Proanthocyanidins in Health Care: Current and New Trends

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Abstract: Polyphenolic compounds are widely distributed in higher plants and are an integral part of the human diet. Recent interest in these substances has been stimulated by their potential health benefits, which are believed to arise mainly from their antioxidant activity. In the past years, the antioxidant activity of flavonoids has been studied in detail. An important but often overlooked group of polyphenols is that of the proanthocyanidins. Therefore, the present review is focused mainly on the antioxidant activity of proanthocyanidins and its relevancy *in vivo*. The three most important mechanisms of their antioxidant action will be discussed, i.e. free radical scavenging activity, chelation of transition metals, and inhibition of enzymes. In addition, the protective role of proanthocyanidins against lipid peroxidation and peroxynitrite, as well as their antimicrobial properties will be discussed.

To study the *in vivo* relevancy of the proanthocyanidin activities, the knowledge of their pharmacokinetic parameters is crucial. Although bioavailability and metabolism data on polyphenols in general and proanthocyanidins in particular are still largely unavailable, the first reports indicate that at least monomers and smaller oligomeric procyanidins are absorbed. There is also considerable scientific and public interest in the important role that antioxidants may play in health care, e.g. by acting as cancer chemopreventive and anti-inflammatory agents and by reducing risk of cardiovascular mortality. Each of these aspects will be discussed, with special attention to the role of proanthocyanidins on apoptosis, gene expression and transcription factors, such as NF-kappa B.

Keywords: Proanthocyanidins, polyphenols, condensed tannins, antioxidant, free radicals, bioavailability, cardiovascular diseases, cancer.

INTRODUCTION

Polyphenolic compounds are widely distributed in higher plants and are an integral part of the human diet. Recent interest in these substances has been stimulated by their potential health benefits, which are believed to arise mainly from their antioxidant activity [1, 2]. In the past years, the antioxidant activity of flavonoids has been studied in detail [3-6]. An important but often neglected group of polyphenolic compounds is that of the tannins, which can be further classified into three major groups, i.e., the hydrolyzable, the condensed, and the complex tannins. This review will focus on condensed tannins, since they are widely distributed in higher plants, while hydrolyzable and complex tannins are of limited distribution in nature.

Since the reviews of Haslam in 1996 [7] and Chung *et al*. in 1998 [8], important advances were made on the knowledge of the biological and pharmacological activities of condensed tannins. Therefore, the purpose of this review is to discuss the current and new trends on condensed tannins, with special attention to their antioxidant and antimicrobial activities and their relevancy *in vivo*.

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DEFINITION AND CLASSIFICATION OF TANNINS

Tannins are substances present in vegetable extracts that are able to convert animal skin into leather. These tanning properties are related to their ability to interact with proteins and to precipitate them. In 1957, Bate-Smith and Swain defined plant tannins as water-soluble phenolic compounds having a molecular weight between 500 and 3000 dalton [9]. In addition, they show the usual phenol reactions and can precipitate alkaloids, gelatin and other proteins. Thereafter, several tannins with higher molecular weights were isolated, so that at present, tannins are classified into three major groups on the basis of their structural characteristics: the hydrolyzable, the complex or partially hydrolyzable, and the condensed or non-hydrolyzable tannins (Fig. (**1**)) [10]. Hydrolyzable tannins are compounds containing a central core of glucose or another polyol esterified with gallic acid (**1**), also called gallotannins, or with hexahydroxydiphenic acid (HHDP) (**2**), also called ellagitannins. Oxidative coupling of two galloyl units to form an HHDP unit will convert gallotannins to their related ellagitannins. In aqueous solution, HHDP spontaneously dehydrates to its lactone form ellagic acid (**3**). Complex tannins are tannins in which a flavan-3-ol unit is bound glycosidically to a gallotannin or an ellagitannin unit. They are only partially hydrolyzable due to the C-C coupling of their flavan-3-ol unit with the glycosidic part. Condensed tannins are oligomers and polymers composed of phenolic flavan-3-ol nuclei. In contrast to hydrolyzable tannins, condensed tannins do not have a polyol nucleus and are not readily hydrolyzed.

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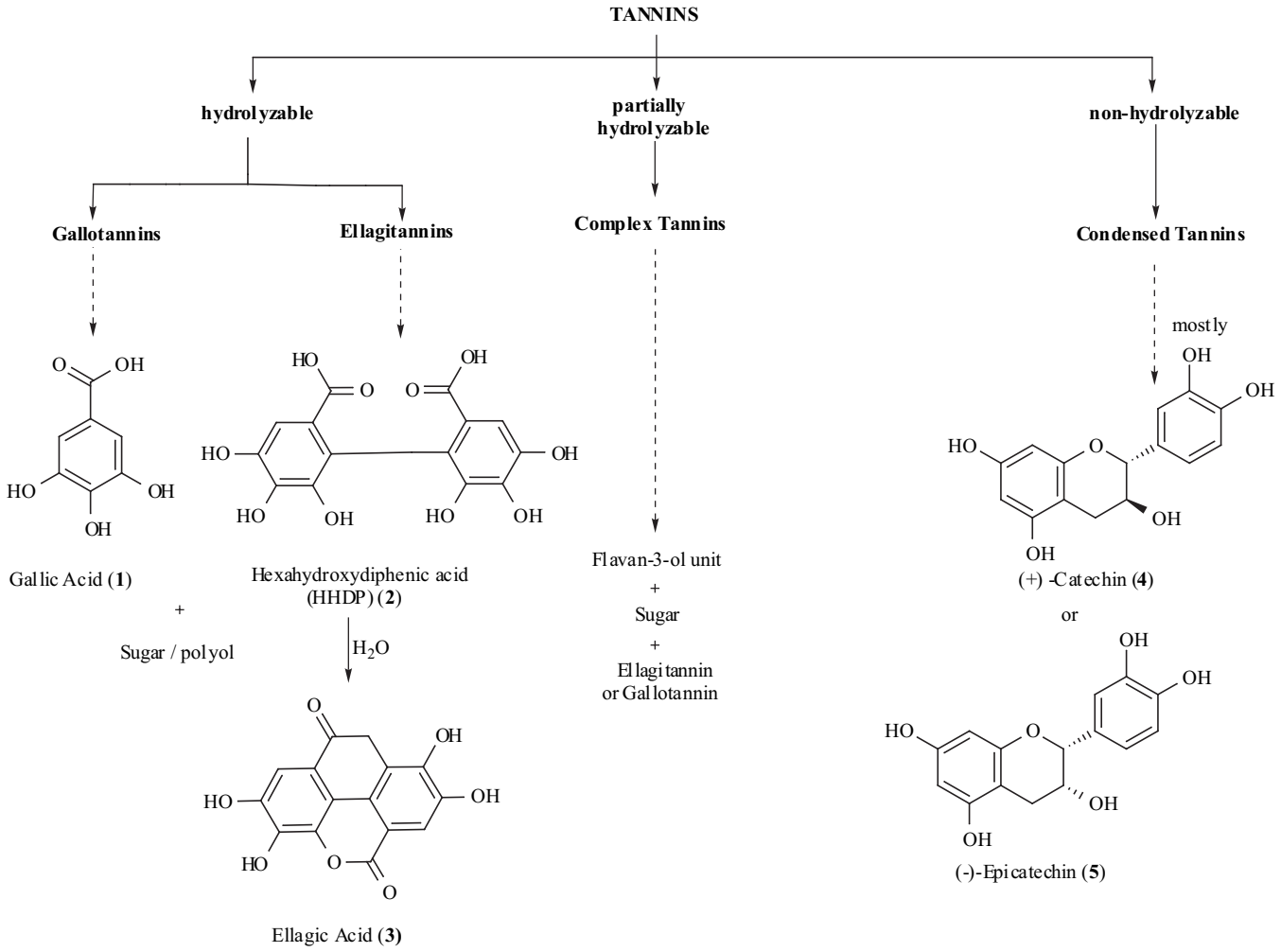


Fig. (1). Classification of tannins. A dotted line means "composed of".

However, upon heating in acidic alcohols condensed tannins produce red anthocyanidin pigments and are therefore also termed proanthocyanidins.

CHEMISTRY AND DIETARY SOURCES OF PROANTHOCYANIDINS

Proanthocyanidins consist of an electrophilic flavanyl unit, generated from a flavan-4-ol or a flavan-3,4-diol (leucoanthocyanidin), and coupled to a nucleophilic flavanyl unit, often a flavan-3-ol [11-13]. The most studied condensed tannins are based on the flavan-3-ols (+)-catechin (**4**) and (-)-epicatechin (**5**) (see Fig. (**1**)). Other important flavan-3-ols are (+)-gallocatechin (**6**), (-)-epigallocatechin (**7**), and (-)-epigallocatechin gallate (**8**) (Fig. (**2**)). The two latter compounds are major phenolic constituents of green teas.

Structural diversity is possible by variation in hydroxylation pattern, stereochemistry at the three chiral centers, and the location and type of interflavan linkage [14]. Furthermore, derivatisations as O-methylation, C- and O-glycosylation, and O-galloylation are frequently reported. Proanthocyanidins are classified according to their hydroxylation pattern into several subgroups, including procyanidins (3,5,7,3',4'-OH), prodelphinidins (3,5,7,3',4',5'-OH), propelargonidins (3,5,7,4'-OH), profisetinidins (3,7,3',4'-OH), prorobinetinidins (3,7,3',4',5'-OH), proguibourtinidins (3,7,4'-OH), proteracacinidins (3,7,8,4'-OH), and promelacacinidins (3,7,8, 3',4'-OH). Procyanidins are the most common group of naturally occurring proanthocyanidins. 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 upper unit and C-6 or C-8 of the lower unit, while proanthocyanidins of the A-type possess an additional ether linkage between C-2 of the upper unit and a 7- and/or 5-OH of the lower unit [12]. The chemical structures of procyanidins A_1 (**9**) and A_2 (**10**), B_1 to B_8 (**11** - **18**), and C_1 (**19**) and C_2 (**20**) are listed in Fig. (**3**).

Proanthocyanidins are present in plants as complex mixtures of polymers with an average degree of polymerization between 4 and 11, usually in association with their composing flavan-3-ols. Predominant food sources are red wine, tea, chocolate and fruits like grapes, apples, pears, and cranberries [15, 16]. Compositional data for proanthocyanidins are scarce due to lack of appropriate methodology suitable for reliable characterization and the absence of commercial standards. Moreover, significant variation in procyanidin content can exist within these foods, due to uneven distribution in plant tissues, seasonal

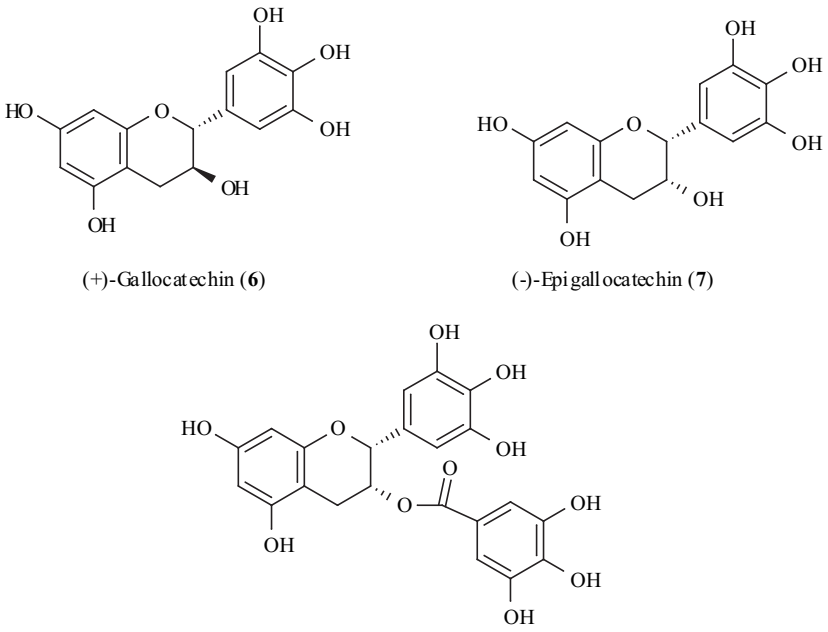


Fig. (2). Chemical structures of some flavan-3-ols.

variation and processing. Recently, Hammerstone *et al.* determined the procyanidin content in chocolate, red wine, cranberry juice and 4 different apple varieties using normal phase HPLC, which allows the resolution of the higher oligomers [16]. On average, apples and chocolate contained the largest procyanidin content per serving.

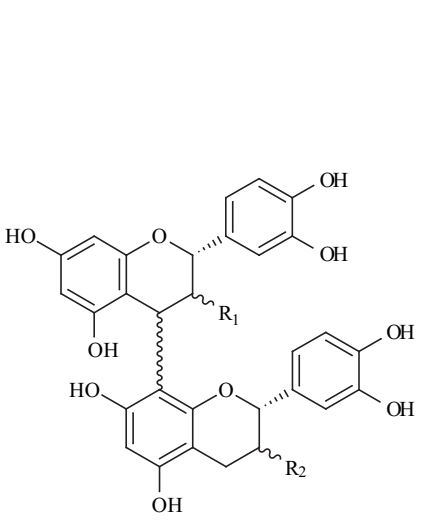
Average intake levels of total polyphenols or of some selected flavonoids have been studied in detail [17, 18], but data on proanthocyanidin intake are still lacking. However, there are indications that the intake of those flavonoids investigated (flavonols, flavones, and isoflavones) is relatively low in comparison with other polyphenols, such as phenolic acids, proanthocyanidins, anthocyanins and oxidized polyphenols [15]. Moreover, average daily intakes of those flavonoids are largely influenced by individual differences like dietary habits and preferences. The incomplete compositional information and dietary intake estimates confound the ability to infer epidemiological relationships with health and disease [16].

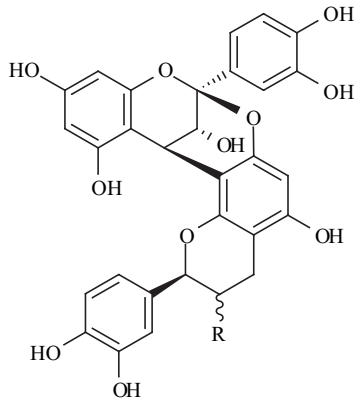
ABSORPTION, BIOAVAILABILITY, AND METABOLISM OF PROANTHOCYANIDINS

In contrast to monomeric flavonoids (see [19] for an excellent review), the number of studies on the bioavailability of proanthocyanidins is still very limited. This is not surprising, since the interest in the health benefits of proanthocyanidins is only recent and bioavailability studies are not easy, especially for such complex molecules as the proanthocyanidins of which purification of sufficient amounts is extremely difficult. Furthermore, it was assumed for a long time that proanthocyanidins are not absorbed due to their high molecular weight. Recently, in a limited number of studies the bioavailability of proanthocyanidins has been reported [20-25].

Experiments obtained from a caco-2 cell line, which is used as an *in vitro* model of the human intestinal epithelium, showed that radiolabeled procyanidin dimers and trimers are absorbed in contrast to procyanidin polymers with an average polymerization degree of 7 [20]. The dimers and the trimers were absorbed to a similar extent as (+)-catechin. One study showed that exposure of procyanidins to gastric juice resulted in a time-dependent decomposition of oligomers (trimer to hexamer) to mixtures of epicatechin monomer and dimers [23]. Furthermore, the higher the polymerization degree, the more readily the oligomers were cleaved. However, in six healthy volunteers, it was demonstrated that cocoa procyanidins are remarkably stable in the stomach environment, suggesting that most of the ingested procyanidins reach the small intestine intact [25]. Nevertheless, degradation of proanthocyanidins can also take place by the human colonic microflora, as suggested by Déprez *et al.* [22]. After 10 hours of incubation in anoxic conditions, approximately 50% of the proanthocyanidins were degraded by human colonic flora, while 48 hours incubation almost totally degraded the proanthocyanidins. Phenylacetic, phenylpropionic, and phenylvaleric acids were identified as metabolites by gas chromatography coupled to mass spectrometry [22].

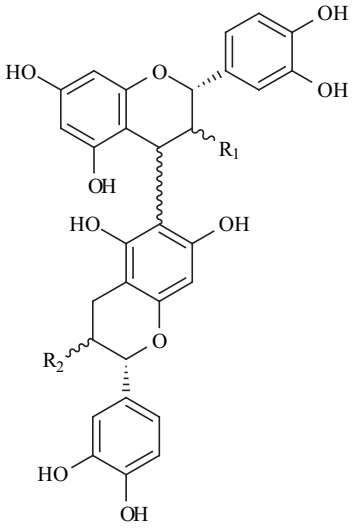
Supporting evidence for the resorption of procyanidins – either unchanged or as flavan-3-ols – is presented by Richelle *et al.* who reported a marked increase in epicatechin plasma concentration after chocolate consumption [26]. Epicatechin concentration reached its maximal level two to three hours after chocolate ingestion. According to Spencer *et al.*, epicatechin is the primary bioavailable form of the procyanidin dimers B_2 and B_5 after transfer across the small intestine [24], while Baba *et al.* demonstrated in rats that procyanidin B_2 is absorbed and excreted in urine and a portion of procyanidin B_2 is degraded to epicatechin [21].



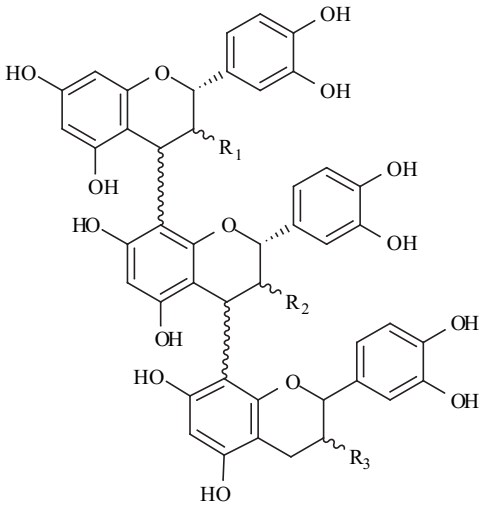


R	
····OH	Procyanidin A_1 or epicatechin-(4β⟶8,2β⟶7)-catechin (**9**)
◄ OH	Procyanidin A_2 or epicatechin-(4β⟶8,2β⟶7)-epicatechin (**10**)

R_1	R_2	interflavan bond	
····OH	◄ OH	◄	Procyanidin B_1 or epicatechin-(4β⟶8)-catechin (**11**)
····OH	····OH	◄	Procyanidin B_2 or epicatechin-(4β⟶8)-epicatechin (**12**)
◄ OH	◄ OH	·····	Procyanidin B_3 or catechin-(4α⟶8)-catechin (**13**)
◄ OH	····OH	·····	Procyanidin B_4 or catechin-(4α⟶8)-epicatechin (**14**)



R_1	R_2	interflavan bond	
····OH	····OH	◄	Procyanidin B_5 or epicatechin-(4β⟶6)-epicatechin (**15**)
◄ OH	◄ OH	·····	Procyanidin B_6 or catechin-(4α⟶6)-catechin (**16**)
····OH	◄ OH	◄	Procyanidin B_7 or epicatechin-(4β⟶6)-catechin (**17**)
◄ OH	····OH	·····	Procyanidin B_8 or catechin-(4α⟶6)-epicatechin (**18**)



R_1	R_2	R_3	interflavan bond	
····OH	····OH	····OH	◄	Procyanidin C_1 or epicatechin-(4β⟶8)-epicatechin-(4β⟶8)-epicatechin (**19**)
◄ OH	◄ OH	◄ OH	·····	Procyanidin C_2 or epicatechin-(4α⟶8)-catechin-(4α⟶8)-catechin (**20**)

Fig. (3). Chemical structures of A-, B-, and C-type proanthocyanidins.

In conclusion, the limited number of studies does not clearly prove whether proanthocyanidins are bioavailable or not. Nevertheless, these studies indicate that absorption of proanthocyanidins is limited to absorption of those with a low polymerization degree and/or of metabolites thereof formed in the colon. Moreover, metabolization and conjugation can alter biological activities dramatically. Further *in vivo* studies are urgently needed to confirm these preliminary observations.

PROANTHOCYANIDINS AS ANTIMICROBIAL COMPOUNDS

Antibacterial and Antifungal Activities

The inhibitory activity of proanthocyanidins on bacteria and fungi has been recognized for a long time. In 1991, Scalbert reviewed the antibacterial and antifungal properties of tannins, including proanthocyanidins [27]. It was concluded that tannins could inhibit the growth of these microorganisms through three major mechanisms. First, inhibition of extracellular microbial enzymes is caused by the typical astringent character of tannins. Second, tannins can exhibit a direct action on microbial metabolism. This was demonstrated for tannic acid through inhibition of oxidative phosphorylation. Third, tannins are able to complex metal ions, which are required for microbial growth.

Recently, a series of proanthocyanidins were evaluated for their activity against several gram-positive and gram-negative bacteria and against the yeasts *Candida albicans* and *Cryptococcus neoformans* [28, 29]. Both studies concluded that all proanthocyanidins tested showed only a moderate to weak antibacterial activity. Interestingly, B-type proanthocyanidins were more active than A-type proanthocyanidins against *C. albicans* and *C. neoformans*, with MIC values ranging from 250 to 1000 μg/ml [28].

Until now, the most interesting antibacterial activity of proanthocyanidins is related to their presence in cranberries (*Vaccinium macrocarpon* Ait.). A number of clinical trials have demonstrated the effectiveness of cranberry consumption in preventing urinary tract infections (UTIs) [30-32]. Although UTIs can be caused by many microorganisms, more than 85% are caused by *Escherichia coli*. The presence of P-fimbriae on *E. coli*, which are proteinaceous fibers on the bacterial cell wall, has been clearly established as a virulence factor, since they are responsible by producing adhesions for adherence to uroepithelial cells. Recently, it was demonstrated that cranberry proanthocyanidins might inhibit P-fimbriated *E. coli* from adhering to uroepithelial cells [33]. The anti-adhesion activity of cranberry juice appears to be related to the presence of proanthocyanidins with at least one A-type linkage [34, 35]. From the proanthocyanidins isolated from cranberries, A-linked proanthocyanidin trimers were more effective inhibitors of adherence than A-linked proanthocyanidin dimers, while a B-linked proanthocyanidin was not active at all [35].

Further studies are underway to determine the preventive and curative capacity of A-linked proanthocyanidins in UTIs and in other infectious diseases [36]. A recent *in vitro* study showed that a cranberry fraction is able to inhibit adhesion of three strains of *Helicobacter pylori*, indicating a potential role in preventing peptic ulcers [37]. Another study demonstrated that a cranberry fraction inhibits several oral bacteria from adherence to teeth [38]. In conclusion, it is generally accepted that the consumption of cranberries can help to prevent UTI. However, more *in vivo* studies are needed to conclude that the A-linked proanthocyanidins are responsible for this effect.

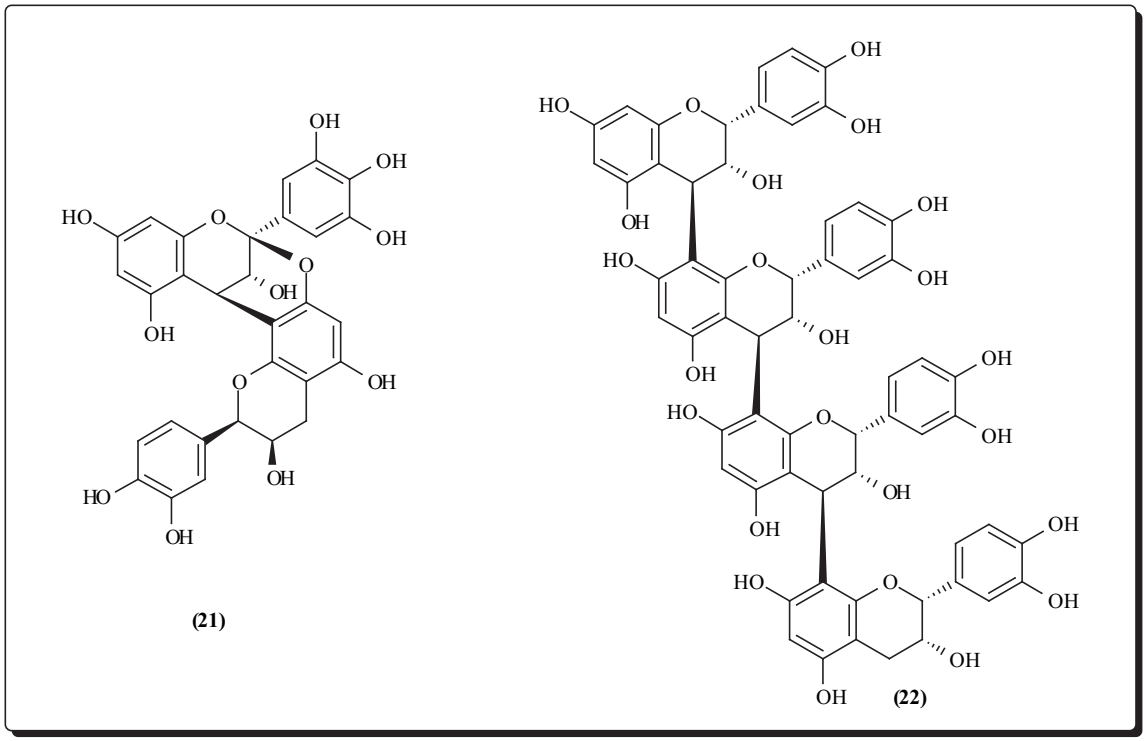
Antiviral Activity

Despite an antiviral repertoire of more than thirty drugs, the demand for new antiviral drugs is still great. First, antiviral drugs are not always efficacious or well tolerated. Second, drug-resistant strains are rapidly emerging. Consequently, further research should be focused on the development of compounds which act by other mechanisms and have fewer side effects than the currently available antiviral drugs [39]. One of the possible approaches is the antiviral screening of products from natural sources, such as fungi, marine fauna and flora, bacteria, and plants. Research on antiviral natural products is mainly focused on plants, since they can be selected on the base of their ethnomedicinal use, e.g. against infections [40].

Recently, several reviews on the antiviral activity of plant-derived compounds against human immunodeficiency virus (HIV) [39, 41-44] and herpes simplex virus (HSV) [39, 44] were published.

A recent study demonstrated that epigallocatechin-(4β→8,2β→7)-epicatechin (**21**) inhibited HIV-1 protease at 70 μg/ml, while procyanidin A_2 (**10**) was not active at concentrations up to 100 μg/ml [45]. However, in a structure-antiviral activity relationship study of a series of proanthocyanidin oligomers, it was shown that procyanidin A_2 (**10**) was the most active anti-HIV-1 compound with a Selectivity index (SI) of 24 [46]. Interestingly, procyanidin A_1 (**9**) exhibited an SI of only ten, which was still larger than the SI for the procyanidins with single linkage. In the same study, the double linked procyanidins A_1 (**9**) and A_2 (**10**), and the 4→6 coupled dimeric procyanidins B_5 (**15**) and B_6 (**16**) showed the highest anti-HSV-1 activity [46]. Although (-)-epigallocatechin had a weak antiherpetic activity, it was still better than some dimers, such as B_3 (**13**) and B_4 (**14**), indicating the importance of a pyrogallol function. Another potentiating factor was the mode of linkage, with A-series procyanidins or 4→6 linkage being preferred. Another study demonstrated that at a maximal non-toxic dose of 100 μg/ml procyanidin C_1 (**19**) and a tetrameric proanthocyanidin, called cinnamtannin A_2 (**22**), exhibited a complete HSV-1 titer reduction, which was ascribed to an extracellular antiviral activity [47].

A few years ago, some interesting results were published on a proanthocyanidin oligomer with a molecular weight of 2100 dalton, also called SP-303, and, which has been isolated from the latex of the plant *Croton lechleri* [48]. It possesses *in vitro* activity against HSV-1 and -2, including thymidine kinase deficient strains, and appeared to act through inhibition of virus penetration into cells. A topical formulation of SP-303 for the treatment of AIDS patients with recurrent genital and perianal herpetic lesions was



(21) **(22)**

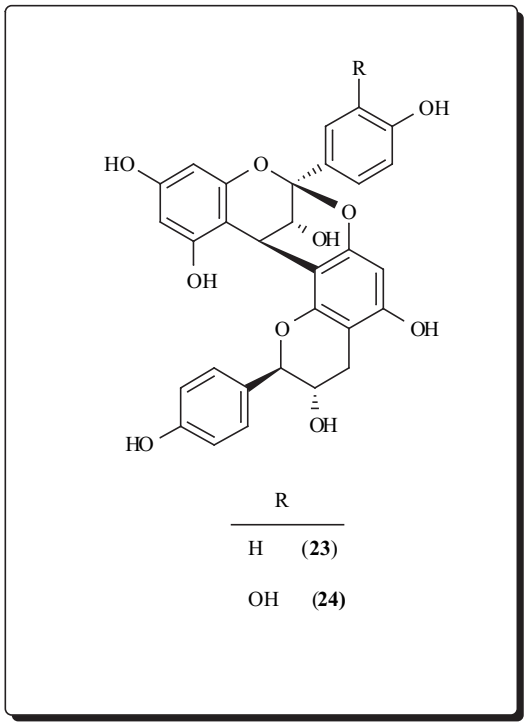
evaluated in a phase II study for safety and effectiveness [49]. However, in 1998 the company decided to suspend the development of Virend®, their topical formulation of SP-303, since the clinical results were disappointing.

Antiprotozoal Activity

Protozoa's, such as *Plasmodium* spp., *Entamoeba histolytica*, *Trypanosoma* spp., and *Leishmania* spp. are major worldwide pathogens and are among the leading causes of disease and mortality in the developing countries. Furthermore, *Giardia lamblia* is a frequent cause of diarrhea in the developing and the western countries. Unfortunately, there are only a limited number of antiprotozoal drugs available.

A small number of studies have evaluated the antiprotozoal activity of proanthocyanidins. Interesting results were obtained in a study on a series of proanthocyanidins tested against *L. donovani* amastigotes and promastigotes *in vitro* [50]. All of the proanthocyanidins tested significantly inhibited the intracellular survival of *L. donovani* amastigotes, with IC_{50} values ranging from 0.7 to 7.7 nM. The antileishmanial drugs sodium stibogluconate (Pentostam®) and amphotericin B were used as positive controls and showed an intracellular antileishmanial activity with IC_{50} values of 10.6 nM and 0.3 nM, respectively. In contrast to these positive controls, none of the proanthocyanidins were active against the extracellular form. A structure-activity relationship study indicated that 4α,8-coupled dimers were more active than their corresponding 4β,8-dimers [50]. An increase in molecular weights, galloylation of constituent units or the presence of predominantly 2,3-cis flavanyl chain extender units enhanced the antileishmanial activity.

A bioassay-guided fractionation of the antiprotozoal extract of *Geranium niveum* resulted in the isolation of two new A-type proanthocyanidins, epiafzelechin-(4β→8, 2β→7)-afzelechin or geranin A (**23**) and epicatechin-(4β→8, 2β→7)-afzelechin or geranin B (**24**). Geranins A and B showed IC_{50} values of respectively 2.4 and 6.0 μg/ml for *G. lamblia* and of respectively 184.7 and 13.6 μg/ml for *E. histolytica* [51, 52]. Metronidazole was used as a positive control and exhibited much lower IC_{50} values of 0.21 and 0.04 μg/ml for respectively *G. lamblia* and *E. histolytica*.



R	
H	(**23**)
OH	(**24**)

REACTIVE OXYGEN SPECIES, ANTIOXIDANTS AND OXIDATIVE STRESS

Reactive oxygen species (ROS) is a collective term for oxygen-derived species, namely oxygen radicals and certain non-radicals that are oxidizing agents and/or easily converted into radicals (Table 1) [53]. A free radical is defined as any species capable of independent existence and containing one or more unpaired electrons. All aerobic organisms possess an antioxidant defense system to protect against ROS-mediated injury. A broad definition of an antioxidant is "any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate" [54]. Oxidizable substrates include DNA, lipids, proteins, and carbohydrates.

Table 1. Reactive Oxygen Species or ROS

	ROS	Symbol
Radicals	Superoxide anion	$O_2^{\bullet-}$
	Hydroxyl	$HO^{\bullet}$
	Alkoxyl	$RO^{\bullet}$
	Peroxyl	$ROO^{\bullet}$
	Nitric oxide	$NO^{\bullet}$
Non-radicals	Hydrogen peroxide	H_2O_2
	Hypochlorous acid	HOCl
	Ozone	O_3
	Singlet Oxygen	$^1\Delta O_2$
	Peroxynitrite	$ONOO^-$

In healthy individuals, the production of ROS is balanced with the antioxidant defense system. Oxidative stress is defined as a disturbance in the balance in favor of the ROS, leading to potential damage [55]. The biomedical literature is full of claims that ROS and oxidative stress are involved in different human diseases. However, the role of oxidative stress in these diseases is not always the same and can be classified into three categories [56]. First, some diseases are caused by oxidative stress. For example, ionizing radiation generates $HO^{\bullet}$ by splitting water molecules and the formation of this highly reactive $HO^{\bullet}$ is recognized as one of the causes of radiation-induced carcinogenesis. Second, oxidative stress is not the cause of the disease, but contributes to the disease pathology. Two extensively studied examples are atherosclerosis and rheumatoid arthritis. Third, in most diseases oxidative stress occurs, but does not contribute to the disease pathology.

PROANTHOCYANIDINS AS ANTIOXIDANTS

Five basic mechanisms of antioxidant activity have been described: (1) free radical scavenging activity, (2) chelation of transition metals, (3) inhibition of enzymes, (4) enzyme-mimetic activity, and (5) quenching of singlet oxygen. The first three mechanisms have been attributed to proanthocyanidins and will therefore be discussed in detail. In addition, special attention will be given to the use of ESR in the study of the antioxidant activity of proanthocyanidins. Finally, the protective role of proanthocyanidins against lipid peroxidation and peroxynitrite will be discussed.

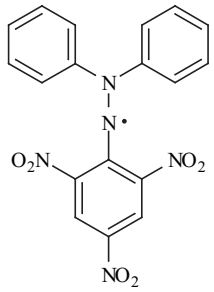
Free Radical Scavenging Activity

The basic concept of free radical scavenging activity of an antioxidant (AH) is a redox transition involving the donation of a single electron (or H-atom, equivalent to donation of an electron and an H^+) to a free radical species ($R^{\bullet}$) [57]. During the course of this electron transfer, the radical character is transferred to the antioxidant, yielding the antioxidant-derived radical ($A^{\bullet}$) (Equation 1).

$$AH + R^{\bullet} \rightarrow A^{\bullet} + RH \qquad \text{(Eq. 1)}$$

To act as an antioxidant, a free radical scavenger should produce a more stable and therefore less harmful compound after reaction with a free radical.

Free radical scavenging activity of a compound is usually determined with a relatively stable free radical. For example, in $DPPH^{\bullet}$ scavenging and Trolox Equivalent Antioxidant Capacity (TEAC) assays, respectively 1,1-diphenyl-2-picrylhydrazyl free radical ($DPPH^{\bullet}$) (**25**) and 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical cation ($ABTS^{\bullet+}$) are used. In the TEAC assay, TEAC is defined as the millimolar concentration of trolox having an antioxidant capacity equivalent to 1 mM of the test compound [58]. Despite the enormous popularity of this assay, it must be emphasized that the TEAC assay is just one particular antioxidant assay [6]. Therefore, measuring radical scavenging activity of a compound with other assays could give different activities. Furthermore, free radical scavenging activity of a compound can also be determined for unstable free radicals, such as $HO^{\bullet}$ and $O_2^{\bullet-}$.



1, 1-diphenyl-2-picrylhydrazyl free radical or $DPPH^{\bullet}$ (25)

A great number of flavan-3-ols and proanthocyanidins were investigated for their $DPPH^{\bullet}$ [59-62], $ABTS^{\bullet+}$ [63], $O_2^{\bullet-}$ [61, 64, 65], and $HO^{\bullet}$ [47] scavenging activities. In several studies, it was found that introduction of a gallic acid function at position 3 increases significantly the radical scavenging activity, while glycosylation of the position 3 decreases the scavenging ability [60, 62, 63]. These effects were demonstrated for flavan-3-ols as well as for procyanidins. It must also be emphasized that the highest

scavenging activity of all proanthocyanidins tested was found for galloylated procyanidins.

With respect to the scavenging activity of B-type procyanidins, the results are ambiguous. One study using the TEAC assay found no significant difference in activity between six B-type procyanidins [63], while in two DPPH$^{\bullet}$ assays procyanidin B_2 was more active than procyanidin B_3 and B_5 [59, 60]. In an $O_2^{\bullet-}$ scavenging assay, procyanidin B_1 and B_3 showed similar scavenging activities [64]. Consequently, further research is needed to compare free radical scavenging activity of B-type procyanidins. There is a better consensus on the degree of polymerization and scavenging activity. Polymerization up to trimers will increase free radical scavenging activity, while further polymerization will decrease scavenging activity [47, 61, 63].

The high free radical scavenging activity of grape seed extracts [66-69] and Pycnogenol® [70, 71] has been related to their relatively high content of procyanidins. Pycnogenol®, which is a water extract from the bark of the French maritime pine tree (Pinus *maritima* Lamk.), contains procyanidins up to 85% of its weight.

Free Radicals and Electron Spin Resonance (ESR)

Electron spin resonance (ESR), also called electron paramagnetic resonance (EPR), is a spectroscopic technique that is particularly useful to study free radicals, since it detects the presence of unpaired electrons. A single unpaired electron can align itself in an external magnetic field either parallel or antiparallel to that field, yielding two possible energy levels. Application of electromagnetic radiation of the proper energy will induce transitions leading to an ESR signal. The basic ESR line of an electron can "split" into two or more "hyperfine" lines as a consequence of interaction of the unpaired electron with nuclei having magnetic moments (e.g. ^1H, ^2H and ^{13}C). This hyperfine splitting, expressed as hyperfine coupling constants, can greatly enhance identification of free radicals through analysis of their ESR spectrum.

Two studies using a direct ESR technique investigated the radical species of proanthocyanidins formed during slower enzymatic catalysis or alkaline autoxidation [72] and in the presence of H_2O_2 [73]. The first study concluded that flavan-3-ol-derived antioxidants, such as proanthocyanidins, are superior antioxidants compared to flavonols [72]. Autoxidation of flavonols, such as quercetin, results in the formation of o-quinones, which are potential prooxidants. This is due to the formation of ROS, such as $O_2^{\bullet-}$ and H_2O_2, by redox cycling [74]. In contrast, flavan-3-ol o-quinones, formed from the initial semiquinone radicals, are capable of producing oligomeric compounds by nucleophilic addition reactions. Consequently, through this coupling reaction the number of hydroxyl groups and thus the scavenging activity will be retained.

In an ESR study on radicals generated from the oxidation of Pycnogenol®, it was found that these radicals are mainly derived from the procyanidin B_3 radical [73]. The latter radical showed a quite high stability, which is partially caused by a hydrogen bond between the O$^{\bullet}$ in one B-ring and an OH-group in the other B-ring.

Chelation of Transition Metals

The transition metals iron and copper are essential cofactors of several enzymes that are involved in oxygen metabolism and are usually bound to proteins, such as lactoferrin and ferritin for iron and ceruloplasmin for copper [53]. However, when these transition metals are present in free state in biological systems, they can catalyze free radical reactions. For example, iron can act as a catalyst in the generation of HO$^{\bullet}$ through the Fenton reaction (Equation 3) and the Haber-Weiss reaction (Equation 4). Agents that complex these transition metals decrease their biological effects dramatically.

$$Fe^{3+} + O_2^{\bullet-} \rightarrow Fe^{2+} + O_2 \quad \text{(Eq. 2)}$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO^{\bullet} \text{ (Fenton reaction)} \quad \text{(Eq. 3)}$$

$$O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + OH^- + HO^{\bullet} \text{ (Haber-Weiss reaction)} \quad \text{(Eq. 4)}$$

There are only a limited number of studies on metal-chelating activity of proanthocyanidins. In one study the metal-chelating capacity of proanthocyanidins are evaluated by testing the relative stability of an aluminum-proanthocyanidin complex [75]. The study concluded that the catechol function of the B-ring is very important for chelating capacity of proanthocyanidins. Furthermore, aluminum-chelating capacity increased with the degree of polymerization [75]. Another study on a procyanidin-rich grape extract showed that it has a strong sequestering ability at the following favorable stoichiometric ratios: Fe^{2+}/procyanidins 2:1 and Cu^{2+}/procyanidins 4:1 [68].

Several investigations with polyphenol-containing foods, such as tea and red wine, have demonstrated that the major types of polyphenols inhibit non-heme iron absorption [76-78]. In a study on rats, it was demonstrated that consumption of tea does not affect iron absorption, unless they are consumed together [79]. In a recent review, it was concluded that tea consumption does not influence the iron status in Western populations in which most people have adequate iron stores [80]. However, in populations of individuals with marginal iron status, there seems to be a negative association between tea consumption and iron status. In conclusion, further studies must focus on the *in vivo* relevancy of the interaction between proanthocyanidins and iron. Several questions remain. First, does this complexation of iron, and subsequently inhibition of the Fenton reaction, protect the gastrointestinal tract against oxidative damage? Second, does the intake of proanthocyanidins influence negatively the iron status of humans?

Inhibition of Enzymes

Proanthocyanidins can also exhibit antioxidant activity through inhibition of prooxidative enzymes. Recently, research has been concentrated on the inhibition of lipoxygenase [81-83]. One study described the inhibition by (-)-epicatechin and cocoa procyanidins of a mammalian reticulocyte-type 15-lipoxygenase, which is an important catalyst of enzymatic lipid peroxidation of biomembranes and plasma lipoproteins [81]. Inhibitory activities decreased

from monomer reaching a minimum at trimers and tetramers and increased again up to decamers. Moreover, (-)-epicatechin and a procyanidin decamer inhibited recombinant human platelet 12-lipoxygenase. In another study, it was demonstrated that procyanidin dimers, and to a lesser extent, trimers, tetramers and pentamers inhibited recombinant human 5-lipoxygenase [82]. In contrast to the former study, hexamers and higher molecular procyanidins were almost inactive.

Endogenously derived $NO^{\bullet}$ is synthesized from L-arginine by nitric oxide synthases (NOS), such as neuronal NOS (nNOS). In a recent study on a series of hop proanthocyanidins, it was found that procyanidin B_2 and B_4 and (-)-epigallocatechin gallate were the most active inhibitors of nNOS, while procyanidin B_3, (+)-catechin, and (-)-epicatechin were inactive [84]. Consequently, dimeric procyanidins having epicatechin as terminal flavan-3-ol unit are stronger inhibitors of nNOS activity than dimers in which catechin represents the terminal unit.

The procyanidin-rich extract Pycnogenol® was able to inhibit the activities of xanthine oxidase, horseradish peroxidase, and lipoxygenase, while it did not affect the activities of glucose oxidase and ascorbate oxidase [83].

Inhibition of Lipid Peroxidation

ROS-initiated autooxidation of polyunsaturated fatty acids, also called lipid peroxidation, is a common mechanism in pathological conditions with oxidative stress. Although extensive investigations have been carried out in the field of lipid peroxidation, it is not yet clear whether lipid peroxidation is the cause or the result of several pathological conditions in humans. However, there is considerable evidence that lipid peroxidation is involved in oxidative modification of low-density lipoproteins (LDL) and that this ultimately leads to the formation of atherosclerotic lesions [85]. Lipid peroxidation also plays an important role in conditions involving prematurely born babies, since they are often exposed to lipid peroxidation-generating conditions (lower antioxidant concentrations and hyperoxia) [86]. Due to its biological significance, inhibition of lipid peroxidation should be one of the first tests to determine the antioxidant activity of a compound.

There is no unambiguous structure-activity relationship of flavan-3-ols and proanthocyanidins as inhibitors of lipid peroxidation [4, 47, 59, 63, 64, 87]. In all these studies lipid peroxidation was initiated through different mechanisms, which could explain the diverse results. Nevertheless, it can be concluded that proanthocyanidins are good inhibitors of lipid peroxidation, with similar or higher inhibitory activities than the standard antioxidants trolox and vitamin E.

Recently, antioxidant activity of proanthocyanidins on oxidation of human LDL was investigated [88]. Using copper ions, which are known to induce formation of radicals in the aqueous phase, antioxidant activity decreased in the following order: cinnamtannin A2 (**22**) ~ procyanidin C1 > procyanidin B2 > (+)-catechin > (-)-epicatechin. Similar results were obtained by the use of a lipid-soluble azo-initiator. It was concluded that the number of hydroxyl groups or the degree of polymerization is related to the antioxidant activity.

Protection Against Peroxynitrite ($ONOO^-$)

$ONOO^-$ is a strong oxidizing species, which is formed from nitric oxide ($NO^{\bullet}$) and $O_2^{\bullet-}$ by the following reaction (Equation 5):

$$NO^{\bullet} + O_2^{\bullet-} \rightarrow ONOO^- \quad \text{(Eq. 5)}$$

The reaction is extremely fast and proceeds with a rate constant of $k = 6.7 \times 10^9\ M^{-1}\ sec^{-1}$, indicating that $ONOO^-$ formation is only diffusion-limited [89]. Excess amounts of $NO^{\bullet}$ and $ONOO^-$ may be implicated in the pathophysiology of neurological disorders, such as stroke, Parkinson's disease, and Huntington's disease [90].

In two studies, a number of procyanidin oligomers were isolated from *Theobroma cacao* L. and were tested for their ability to protect against $ONOO^-$-dependent oxidation of dihydrorhodamine 123 and nitration of tyrosine [91, 92]. By molarity, procyanidin oligomers were more effective than (-)-epicatechin. From all oligomers tested (monomer through nonamer), the tetramer showed the highest protecting activity against oxidation and nitration reactions [91]. It was suggested that procyanidins do not react directly with $ONOO^-$, but most likely react with oxidizing/nitrating intermediates [92]. In a study on Pycnogenol®, it was shown that Pycnogenol® could protect endothelial cells from $ONOO^-$-induced α-tocopherol loss [93]. It was therefore suggested that procyanidins present in Pycnogenol® participate in the cellular antioxidant network.

PROANTHOCYANIDINS AS ANTI-CANCER AGENTS

Cell death can follow different pathways, including apoptosis and necrosis. The latter is characterized by cell swelling and rupture of the plasma membrane, with the release of cellular content. This can cause further tissue damage. Apoptosis is a form of programmed cell death that is characterized by cell shrinkage, chromatin condensation, internucleosomal DNA fragmentation, and formation of apoptotic bodies [94]. In contrast to necrosis, apoptosis minimizes the leakage of cellular constituents from dying cells. The apoptotic death process is regulated by several genes, some of which promote (Bax, Bcl-xs, c-myc, p53, c-fos) and other inhibit (Bcl-2, Bcl-x_L) apoptosis. Improper regulation of apoptosis contributes to disorders, such as cancer, viral infection, and stroke [95]. Studies have also shown that several cancer chemopreventive agents may induce apoptosis, whereas several tumor-promoting agents inhibit apoptosis.

Oxidative stress is involved in the activation of transcription factors and the triggering of apoptosis [96, 97]. These alterations may result in the initiation of apoptosis signaling leading to cell death, or to the activation of several proto-oncogenes and/or the inactivation of some tumor suppressor genes. Consequently, oxidative stress has been implicated in both apoptosis and the pathogenesis of cancers, and thus, antioxidants, including

proanthocyanidins, may be useful as cancer chemopreventive and/or anticarcinogenic agents.

In a comparative study of different tannins, it was found that apoptosis-inducing activity is generally higher for hydrolyzable tannins than for proanthocyanidins [98]. Nevertheless, an increasing number of studies have been published on apoptotic-modulating and anticarcinogenic properties of flavan-3-ols and proanthocyanidins. Two studies on (-)-epigallocatechin-3-gallate showed that it induces apoptosis in cancer cells, but not in normal cells [99, 100]. (-)-Epigallocatechin-3-gallate enhanced serum-induced expression of c-fos and c-myc genes in SV40 virally transformed human fibroblasts, but not in normal human fibroblasts [100]. Recent studies have demonstrated dose- and time-dependent increases in production of free radicals, DNA damage, lipid peroxidation, and apoptotic cell death of human oral keratinocytes after treatment with a smokeless tobacco extract (STE) [101]. Grape seed extract showed a higher protection than vitamins C and E against STE-induced oxidative stress and apoptosis. Moreover, an increased Bcl-2 mRNA expression was observed when cells were preincubated with grape seed extract before STE treatment [102]. Preincubation of cells with grape seed extract decreased p53 expression [102, 103]. It was suggested that the chemoprotection of grape seed extract against STE-induced cellular injury, which is associated with an increased risk of oral cancers, might involve modulation of the cellular regulatory genes Bcl-2 and p53. Grape seed extract also increased Bcl-x_L expression in hepatic tissue and prevented acetaminophen-induced lethality, DNA fragmentation and apoptotic cell death in mouse liver tissue [104].

In several studies, an inhibition of 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced mouse skin tumor promotion was demonstrated for proanthocyanidin-rich extracts [105-108]. These protective effects of proanthocyanidins were linked to their antioxidant activity [105] and their ornithine decarboxylase (ODC) inhibitory activity [106-108]. The latter was also shown for procyanidin-enriched extracts on human colonic cancer cells [109]. ODC is a rate-limiting enzyme in the biosynthesis of polyamines and a high expression of ODC is an important characteristic of tumor cells and tumor development. Procyanidins with different degrees of polymerization were tested for their ODC inhibitory activity and activity decreased in the following order: trimer (procyanidin C_1) > dimer (procyanidins B_1, B_2, and B_4) > monomer ((+)-catechin and (-)-epicatechin) [107].

The effects of cacao liquor proanthocyanidins on 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine-induced mutagenesis *in vitro* and on *in vivo* carcinogenesis in female Sprague-Dawley rats were investigated [110]. In the Ames assay, proanthocyanidins showed strong antimutagenic effects when assayed in the presence of S-9 mixture. They also inhibited significantly rat pancreatic carcinogenesis in the initiation stage, but not mammary carcinogenesis.

In conclusion, a great number of studies indicate that proanthocyanidins and flavan-3-ols may have interesting cancer chemopreventive properties through different mechanisms of action, including apoptotic-modulating, antioxidant, antimutagenic, and ODC inhibitory activities. (-)-Epigallocatechin-3-gallate and proanthocyanidins do not exhibit a well-defined mechanism of anticarcinogenic action, and thus at present, most of the research should be focused on their cancer chemopreventive properties.

PROANTHOCYANIDINS AS ANTI-INFLAMMATORY AGENTS

The transcription factor nuclear factor-κB (NF-κB) is an important regulator of gene expression and promotes transcription of cytokines, such as interleukin (IL)-1, IL-2, IL-6, IL-8, tumor necrosis factor (TNF)-α, cell adhesion molecules such as E-selectin, intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1. NF-κB is mainly an inducer of inflammatory cytokines and has been shown to play a pivotal role in the mammalian innate immune response and chronic inflammatory conditions, such as rheumatoid arthritis [111]. Thus, NF-κB is a suitable target to prevent or to reduce inflammatory response.

The effect of (-)-epigallocatechin gallate on lipopolysaccharide (LPS)-induced TNF-α production and lethality in a murine model was investigated [112]. (-)-Epigallocatechin gallate inhibited LPS-induced TNF-α mRNA expression and NF-κB-binding activity in a macrophage cell line. In a male BALB/c mice model, oral intake of 0.5 g green tea polyphenols/kg body weight reduced serum TNF-α by 80% compared to the control group, while LPS-induced lethality was completely inhibited. It was concluded that the mechanism of anti-inflammatory action of (-)-epigallocatechin gallate is partly mediated through down regulation of TNF-α gene expression by blocking NF-κB activation.

In interferon (IFN)-γ-stimulated macrophages, (+)-catechin, (-)-epicatechin, procyanidins B_1 and B_2 decreased, while procyanidin C_2 (**20**) and Pycnogenol® increased NF-κB-dependent gene expression [113]. Similar results were obtained for $NO^{\bullet}$ production and TNF-α secretion. These results clearly demonstrate the selective activity of procyanidins on NF-κB-dependent gene expression, indicating also the importance of degree of polymerization in determining their activity. Interestingly, in another study Pycnogenol® inhibited UV-induced NF-κB-dependent gene expression, but NF-κB-DNA-binding activity was not prevented [114]. Moreover, oral administration of Pycnogenol® to human volunteers reduced significantly UV-induced erythema in the skin.

In a recent study, a grape seed extract down regulated TNF-α-induced VCAM-1 expression, but not ICAM-1 expression in human umbilical vein endothelial cells [115]. In contrast to other studies, inhibition of inducible VCAM-1 gene expression by the grape seed extract was not through an NF-κB-dependent pathway. Two studies demonstrated that procyanidins, but not the monomers, suppress phytohemagglutinin-stimulated human peripheral blood mononuclear cells proliferation [116, 117].

It can be concluded that proanthocyanidins exhibit interesting anti-inflammatory properties. Until now, most of the research is focused on their modulation of the NF-κB-dependent gene expression and their antioxidant activity (cfr.

supra). However, one must keep in mind that the inflammatory process is a very complex process, and thus, other mechanisms of anti-inflammatory action must also be studied for proanthocyanidins.

PROANTHOCYANIDINS AND CARDIOVASCULAR DISEASES

Proanthocyanidins exhibit cardioprotective properties through different mechanisms of action, including inhibition of LDL oxidation, endothelium-dependent relaxation of blood vessels, inhibition of platelet aggregation and thrombosis, and protection against ischemia-reperfusion injury [118]. In this review each mechanism of action will be discussed.

Inhibition of LDL Oxidation

It is now generally accepted that oxidative stress plays an important role in the development of atherosclerosis, as stated in the so-called "oxidation theory": Oxidized LDL is rapidly taken up by macrophages, converting the macrophages into foam cells that serve as precursors of fibrous plaques [119]. As discussed previously, proanthocyanidins are able to inhibit LDL oxidation *in vitro*. Human studies on the intake of proanthocyanidin-rich diets, such as red wine [120] and chocolate [121], have demonstrated that the blood taken from the volunteers is more resistant to LDL oxidation. Further studies must determine the *in vivo* significance of proanthocyanidins on LDL oxidation.

Endothelium-Dependent Relaxation (EDR) of Blood Vessels

$NO^{\bullet}$, termed as endothelium-derived relaxing factor, is synthesized from the amino acid L-arginine by the action of NOS. Besides its vasodilatating effect on vascular endothelium, $NO^{\bullet}$ may protect LDL from oxidation, inhibit platelet aggregation, and decrease adhesion of leukocytes to the endothelium [118].

A great number of *in vitro* studies have demonstrated for procyanidins an interesting EDR activity in blood vessels [122-127]. The activity of the procyanidins increased with polymerization degree, epicatechin content, and number of galloyl units [122, 123]. Vasodilatation was due to stimulation of $NO^{\bullet}$ production by the endothelial cells, because addition of an NOS inhibitor reversed the relaxation caused by the procyanidins [125, 126].

The renin-angiotensin system (RAS) is a bioenzymatic cascade that plays an important role in cardiovascular homoeostatis by influencing vascular tone, fluid and electrolyte balance and the sympathetic nervous system (Figure **4**) [128]. The biological actions of the RAS are mediated primarily by the highly active peptide angiotensin II (Ang II). The RAS is a circulating endocrine system, whereby renin released from the juxtaglomerular cells of the kidney cleaves the liver-derived macroglobulin precursor angiotensinogen, to produce the inactive decapeptide angiotensin I (Ang I), which is then converted into the active octapeptide Ang II by angiotensin converting enzyme (ACE) within the pulmonary circulation. The common points of potential therapeutic intervention are the inhibition of ACE and the angiotensin receptor antagonists. Both mechanisms of action have been described for proanthocyanidins [129-132]. The ACE inhibitory activity of procyanidins increased with the polymerization degree. The trimeric procyanidin C_1 was almost twice as active as the dimeric procyanidin B_2 [129]. Recently, our research group demonstrated *in vitro* that proanthocyanidins could inhibit Ang II binding to the AT_1 receptor [132]. Inhibitory activity increased with the degree of polymerization to a maximal activity for pentamers and hexamers.

Inhibition of Platelet Aggregation and Thrombosis

Platelets are essential for hemostasis by forming a plug that temporarily seals the break in the vessel wall. A healthy endothelium secretes $NO^{\bullet}$ and prostacyclin, which prevents platelet adhesion. However, when endothelium is damaged and underlying collagen fibers are exposed, platelets become activated and adhere to the endothelium. Therefore, platelets contribute to atherosclerosis by incorporation into the developing lesion.

Several human studies investigated the inhibitory activity of procyanidin-rich cocoa on platelet activation [133-136]. Procyanidin-rich cocoa suppressed ADP- or epinephrine-stimulated platelet activation and platelet microformation [133-135]. In addition, a high-procyanidin chocolate diet increased plasma prostacyclin and decreased plasma leukotrienes, indicating that procyanidins can favorably alter eicosanoid synthesis in humans [136].

Protection Against Ischemia-Reperfusion Injury

ROS play an important role in pathogenesis of ischemia-induced tissue injury. Early restitution of blood flow to ischemic tissues is essential to halt the progression of cellular injury associated with decreased oxygen and nutrient delivery. However, reperfusion of ischemic tissues initiates a complex series of reactions that injures tissues.

Some studies showed a reduction in ischemia-reperfusion damage when rats were supplemented for three weeks with procyanidins from grape seeds [137-139] The animals were sacrificed, the hearts were excised and then typical reperfusion experiments were performed. One study suggested that the cardioprotective properties of grape seeds might be partially attributed to its ability to inhibit the proapoptotic factors JNK-1 and c-Jun [137].

CONCLUDING REMARKS

According to *in vitro* studies discussed above, it can be concluded that proanthocyanidins act as antioxidants through different mechanisms. Proanthocyanidins have the advantage over the flavonols quercetin and myricetin that they do not act as potential pro-oxidants as shown by ESR autooxidation studies. However, convincing studies on the *in vivo* antioxidant activity of proanthocyanidins are still lacking, since purification of sufficient amounts is extremely

difficult. Therefore, most *in vivo* studies, including bioavailability studies, are using proanthocyanidin-rich extracts, such as cocoa, grape seeds, and Pycnogenol®. It is still not clear whether proanthocyanidins are bioavailable or not, but a limited number of studies indicate that absorption of proanthocyanidins is restricted to absorption of those with low polymerization degree and/or of metabolites thereof formed in the colon. Consequently, further *in vivo* studies on their bioavailability are urgently needed.

Besides their antioxidant activity, several proanthocyanidins exhibit other biological and pharmacological activities, such as anti-inflammatory, cancer chemopreventive, and antimicrobial activities, so that these

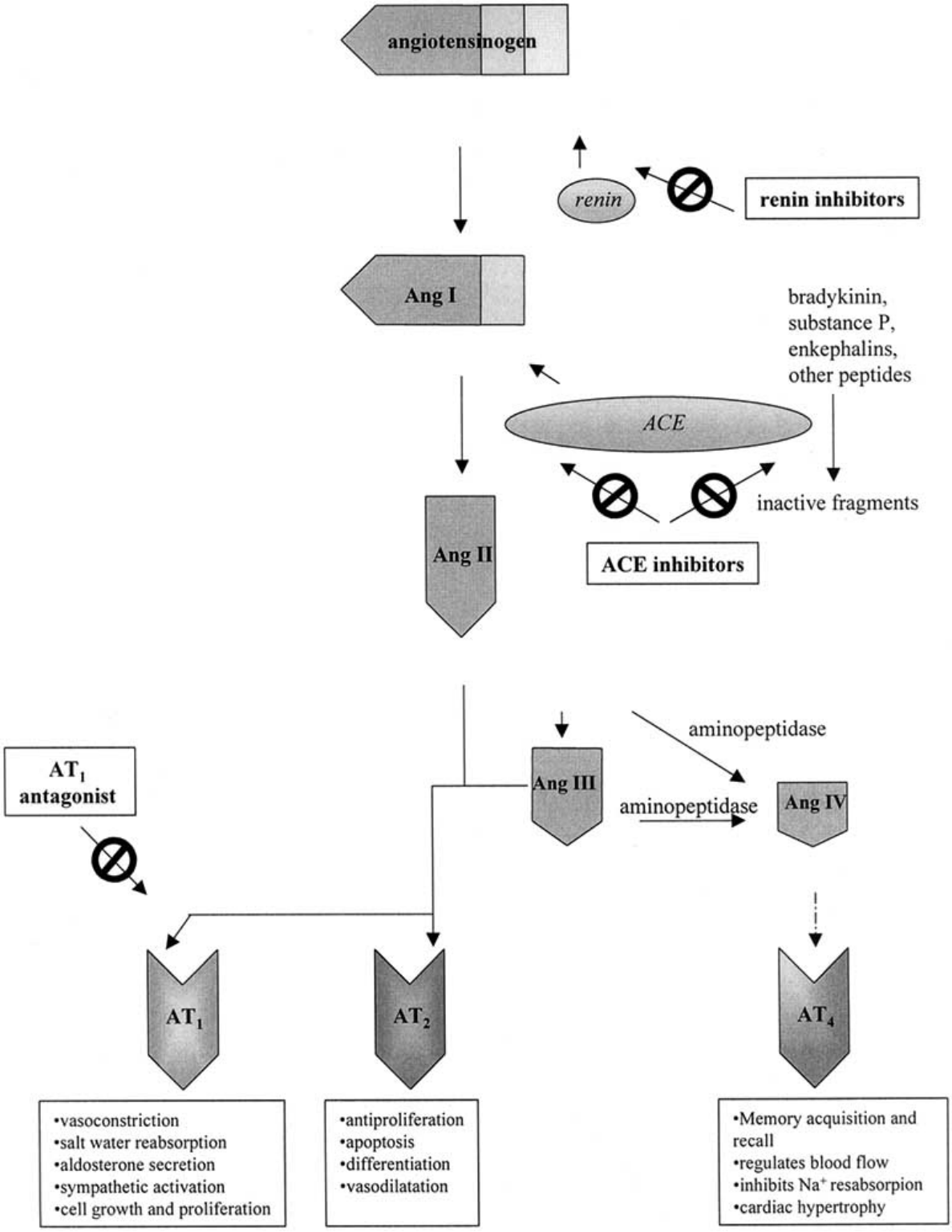


Fig. (4). Bioenzymatic cascade of the renin-angiotensin system [140].

molecules could be very useful in the future in the treatment of some diseases due to their combined activities.

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ABBREVIATIONS

$A^{\bullet}$	=	Antioxidant-derived radical
ACE	=	Angiotensin converting enzyme
Ang I	=	Angiotensin I
Ang II	=	Angiotensin II
EPR	=	Electron paramagnetic resonance
ESR	=	Electron spin resonance
HIV	=	Human immunodeficiency virus
HSV	=	Herpes simplex virus
ICAM	=	Intercellular cell adhesion molecule
IFN	=	Interferon
IL	=	Interleukin
LDL	=	Low-density lipoprotein
NF-κB	=	Nuclear factor-κB
NOS	=	Nitric oxide synthase
nNOS	=	neuronal nitric oxide synthase
ODC	=	Ornithine decarboxylase
RAS	=	Renin-angiotensin system
SI	=	Selectivity index
TEAC	=	Trolox equivalent antioxidant capacity
TI	=	Therapeutic index
TNF	=	Tumor necrosis factor
UTI	=	Urinary tract infection
VCAM	=	Vascular cell adhesion molecule

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