumor necrosis factor-alpha
TPA	=	12-O-tetradecanoylphorbol-13-acetate

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