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Antioxidant protection of low density lipoprotein by procyanidins: structure/activity relationships

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Abstract

The antioxidant activity of catechins and oligomeric procyanidins against low density lipoproteins peroxidation was studied by means of three distinct methods: *cis*-parinaric acid fluorescence decay, conjugated-dienes detection, and oxygen consumption. A relationship between the radical trapping efficiency of procyanidins and their structure was investigated. The results indicated that: (i) interflavan linkage type (C4–C6 or C4–C8) exerts a significant effect upon radical-trapping antioxidant activity of procyanidins. It is suggested that the conformation adopted by each procyanidin in aqueous solution influence their hydrophilic character, hence affecting their interaction with the peroxyl radicals present in aqueous phase and those in LDL particle (lipidic nature); (ii) antioxidant activity increase with the degree of polymerization for the compounds with (−)-epicatechin (epi) as structural unit (epi, dimer B2 (epi–epi) and trimer C1 (epi–epi–epi)); (iii) galloylation increases antioxidant activity of procyanidins, specially in the case of B2-3′′-*O*-gallate dimer, which revealed the maximal trapping efficiency.

Keywords: Procyanidins; Antioxidant; Low density lipoproteins (LDL); *cis*-Parinaric acid; Conjugated dienes

1. Introduction

A high consumption of dietary phenolic compounds has been consistently correlated with reduction of coronary heart disease [19]. These phenolic compounds were shown to exhibit a powerful antioxidant activity in several lipid systems and in particular against oxidation of low density lipoproteins (LDL), an event that is thought to play a crucial role in the development of atherosclerotic lesion [7,12,21,34,35].

Procyanidins, or condensed tannins, are a complex family of polyphenol polymers widespread in nature that occur in processed products such as red wine, green and black tea, and fruit juices. These polyphenols are made of monomeric flavan-3-ol units ((+)-catechin and/or (−)-epicatechin) and have a 3,4-dihydroxy substitution on the B-ring (catechol unit). In the condensed forms (dimers, trimers), monomeric units are linked by an interflavan linkage established between the carbon C4 of the upper unit and the carbon C8 or C6 of the lower unit (Fig. 1). Procyanidins are powerful antioxidant agents since the correspondent oxidized forms acquire additional stabilization due to the extensive electron delocalization induced by the catechol unit on the aromatic B-ring [31]. Beside, the several *o*-dihydroxy phenolic groups in such high molecular weight structure give to the procyanidins a high capacity to complex metal ions (Fe(III), Cu(II), Al(III)) and proteins. These are the major features responsible for the nutritional and biological interest of procyanidins [7,26,30,32,33]. Several biological effects are attributed to procyanidins: antibacterial, antiviral, anticarcinogenic, antiinflammation, antiallergic, and also a vasodilating action in the organism [20,24,32]. However, a special interest has been devoted to its antioxidant activity since procyanidins can exert a protective action in the organism, against protein and lipid oxidation, reducing

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Abbreviations: LDL, low density lipoproteins; PnA, *cis*-parinaric acid; AAPH, 2,2′-azobis(2-amidinopropane hydrochloride).

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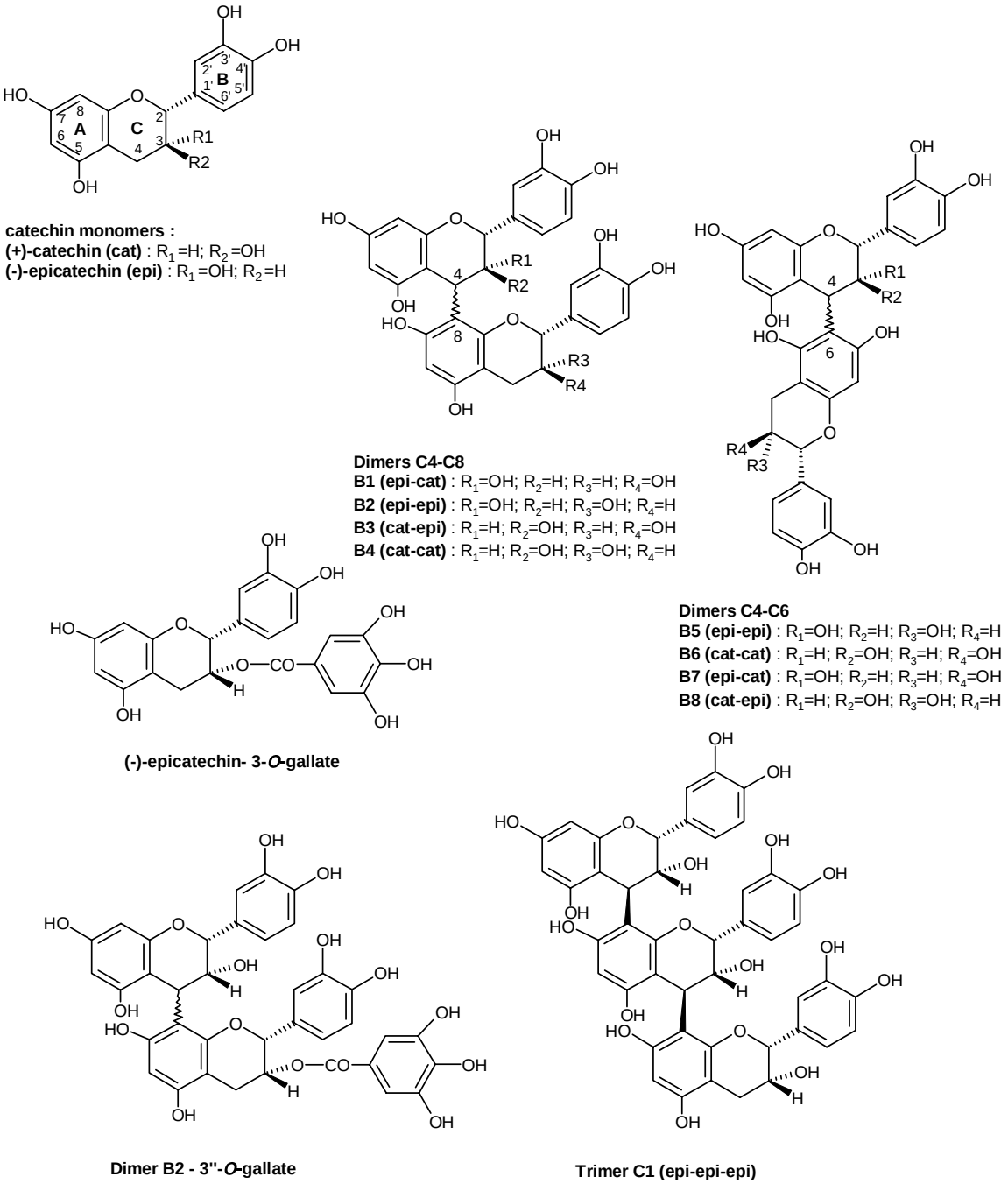

Fig. 1. Structure of catechins and oligomeric procyanidins.

the risk of chronic diseases such as coronary heart disease [2,6,11–14,16,19,20] and certain types of cancer [2,3], which are major causes of mortality in the world.

The mechanisms proposed to explain the potential antioxidant effect of polyphenols relies on thermodynamic and kinetic parameters and structural features in polyphenol structure that maximize antioxidant activity have been identified [5]. However, the potential for polyphenols to behave as effective antioxidants in lipid systems, such as the LDL, appears to be also dictated by location of the phenol at the lipid–water interface. This has been critically suggested for caffeic acid, against LDL oxidation [11,24]. At the LDL/water interface, the antioxidant activities of caffeic acid may include the modification of binding sites for transition metals in LDL and, also, the regeneration of α-tocopherol in the lipoprotein particles [23,29].

The *in vitro* antioxidant activity of many polyphenols has been largely studied using a great deal of different techniques such as EPR [24], fluorescence [20,22,23], and absorption spectroscopy [1,4,17,29,35]. Nevertheless, the influence of the procyanidin structure in determining the

antioxidant activity as well as the mechanistic details of their action require further studies.

The purpose of this work was to investigate the influence of different structural factors of 3-flavanols, such as catechin structure units ((+)-catechin and (−)-epicatechin), interflavan linkage, gallic acid esterification and degree of polymerization, in their relative antioxidant activity upon *in vitro* LDL oxidation.

2. Material and methods

2.1. Isolation of flavanols

(+)-Catechin and (−)-epicatechin were purchased from Aldrich®. Procyanidin dimers B1, B2, B5, and B7 were synthesized as previously described [18,27]. The remaining procyanidins B3, B4, B6, B8, trimer C1, and esters epi-3-*O*-gallate and B2-3′′-*O*-gallate were extracted from grape seeds from *Vitis vinifera* species. Grape seeds (*V. vinifera*) were extracted with 80% aqueous methanol by contact under stirring during 24 hr, in the dark at room temperature, and in the presence of sodium metabisulfite (0.5 g/L) to avoid oxidation. The resulting solution containing the flavanol compounds was filtered and the methanol was removed by a rotatory evaporator under reduced pressure at 30°. Flavanols were extracted from the resulting aqueous solution with ethyl acetate. The oligomeric fraction of procyanidins was isolated by selective precipitation with hexane according to the procedure described in the literature [28].

Flavanol extracts (from synthesis and grape seeds) were applied on a 250 mm × 16 mm i.d. TSK Toyopearl HW-40(s) gel column (Tosoh®) using methanol as eluant at 0.8 mL/min to give different fractions containing catechin monomers and procyanidin dimers, according to a procedure described before [9]. Afterwards, individual flavanol compounds were isolated by HPLC semipreparative (Merck-Hitachi® L-7100) on a 250 mm × 4.6 mm i.d. reversed-phase C18 column (Merck®). The elution system consisted of two solvents, A: 2.5% HOAc in H_2O, B: 80% CH_3CN in A and the following gradients; elution starting with 7% B in A isocratic for 5 min; 7–20% B in A, 5–90 min; 20–100% B in A, 90–95 min; 100% B, 95–100 min (isocratic); followed by washing (100% B over 10 min) and reconditioning of the column (100–7% B in A over 5 min). The analysis was carried out at room temperature at 1 mL/min. Detection was conducted with a diode-array detector (Merck-Hitachi® L-7450A) at 280 nm.

The purity of the obtained compounds was assessed by HPLC-DAD analysis by comparison with standards previously determined on basis of NMR and mass spectrometry studies [9,10]. The retention times (R_t, min) of the studied flavanols were the following: (+)-catechin, 29.5; (−)-epicatechin, 48.6; (−)-epicatechin-3-*O*-gallate, 74.7; dimers B1, 21.8; B2, 37.7; B3, 23.4; B4, 32.2; B5, 83.7; B6, 39.9; B7, 59.3; B8, 47.0; B2-3′′-*O*-gallate, 52.5; trimer C1, 50.8.

Individual procyanidins were kept at −20° under argon atmosphere until further analysis.

2.2. Preparation of LDL

Blood was collected by venepuncture into heparinized tubes from a human volunteer. Plasma was recovered by centrifugation at 3000 *g* for 15 min at 15°. LDL were isolated from fresh plasma by differential density ultracentrifugation, at 4°/65,000 rpm, in a Beckman L8-70 ultracentrifuge equipped with a 50.2 Ti rotor, as described elsewhere [36]. LDL were concentrated and dialyzed by vacuum filtration through a collodium bag (sartorius cellulose nitrate ultra filter of 12,000) for 3 hr at 4° in the dark against phosphate buffer (110 mM NaCl, 20 mM phosphate, pH 7.4). LDL solution obtained was filtered through a 0.22 μm pore size filter and stored at −70°, under argon atmosphere until its use. The LDL protein content was determined by Lowry method using bovine serum albumin as standard [25]. This rapid isolation and concentration procedure allows to obtain the LDL fraction containing high amounts of endogenous Vitamin E and very low level of oxidation, thereby suitable for oxidation/antioxidation studies [23].

2.3. Measurement of parinaric acid fluorescence

cis-Parinaric acid (PnA) fluorescence was monitored in a Perkin-Elmer LS 50 spectrofluorometer provided with a thermostated cuvette containing a magnetic stirring device. The excitation and emission wavelengths were 324 and 413 nm, respectively (slit widths: 3.5 nm).

Preliminary studies were performed in order to determine the concentration of LDL and PnA that insure totally incorporation of the probe into LDL and a linear fluorescent response with PnA concentration.

The assays were performed at 37° in 2 mL of phosphate buffer (110 mM NaCl, 20 mM phosphate, pH 7.4) containing 45 μg of LDL protein and an aliquot of ethanolic solution of PnA (1.5 μM final concentration). The incorporation of the probe was made by gentle stirring for 1 min. The polyphenol was added to the mixture and the oxidation reaction was initiated by addition of AAPH (15 mM final concentration). Three different concentrations were tested for each polyphenol. The inhibition period (t_i) was determined from the chart recorded as the intersection of tangents to the inhibited and uninhibited rates of oxidation (Fig. 2). The capacity of procyanidins to protect LDL from oxidation was determined by the increase in the inhibition period caused by compounds added relatively to the control assay [22,25]. The inhibition periods induced by each procyanidin were graphically expressed as a function of concentrations, and molar inhibition periods (T_i) were

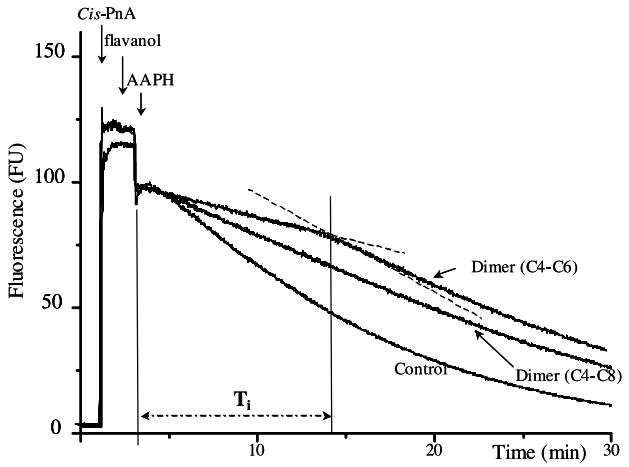

Fig. 2. PnA oxidation rates in LDL particles (45 μg) induced by AAPH (5 mM), in the absence of antioxidants (control assay) and presence of C4–C6 and C4–C8 procyanidin dimers.

calculated as the slope of oxidation curve tangent. Oxidation rate (k_{ox}) was also calculated as the slope of tangent to oxidation phase for assays that did not revealed induction period, and then expressed this parameter as a function of concentration tested, obtaining the molar oxidation rate (K_{ox}) as the slope of this graphic.

2.4. Conjugated-diene measurements

The conjugated-diene hydroperoxides measurements were carried out at 37° in a Shimadzu UV-265 spectrophotometer at 234 nm. Preliminary studies were performed to determine the concentrations of LDL, radical, and phenol that induced a significant inhibition period but not an assay too long. A solution of 5 mM of AAPH in phosphate buffer (110 mM NaCl, 20 mM phosphate, pH 7.4) was prepared and an aliquot of 2 mL was immediately transferred to reference and sample cuvettes. After running a background correction, polyphenol (1.5 μM final concentration) and 23 μg of LDL were rapidly added to the sample cuvette. The increase of the absorbance at 234 nm corresponding to the conjugated-diene hydroperoxides formation resulting from LDL oxidation was recorded in time. During assays, reference and sample cuvettes were maintained closed, in the dark at 37° without stirring [23]. The inhibition period was graphically determined as described for the fluorescence assays and the results were expressed in terms of ratio between procyanidins and trolox inhibition periods. Assays were performed in triplicate.

2.5. Oxygen consumption

Measurements of oxygen consumption were made using a Clark-type electrode (Hansatech®) in a closed glass vessel, protected from light, thermostated at 37° and provided with a stirrer. LDL solution containing 36 μg of LDL protein was incubated with 1 mL of phosphate buffer (110 mM NaCl, 20 mM phosphate, pH 7.4). Reaction was started by addition of AAPH (10 mM final concentration). After a convenient period of oxidation, phenol was added (1.5 μM final concentration) and the oxygen consumption recorded in time [22,23]. The resulting inhibition period was estimated as previously described for PnA fluorescence and conjugated-dienes assays. All solutions of AAPH were freshly prepared in phosphate buffer, and stored under an argon atmosphere until the use. Assays were made in triplicate.

3. Results and discussion

The relative antioxidant activity of the following flavanol compounds upon *in vitro* LDL oxidation was tested: (i) monomers: (+)-catechin (cat) and (−)-epicatechin (epi); (ii) dimers B1 to B4 (C4–C8 interflavan linkage) and B5 to B8 (C4–C6 interflavan linkage); (iii) trimer C1 (epi–epi–epi); (iv) esters-gallate: epi-3-*O*-gallate and B2-3′′-*O*-gallate.

The LDL oxidation was initiated using AAPH (2,2′-azobis(2-amidinopropane hydrochloride)). Despite the fact that AAPH is not a biological relevant molecule, several authors have been using this compound as a peroxidation initiator since it undergoes thermal decomposition at 37° and generates peroxyl radicals at a known and constant rate, thus making possible a reproducible and quantitative analysis of procyanidin antioxidant activity. For instance, accepted methods for determination of total antioxidant activity of human plasma use AAPH-derived peroxyl radicals as radical initiators. Antioxidant activities were evaluated by three distinct methods: PnA fluorescence decay, conjugated-dienes detection and oxygen consumption (Table 1).

4. Parinaric acid fluorescence

The PnA (9,11,13,15-octadecatetraenoic acid) is a fluorescent polyunsaturated fatty acid that was used as a probe to evaluate lipid peroxidation. This acid contains four double bonds making it a preferential substrate for oxidation. Due to its hydrophobic chain, this molecule can be inserted in LDL membrane, and its degradation (fluorescence decay) is indicative of LDL fatty acids oxidation. It is fast, reliable, and high sensitive method [22,23].

Dimer B2-3′′-*O*-gallate presented the higher molar inhibition period (T_i, 5.52), followed by trimer C1 (2.35) and monomers (1.77 and 1.33 for cat and epi, respectively). Dimers C4–C6 were less effective in preventing the PnA degradation, especially dimers B5 and B6.

The parinaric acid fluorescence decay observed for all procyanidin dimers of C4–C8 series (B1 to B4) was different from that observed for the analogue C4–C6

Table 1
Catechin and procyanidins antioxidant activities upon LDL oxidation

Compound	PnA		t_i (plasma)/t_i (trolox)	t_i (plasma)/t_i (trolox)
	$T_i \times 10^{-8}$ (M s)	K_{ox} (M^{-1} s^{-1})	Dienes	O_2 consumption
Catechin monomers				
(+)-Catechin	1.77	–	2.21 ± 0.10	3.0 ± 0.5
(−)-Epicatecbin	1.33	–	2.08 ± 0.13	2.9 ± 0.3
Dimers C4–C8				
B1 (epi–cat)	0.00	0.0057	2.14 ± 0.31	5.9 ± 0.6
B2 (epi–epi)	0.00	0.0059	2.55 ± 0.15	6.6 ± 0.5
B3 (cat–cat)	0.00	0.0071	2.99 ± 0.15	10.7 ± 1.4
B4 (cat–epi)	0.54	0.0063	2.75 ± 0.41	6.9 ± 1.0
Dimers C4–C6				
B5 (epi–epi)	0.71	–	1.35 ± 0.12	2.0 ± 0.4
B6 (cat–cat)	0.01	–	0.97 ± 0.08	0.0 ± 0.0
B7 (epi–cat)	1.29	–	1.12 ± 0.06	6.2 ± 0.6
B8 (cat–epi)	1.26	–	2.88 ± 0.51	6.9 ± 0.6
Trimers				
EEC (epi–epi–cat)	0.01	–	1.95 ± 0.15	4.8 ± 0.4
C1 (epi–epi–epi)	2.35	–	3.47 ± 0.18	13.9 ± 1.9
Esters-gallate				
B2-3′′-*O*-gallate	5.52	–	6.10 ± 0.42	26.0 ± 2.3
(−)-Epicatechin-3-*O*-gallate	1.70	–	1.91 ± 0.07	4.0 ± 0.9
Standards				
Trolox	–	–	1.00 ± 0.03	1.0 ± 0.1
Ascorbic acid	–	–	1.26 ± 0.18	0.4 ± 0.1
Caffeic acid	–	–	1.58 ± 0.05	2.4 ± 0.2

(B5 to B8) dimers, since the former group did not induce an inhibition period but simply slowed down the oxidation rate (Fig. 2). This behavior was not observed with the other two methods probably due to the higher sensitivity of parinaric acid methodology over oxygen and conjugated-dienes measurements in discriminating different reactivities of the phenols against the peroxyl radicals. Also distinct locations of the dimers, i.e. LDL surface vs. water phase, may influence its behavior towards PnA protection from oxidation (see below).

The molar oxidation rate (K_{ox}) of the procyanidin dimers B3 and B4 containing the interflavan bond C4–C8 are greater than those of the procyanidin dimers B1 and B2.

4.1. Conjugated-dienes detection and oxygen consumption

The detection of conjugated-dienes hydroperoxides, primary products of lipid peroxidation, was made by monitoring the characteristic absorbance of these compounds at 234 nm. This method constitutes a real index of oxidative state of LDL fatty acids in opposition to parinaric acid that is a molecular probe, and hence an indirect method of evaluation. Catechins and procyanidins trap AAPH-derived peroxyl radicals inhibiting the initiation of lipid peroxidation, but, if located at the LDL surface, may also quench LDL-derived peroxyl radicals, inhibiting the chain propagation. Consequently, formation of lipid hydroperoxides and appearance of conjugated dienes were inhibited until exhaustion of polyphenol.

In addition to procyanidins, we evaluated the antioxidant activity of antioxidants usually used as standards, such as trolox (water-soluble analogue of Vitamin E), ascorbic acid and caffeic acid. Results were expressed in terms of ratio between procyanidins and trolox inhibition periods (Table 1).

Under the experimental conditions described, the phenolic compounds showed the following order of relative inhibition periods compared with trolox: B2-3′′-*O*-gallate, 6.10; trimer C1, 3.47; average of dimers C4–C8, 2.61; average of monomers, 2.15; epi-3-*O*-gallate, 1.91; average of dimers C4–C6, 1.58. Dimers C4–C6 were shown to be less effective upon LDL oxidation as compared with the corresponding C4–C8 dimers. The only exception was dimer B8 which was shown to have a similar or slightly higher antioxidant activity against LDL oxidation comparatively to dimers C4–C8 (B1, B2, B3, and B4). The higher antioxidant activity of dimer B8, as compared to dimers B2, B3, and B4 upon LDL oxidation induced by Cu(II), was already reported in literature by Teissedre *et al.* [35]. Additionally, these authors have observed that trimer C1 presented the higher antioxidant activity, and dimer B6 was the less effective antioxidant, which is in agreement with the results presented herein for peroxyl radicals-dependent LDL oxidation.

O_2 measurements suggest that procyanidins act also by quenching LDL-derived peroxyl radicals since they were added during the propagation phase of LDL oxidation. They revealed a relative antioxidant activity similar to the one obtained by the conjugated-dienes detection assay, but shorter inhibition periods were noticed. The main difference is that, in this assay, the procyanidin dimer B7 of C4–C6 series showed an antioxidant activity comparable to the one of dimers C4–C8.

It is also interesting to note that the polyphenols studied, with the exception of dimers B6 and B7, exhibited an antioxidant activity higher than that of caffeic acid and ascorbic acid.

5. Conclusions

This study was focused on the influence of the flavanol structure on the antioxidant activity in connection with the inhibition of LDL oxidation induced by AAPH. The following major conclusions can be withdrawn:

(i) Monomers (+)-catechin and (−)-epicatechin have an identical antioxidant activity suggesting that the stereochemistry of the pyranic ring C does not influence importantly the monomers antioxidant activity upon LDL oxidation.

(ii) Antioxidant activity increases with degree of polymerization for compounds constituted by (−)-epicatechin as the monomer unit, namely epi, dimer B2 (epi–epi) and trimer C1 (epi–epi–epi). This can be explained by: (a) the augmentation of the number of free hydroxyls, potential donors of hydrogen atoms, increasing the antioxidant effectiveness in aqueous phase; (b) the increase of the partition coefficient of these compounds to lipid phase with the degree of polymerization [31]. Data reported in literature allowed to establish a relationship between the degree of polyphenol polymerization and antioxidant efficiency suggesting an increase of the antioxidant activity with the polymerization degree in aqueous systems and an inverse relationship in lipid phase [36]. Other works demonstrated an increase of aqueous phase scavenging activity with the degree of polymerization of procyanidins up to trimers [29] and an opposite trend for compounds containing more than three monomeric units [17].

(iii) Under these experimental conditions, the antioxidant activity of procyanidin dimers containing the interflavan bond C4–C8 and C4–C6 were distinct, suggesting that interflavan linkage contributes importantly to the antioxidant activity of procyanidin dimers. This fact can be explained by the distinct conformation that these procyanidins adopt in aqueous solution. Indeed, molecular studies showed that C4–C8 dimers adopt a more closed conformation, whereas C4–C6 dimers a more elongated one [8–10,15] (Fig. 3). On the other hand, C4–C8 dimers seems to have a slightly higher hydrophobic character than dimers C4–C6 [8], thereby having a higher affinity to LDL surface and interacting more strongly with LDL-derived peroxyl radicals ($LOO^{\bullet}$) than dimers C4–C6. Therefore, C4–C8 dimers exert a more pronounced antioxidant effect in the stages involving LDL lipid radicals (propagation stage as evaluated by UV absorbance and oxygen consumption) and C4–C6 dimers a more powerful action against aqueous radicals. This aspect could explain the different behavior between these two series of procyanidin dimers relatively to PnA fluorescence assay since PnA aligned with the phospholipid acyl chains at the LDL monolayer is readily oxidized by AAPH-derived peroxyl radicals, i.e. it does not require an ongoing oxidation chain for complete oxidation as unsaturated acyl chains do [23].

(iv) Antioxidant activity is increased by esterification of the C3 hydroxyl group with gallic acid, probably due to the increase of free hydroxyl groups (Fig. 4). However, galloylation caused an exceptional increase of antioxidant activity for dimer B2-3′′-*O*-gallate in comparison to (−)-epicatechin-3-*O*-gallate. Nevertheless, in the conjugated-dienes assay (−)-epicatechin did not suffer an increase in its antioxidant activity by esterification. It is important to refer that B2-3′′-*O*-gallate was shown to be the more powerful antioxidant in the three methods used. Previous molecular studies concerning the estimation of the flavan-3-ol conformation in solution using molecular mechanisms and NMR techniques showed a possible "π−π" stacking arrangement between the aromatic gallate and the catechol B-ring of dimer B2-3′′-*O*-gallate yielding a compact structure (Fig. 3). Similar interaction is absent in the analogues dimer B2 and (−)-epicatechin-3-*O*-gallate [10]. The extraordinary enhancement of antioxidant activity of dimer B2 by addition of a galloyl group could be explained through the stabilization of the respective phenoxyl radical by the additional deslocalization of the unpaired electron between the π orbitals of galloyl and catechol B-rings.

Procyanidins represent a significant percentage of polyphenol intakes in our diet and have revealed, under the conditions described using a biologically relevant target (the LDL particles), an antioxidant activity greater than standard antioxidants like trolox and ascorbic acid. Nevertheless, these conclusions cannot be extrapolated to *in vivo* conditions due to the limited knowledge regarding the bioavailability of these polyphenols, especially dimers and trimers in the organism. However, they provide a rationale for the biomedical use of these compounds in the optimization of nutrition or as pharmacological agents in the prevention or decrease of the risk of atherosclerosis.

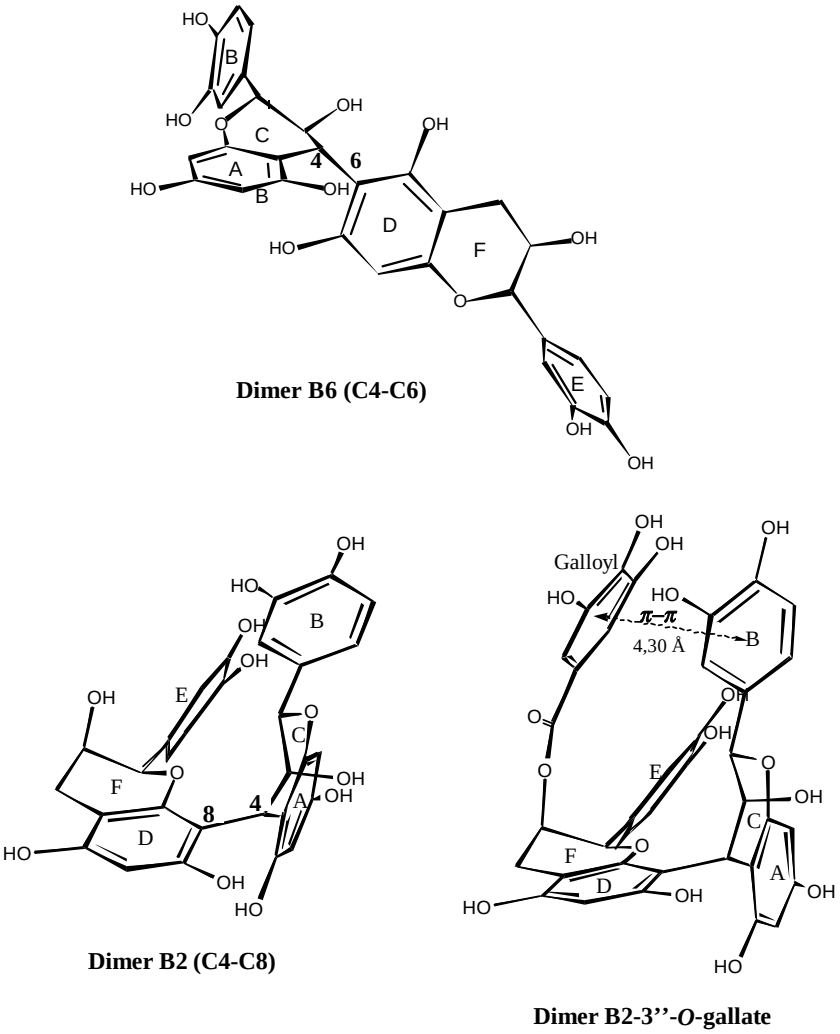

Fig. 3. Preferred conformation of dimer B6, dimer B2, and dimer B2-3′′-*O*-gallate, determined using Allinger's MM2* force field parameters [8,10].

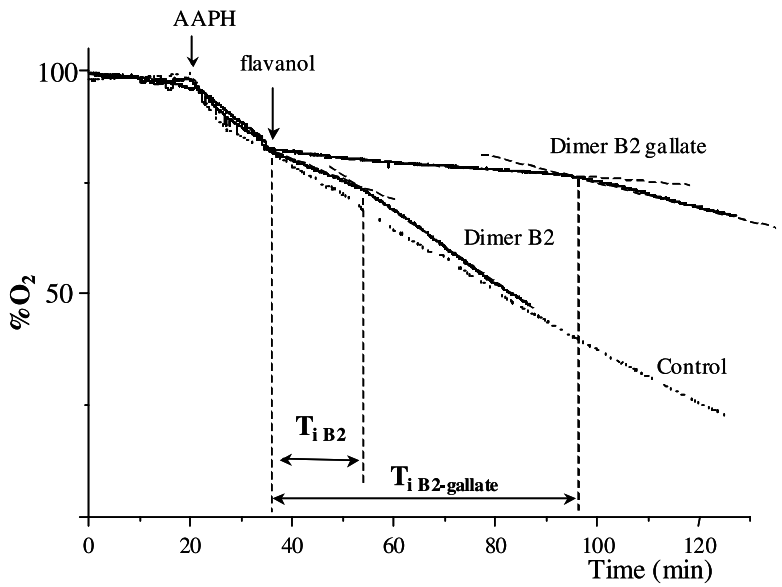

Fig. 4. Oxygen consumption during LDL particles (45 μg) oxidation induced by AAPH (5 mM), in the absence of antioxidants (control assay) and presence of dimer B2 and B2-3′′-*O*-gallate.

References

[1] Ariga T, Hamano M. Radical scavenging action and its modo in procyanidins B-1 and B-3 from azuki beans to peroxyl radicals. Agric Biol Chem 1990;54:2499–504.

[2] Block G. A role for antioxidants in reducing cancer risk. Nutr Rev 1992;50:207–13.

[3] Block G, Langseth L. Antioxidant vitamins and disease prevention. Food Tech 1994;July:80–4.

[4] Blois MS. Antioxidant determinations by the use of stable free radical. Nature 1958;181:1199–200.

[5] Bors W, Heller W, Mitchel C, Saran M. Flavonoids as antioxidants: determination of radical-scavenging efficiencies. Methods Enzymol 1990;186:343–55.

[6] Carbonneau MA, Descomps B, Lèger CL. Consommation de polyphènols du vin par des volontaires sans: amélioration de leur status antioxydatif et potentiellement antiathérogène. Prog Agric Vit 1999; 116:204–14.

[7] Chung K-T, Wong TY, Huang Y-W, Lin Y. Tannins and human health: a review. Crit Rev Food Sci Nutr 1998;38:421–64.

[8] De Freitas VAP, Glories Y, Laguerre M, Vercauteren J. Analyse conformationelle et propriétés physico-chimiques des procyanidines dimères. In: Vercauteren J, Cheze C, Dumon MC, Weber JF, editors. Polyphenols Communication 96, Proceedings of the 18th International Conference on Polyphenols, Bordeaux, France, 15–18 July 1996. Groupe polyphénols; 1996. p. 75–6.

[9] De Freitas VAP, Glories Y, Bourgeois G, Vitry C. Characterisation of oligomeric and polymeric procyanidins from grape seeds by Liquid Secondary Ion Mass Spectrometry (LSIMS). Phytochemistry 1998;49:1435–41.

[10] De Freitas VAP, Glories Y, Laguerre M. Incidence of molecular structure in oxidation of grape seeds procyanidins. J Agric Food Chem 1998;46:376–82.

[11] Esterbauer H, Gebicki J, Puhl H, Jürgens G. The role of lipid peroxidation and antioxidants in oxidative modification of LDL. Free Radic Biol Med 1992;3:341.

[12] Frankel EN, Kanner J, German JB, Parks E, Kinsella JE. Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. Lancet 1993;341:454–7.

[13] Frei B. Cardiovascular disease and nutrient antioxidants: role of low-density lipoprotein oxidation. Crit Rev Food Sci Nutr 1995;35:83–98.

[14] Frei B, Gaziano JM. Content of antioxidants, preformed lipid hydroperoxides and cholesterol as predictors of the susceptibility of human LDL to metal ion-dependent and -independent oxidation. J Lipid Res 1993;34:2135.

[15] Fronczek FR, Gannuch G, Mattice WL, Hemingway RW, Chiari G, Tobiaso FL, Houglum K, Shanafelta A. Preference for occupancy of axial position by substituents bonded to the heterocyclic ring in penta-*o*-acetyl-(+)-catechin in the crystalline state. J Chem Soc Trans 1985; II:1383–6.

[16] Fuster V, Lewis A. Conner Memorial Lecture. Mechanisms leading to myocardial infarction: insights from studies of vascular biology. Circulation 1994;90:2126.

[17] Gaulejac NSC, Vivas N, de Freitas VAP, Bourgeois G. The influence of various phenolic compounds on scavenging activity assessed by an enzymatic method. J Sci Food Agric 1999;79:1018–90.

[18] Geissman TA, Yoshimura NN. Synthetic proanthocyanidin. Tetrahedron Lett 1996;24:2669–73.

[19] Hertog MGL, Feskens EJM, Hollman PCH, Katan MB, Kromhout D. Dietary antioxidant flavonoids and risk of coronary disease. Lancet 1993;342:1007–11.

[20] Ivanov V, Carr AC, Frei B. Red wine antioxidants bind to human lipoproteins and protect them from metal-dependent and -independent oxidation. J Agric Food Chem 2001;49:4442–9.

[21] Kanner J, Frankel E, Granit R, German B, Kinsella JE. Natural antioxidants in grapes and wines. J Agric Food Chem 1994;42: 64–9.

[22] Laranjinha JAN, Almeida LM, Madeira VMC. Reactivity of dietary phenolic acids with peroxyl radicals: antioxidant activity upon low density lipoprotein peroxidation. Biochem Pharmacol 1994;48: 487–94.

[23] Laranjinha JAN, Almeida LM, Madeira VMC. Lipid peroxidation and its inhibition in low density lipoproteins: quenching of *cis*-parinaric acid fluorescence. Arch Biochem Biophys 1992;297:147–54.

[24] Laranjinha JAN, Cadenas E. Redox cycles of caffeic acid, α-tocopherol and ascorbate: implications for protection of low-density lipoproteins against oxidation. Life 1999;48:57–65.

[25] Lowry OH, Rosebrough AL, Farr MJ, Randall RS. Protein measurement with Folin phenol reagent. J Biol Chem 1951;193:265–75.

[26] Masquelier J. Effects physiologiques du vin: sa part dans l'alcoolisme. Bull OIV 1988;689/690:557–78.

[27] Michaud MMJ, Masquelier J, Roudge AM. Synthèse des procyanidines naturelles. Ann Pharmaceutiq Françaises 1973;31:385–95.

[28] Michaud MMJ, Lacaze P, Masquelier J. Fraccionnement des oligomères flavanoliques du raisin. Bull Soc Pharm 1971;110:111–6.

[29] Plumb GW, Pascual-Teresa S, Santos-Buelga C, Cheynier V, Williamson G. Antioxidant properties of catechins and proanthocyanidins: effect of polymerisation, galloylation and glycosilation. Free Radic Res 1998;29:351–8.

[30] Ricardo da Silva JM, Darmon N, Fernandez Y, Mitjavila S. Oxygen free radical scavenger capacity in aqueous models of different procyanidins from grape seeds. J Agric Food Chem 1991;39:1549–52.

[31] Rice-Evans CA, Miller NJ, Paganga G. Structure–antioxidant activity relationships of flavonoids and phenolic acids. Free Radic Biol Med 1996;20:933–56.

[32] Santos Buelga C, Scalbert A. Proanthocyanidins and tannin-like compounds-nature, occurrence, dietary intake and effects on nutrition and health. J Sci Food Agric 2000;80:1094–117.

[33] Serafini M, Laranjinha JAN, Almeida LM, Maiani G. Inhibition of human LDL lipid peroxidation by phenol-rich beverages and their impact on plasma total antioxidant capacity in humans. J Nutr Biochem 2000;11:2–7.

[34] Steinberg DMD, Parthasarathy S, Carew TE, Khoo C, Witztum J. Beyond cholesterol: modifications of low density lipoprotein that increase its atherogenicity. N Engl J Med 1989;320:915–24.

[35] Teissedre PL, Frankel EN, Waterhouse AL, Peleg H, German JB. Inhibition of in vitro human LDL oxidation by phenolic antioxidants from grapes and wines. J Sci Food Agric 1996;70:55–61.

[36] Vieira OV, Laranjinha JAN, Madeira VMC, Almeida LM. Rapid isolation of low density lipoproteins in a concentrated fraction free from water-soluble plasma antioxidants. J Lipid Res 1996;37: 2715–21.