Plant polyphenols: free radical scavengers or chain-breaking antioxidants?

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Abstract

There is increasing interest in the biological effects of tea- and wine-derived polyphenols and many studies *in vitro* and *in vivo* are demonstrating their anti-oxidant properties. Tea is a major source of dietary polyphenols and an even richer source of the flavanols, the catechins and catechin/gallate esters. Although there are limited studies on the bioavailability of the polyphenols, the absorption of flavanols in humans has been shown. The studies described in this chapter discuss the relative antioxidant potentials of the polyphenolic flavonoids *in vitro* against radicals generated in the aqueous phase in comparison with their relative effective-ness as antioxidants against propagating lipid peroxyl radicals, and how their activity influences that of α-tocopherol in low-density lipoproteins exposed to oxidative stress.

Introduction

There is considerable evidence from chemical, biological, human and epidemiological studies for a role for antioxidant nutrients (vitamins E and C and β-carotene) in the maintenance of health and in contributing to protection from cancer, cataract and cardiovascular disease. One epidemiological study of particular interest is the WHO cross-cultural European epidemiological study showing a highly significant inverse correlation between mortality from ischaemic heart disease and vitamin E levels in the blood (lipid-normalized) with $r^2 = 0.62$ [1,2]. The study also reveals a slope across Europe, with relatively higher blood vitamin E levels and enhanced protection from heart diseases in the population of the countries of Southern Europe and vice versa for Northern Europe. In addition, intake of vitamin E supplements has been correlated with a reduced risk of cardiovascular disease [3,4].

There is a considerable amount of epidemiological evidence (reviewed in [5]) revealing an association between those with diets rich in fresh fruit and vegetables and a decreased risk of cardiovascular disease and certain forms of cancer. Until relatively recently it was generally assumed that the active dietary constituents contributing to these protective effects are the antioxidant nutrients. But more recent work is highlighting the additional role of the polyphenolic components of the higher plants [6,7] which may act as antioxidants or agents of other mechanisms contributing to anti-carcinogenic or cardioprotective actions.

Flavonoids constitute a large class of compounds, ubiquitous in plants, containing a number of phenolic hydroxyl groups attached to ring structures, conferring the antioxidant activity [6]. Plant polyphenols are multifunctional and can act as reducing agents, hydrogen-donating antioxidants, metal chelators and singlet oxygen quenchers. Estimates of our daily intake range from about 20 mg to 1 g [7]. Table 1 shows some of the dietary sources of flavonoids. The flavanols, particularly the catechin family, and the flavonols quercetin, kaempferol and their glycosides are major constituents of the beverages green and black tea and red wine. Quercetin is also a predominant component of onions and apples, and myricetin and quercetin of berries. The flavanones are mainly found in citrus fruits. Little is known about the bioavailability and absorption and metabolism in man and it is likely that different groups of flavonoids have different pharmacokinetic properties. In this article we shall focus on the catechins and the flavonol quercetin.

Table 1. Some dietary sources of polyphenolic flavonoids.

Flavonoid	Source
Flavanols	
Catechin	Green and black tea, red wine.
Epicatechin	
Epigallocatechin	
Epicatechin gallate	
Epigallocatechin gallate	
Flavanones	
Naringenin	Eucalyptus
Taxifolin	Citrus fruits
Flavones	
Chrysin	Fruit skins
Apigenin	Parsley, celery
Flavonols	
Kaempferol	Endive, leak, broccoli, radish, teas, grapefruit
Myricetin	Cranberry, grapes (white and black), red wine
Quercetin	Onion, lettuce, broccoli, tomato, cranberry, berries, apple skin, grapes, olive oil, teas
	Red wine* (Chianti > Pinot Noir > Rioja = Bordeaux) [7]

*1990 vintage.

Table 2. French Paradox. From ref. [8].

Region	Plasma cholesterol (mg/dl)	Mortality from coronary heart disease (per 10^4)
France (General)	216	102
Toulouse region	224	78
U.S.A. (Stanford)	209	182
U.K.	240	380

The French paradox

The constituents of red wine are factors of particular interest due to the intrigue created by the French paradox. The Southern French have a very low incidence of coronary heart disease despite their high-fat diet and smoking tendencies [8] (Table 2). One of the features that has been highlighted relates to the high consumption of red wine by the French and the question as to whether the polyphenolic antioxidants from this dietary source contribute to the protection from coronary heart disease along with the antioxidants in olive oil and high intake of antioxidant nutrients from the fresh fruit- and vegetable-rich Mediterranean diet. Indeed, red wine has been shown to inhibit the oxidation of low-density lipoproteins (LDLs) in *in vitro* studies [9]. Of course, sight should not be lost of the fact that the high intake of monounsaturated fatty acids also decreases plasma cholesterol and has been reported to reduce the oxidizability of LDL (*ex vivo*).

Zutphen study

In support of flavonoids exerting a protective effect *in vivo* are the findings of a Dutch epidemiological study showing that coronary heart disease in elderly males is inversely correlated with their intake of flavonoids [7]. Most of their dietary flavonoids derived from tea (48% of the flavonoid intake), onions (29%), apples (7%) and red wine (1%). The risk of death from coronary heart disease in the lower tertile of flavonoid intake was about 2.4 times that of the upper tertile. It still remains to be established to what extents the antioxidant and anti-thrombotic properties of the polyphenols contribute to this protection.

Determinants of radical scavenging potential

The chemical properties of polyphenols in terms of the availability of the phenolic hydrogens as hydrogen-donating radical scavengers defines their anti-oxidant activity. The two basic conditions that must be satisfied for a polyphenolic substance to be defined as an antioxidant are: (i) when present in low concentrations relative to the substrate to be oxidized they can delay, retard or prevent the autoxidation or free radical-mediated oxidation [10]; and (ii) the resulting radical formed after scavenging must be stable; polyphenols can be stabilized through intramolecular hydrogen bonding or by further oxidation [11].

Three criteria for effective radical scavenging by the polyphenols [12,13] are: (i) the *o*-dihydroxy structure in the B ring which confers higher stability to the
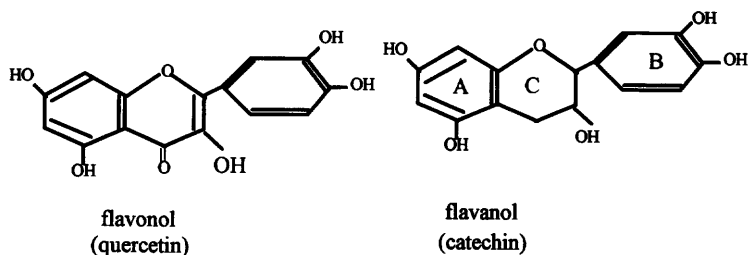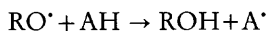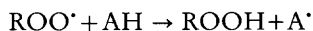
flavonol
(quercetin)

flavanol
(catechin)

**Fig. 1. Comparative structures of representative flavonoids, quercetin
a typical flavonol and catechin a typical flavanol.**

radical form and participates in electron delocalization; (ii) the 2,3 double bond in
conjugation with 4-oxo function in the C ring responsible for electron delocaliza-
tion from the B ring; and (iii) the 3- and 5-OH groups with 4-oxo function in A
and C rings for maximum radical scavenging potential.

Thus, as indicated in Fig. 1, it is reasonably expected that the flavonols (e.g.
quercetin) are more effective antioxidants than the flavanols (e.g. catechin) since
quercetin satisfies all the above determinants whereas catechin lacks aspects of the
structural advantages of quercetin and other flavonols and only satisfies determi-
nant (i).

The phenoxyl radical formed by reaction of a phenolic antioxidant with a lipid
radical is stabilized by delocalization of unpaired electrons around the aromatic
ring. The o-dihydroxy substitution in the B ring is important for stabilizing the
resulting free radical form (as well as for metal chelating activity). The possibility
exists for stabilization of radical forms though the 3-OH, 5-OH and 4-oxo groups
and conjugation from the A ring to the B ring through the additional 2,3 unsatura-
tion in the C ring. This type of reaction is mainly seen with peroxyl (aliphatic)
radicals reacting with phenolic antioxidants, as pointed out by Bors *et al.* [12].
Polyphenols, depending on their precise structure and the proximity or adjacence
of hydroxyl groups, also have the possibility of chelating metal ions and
preventing metal-catalysed formation of initiating radical species. The two points
of attachment of metal ions, such as copper, to the flavonoid molecule are the
o-diphenol in the 3',4'-dihydroxy position in ring B and the ketol structure, 4-oxo,
3-OH, in the C ring of the flavonols [14].

Thus, the specific mode of inhibition of oxidation by the individual poly-
phenols is not clear but they may act by (i) chelating metal ions via the
o-dihydroxy phenolic structure; (ii) scavenging lipid alkoxyl and peroxyl radicals
by acting as chain-breaking antioxidants, e.g. as hydrogen donors

$$ROO^{\cdot} + AH \rightarrow ROOH + A^{\cdot}$$

$$RO^{\cdot} + AH \rightarrow ROH + A^{\cdot}$$

or (iii) by regenerating α-tocopherol through reduction of the α-tocopheroxyl
radical.

Biological properties of bioflavonoids

Flavonoids and other plant phenolics are reported to have multiple biological activities [15,16] including anticarcinogenic, anti-inflammatory, antibacterial, immune-stimulating, anti-allergic, antiviral, and oestrogenic effects, and as inhibitors of cyclo-oxygenase, lipoxygenase and phospholipase A_2 [17–20]. The chemistry of the flavonoids is predictive of their free radical-scavenging activity since the reduction potentials of flavonoid radicals are lower than those of alkyl peroxyl radicals and the superoxide radical, which means that flavonoids may inactivate these oxyl species and prevent the deleterious consequences of their reactions [20,21]. Their antioxidant activity is also reported: as scavengers of superoxide radical [22–25] although there is conflicting evidence [12,26]; as peroxyl radical scavengers [27,28]; inhibitory effects on lipid peroxidation [29–31]; and inhibition of LDL oxidation induced by copper ions and macrophages [32,33] with half-maximal inhibition induced by compounds ranging in concentration from 1–10 μM. In studies on model systems (erythrocyte membranes and rat liver microsomes), the catechins from tea have revealed high activity, with greatest protection from lipid oxidation by epigallocatechin gallate and epicatechin gallate, the latter being ten times more effective than vitamin E [34]. Others have evaluated the antioxidant activity of flavonoids by investigating their effects in cells in culture. In their studies, pretreatment of cells followed by exposure to reactive oxygen species resulted in the proposed concentrations required for protection being quercetin < kaempferol < catechin. Higher concentrations of quercetin and kaempferol induced toxicity in cells.

Catechin, epicatechin and quercetin have been shown to have powerful antioxidative capacities to approximately the same extents in phospholipid bilayers exposed to aqueous oxygen radicals [31], although the electron-donating ability of catechin is lower than that of quercetin. On the other hand, quercetin is more effective than catechin as an antioxidant in protecting LDLs from oxidation in copper-mediated peroxidation systems (B. Scott, G. Paganga, B. Halliwell and C. Rice-Evans, unpublished work). Furthermore, these flavonoids have been shown to conserve endogenous α-tocopherol in LDL and quercetin is the most effective of the compounds studied [33]. It has been proposed that flavonoids located near the surface of phospholipid structures are ideally located for scavenging oxygen radicals generated in the aqueous phase.

Another property of polyphenols is their metal-chelating potential which may also play a role in the protection against iron- and copper-induced free radical reactions and preventing metal-catalysed formation of initiating radical species [35,36]. There are reports of the pro-oxidant activity of some polyphenols in which high concentrations (25–100 μM) accelerate hydroxyl radical production and DNA damage *in vitro*, mediated by iron-EDTA but not by iron-ADP or iron itself [35,37]. Others have shown that high concentrations (100 μM) of the flavonols gossypetin and myricetin can modify LDL through non-oxidative processes probably involving covalent modification of the apolipoprotein B_{100} [38]. These are very high relative concentrations of flavonoid:LDL and it is very unlikely that these compounds achieve such high concentrations *in vivo*.

There is increasing interest in the biological effects of other wine- and tea-derived polyphenols and many *in vitro* and *in vivo* studies are demonstrating their

antioxidant properties [39,40]. Tea is a rich source of dietary polyphenols and a main dietary source of flavonol glycosides. Tea is an even richer source of the flavanols, the catechins, and it has been shown that green tea constituents [(+)-catechin], are absorbed though the human gut [41,42]. The studies described in this chapter discuss the relative antioxidant potentials of the polyphenolic flavonoids against radicals generated in the aqueous phase in relation to their relative effectiveness as antioxidants against propagating lipid peroxyl radicals, and how their activity influences that of α-tocopherol.

Antioxidant potentials of polyphenols

Antioxidant activity against radicals in the aqueous phase

The total antioxidant activity (TAA) or the Trolox equivalent antioxidant activity (TEAC) is defined as the concentration of Trolox solution with equivalent antioxidant potential to a 1 mM concentration of the compound under investigation [43,44]. The chemical basis of the assay is the generation of a long-lived specific radical cation chromophore based on the peroxidative activity of metmyoglobin and the interaction with the phenothiazine compound, 2,2′-azinobis-(3-ethyl benzthiazoline-6-sulphonic acid) (ABTS) in the presence of hydrogen peroxide to form the ABTS$^{\cdot+}$ radical cation with an absorption maximum at 734 nm. The TEAC reflects the ability of the putative antioxidant to scavenge the ABTS$^{\cdot+}$ radical cation compared with that of Trolox, the water-soluble vitamin E analogue.

The structures of the catechins and gallate/catechin compounds are shown in Fig. 2. These are the main catechins found in fresh leaf and green tea, mostly in the epi-configuration. Catechins with three hydroxyl groups on the B ring are referred to as gallocatechins. Catechin gallates contain gallic acid and are esterified at the OH group on the pyran (C) ring. The antioxidant potentials of the catechin/gallate family of polyphenols are given in the form of their TEAC values in Table 3. The results show that for this series of structures the compounds with
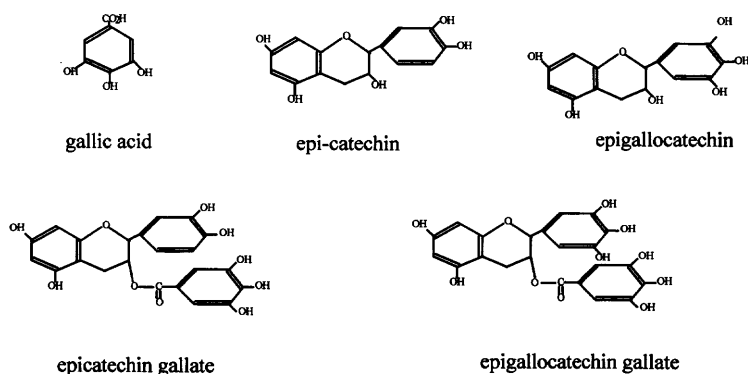

gallic acid   epi-catechin   epigallocatechin

epicatechin gallate   epigallocatechin gallate

Fig. 2. Chemical structures of the catechins and catechin-gallate esters.
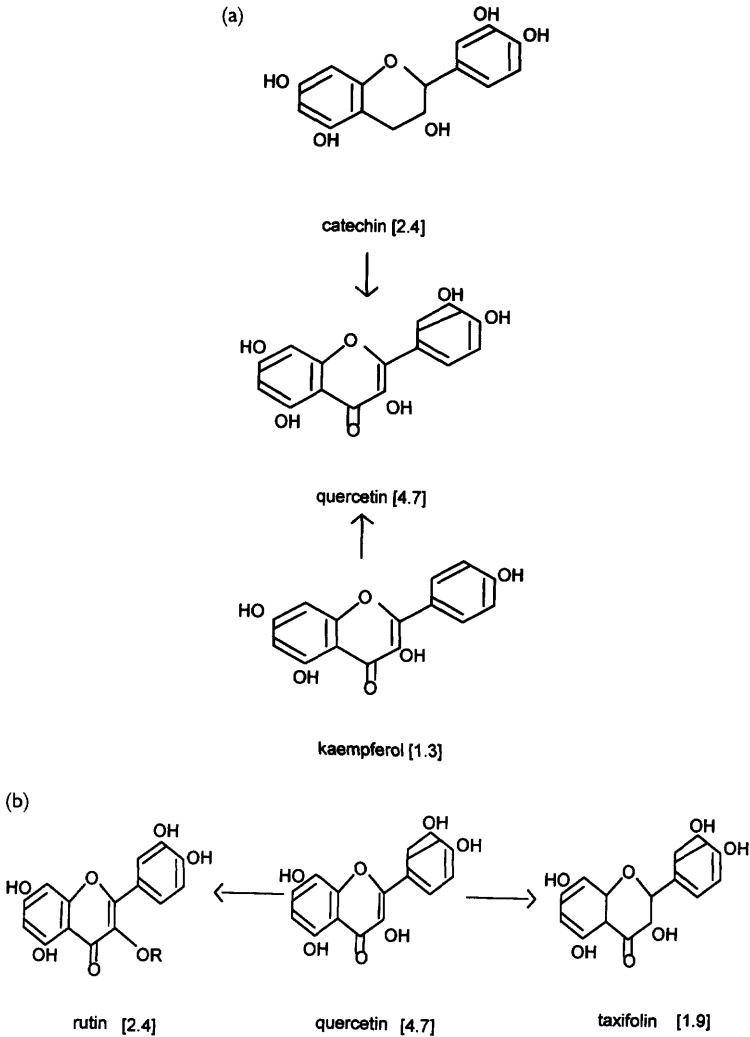
Table 3. Relative antioxidant potentials of the catechins and catechin/gallate esters in comparison with vitamins E and C.

	TEAC (mM)	Number of OH groups
Catechin	2.40 ± 0.05 (9)	3,5,7,3',4'
Epicatechin	2.50 ± 0.02 (6)	3,5,7,3',4'
Gallic acid	3.01 ± 0.05 (7)	3,4,5
Epigallocatechin	3.82 ± 0.06 (6)	3,5,7,3',4',5'
Epigallocatechin gallate	4.75 ± 0.06 (9)	5,7,3',4',3",4",5"
Epicatechin gallate	4.93 ± 0.02 (9)	5,7,3',4',3",4",5"
Vitamin C	0.99 ± 0.04 (5)	
Vitamin E	0.97 ± 0.01 (5)	

the most hydroxyl groups exert the greatest antioxidant activity, with the catechin isomers at 2.4 mM and 2.5 mM, more than twice as effective as vitamins E and C (TEAC = 1 mM) [45]. The values for the catechin-gallate esters reflect the additional contribution from the tri-hydroxy phenolic component of gallic acid. Quercetin (Scheme 1a) has an identical number of hydroxyl groups in the same positions as catechin, but also contains the 2,3-double bond in the C ring and the 4-oxo group. This structural advantage confers an enhancement of the TEAC value to 4.7 ± 0.10 mM ($n = 6$). Thus the catechin structure with a TEAC value of 2.4 ± 0.05 mM ($n = 9$), still more than twice as effective as α-tocopherol or ascorbate, can be modified to enhance its antioxidant potential to 4.7 by two different types of structural modification: as in quercetin by incorporation of the 2,3-double bond and the 4-oxo function both in the C ring, allowing electron delocalization and stabilization of the radical form; and as in epigallocatechin gallate (4.75 ± 0.06 mM, $n = 9$) by ester linkage via the 3-OH group to gallic acid carboxylate group enhancing the number of available hydrogens and incorporation of an additional 5'-OH group in the B ring (Fig. 2). On the other hand, kaempferol (TEAC 1.34 ± 0.08 mM, $n = 6$) lacks the o-dihydroxy structure in the B ring (Scheme 1b), decreasing the hydrogen-donating potential; in this case the presence of the 2,3-double bond in the C ring is less relevant since the monophenolic B ring is not such an effective hydrogen donor. Blocking the 3-hydroxyl group in the C ring as a glycoside, as in rutin (quercetin rutinoside) decreases the antioxidant activity to a value of 2.4 ± 0.06 mM ($n = 7$) (Scheme 1b). Taxifolin, lacking the 2,3-double bond, responds almost as catechin with a TEAC of 1.9 ± 0.03 mM ($n = 6$) as predicted.

Antioxidant activity against radicals generated in the lipophilic phase

The oxidation of LDLs is used as a model for investigating the efficacy of the polyphenols as chain-breaking antioxidants. Free radical-mediated peroxidation of polyunsaturated fatty acids leads to the formation of lipid hydroperoxides through a chain reaction of peroxidation (Scheme 2). Oxidative and reductive decomposition of peroxides mediated by haem-proteins or transition metal ions can amplify the peroxidation process (Scheme 3). The presence of chain-breaking antioxidants
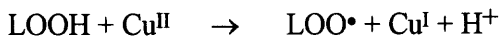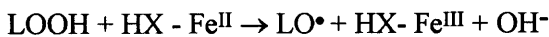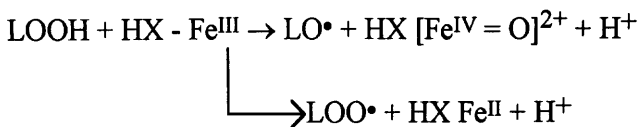
(a)

catechin [2.4]

quercetin [4.7]

kaempferol [1.3]

(b)

rutin [2.4]   quercetin [4.7]   taxifolin [1.9]

Scheme I. Structure relationships and total antioxidant potentials of quercetin in comparison with catechin and kaempferol (a) and with rutin and taxifolin (b).

can intercept this peroxidation process by reducing the alkoxyl or peroxyl radicals to alkoxides or hydroperoxides, the hydroperoxides re-entering the cycle until the antioxidants are consumed. It has been proposed that alkoxyl radicals rearrange through their own reactivity to epoxides [46]. The oxidative interaction of LDL with haem proteins is hydroperoxide-dependent [47], and, without the addition of initiating species, these agents will slowly cycle the endogenous peroxides within the LDL and amplify the peroxidation process (Scheme 4). In order to study the antioxidant activity of polyphenols as scavengers of propagating lipid peroxyl radicals, no initiating species were added, but metmyoglobin was applied to
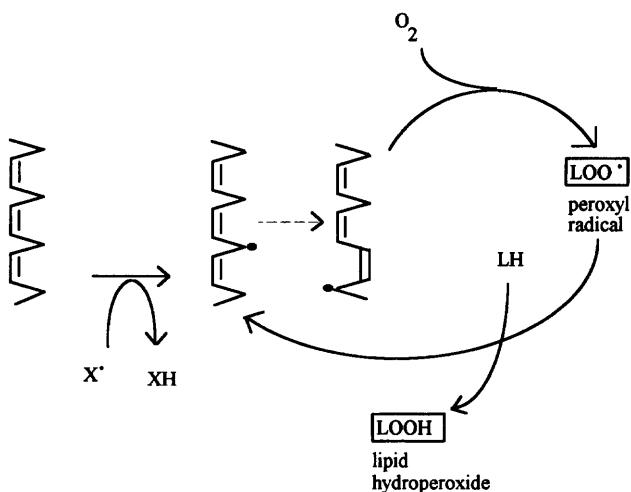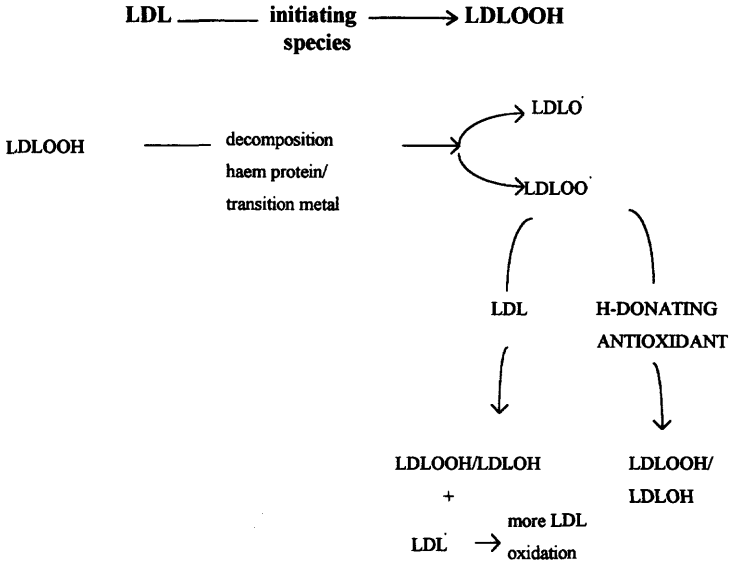
Scheme 2. The initiation of the peroxidation of a polyunsaturated fatty acid.

$$LOOH + HX - Fe^{III} \rightarrow LO^\bullet + HX\ [Fe^{IV} = O]^{2+} + H^+$$

$$\phantom{LOOH + HX - Fe^{III} \rightarrow} \rightarrow LOO^\bullet + HX\ Fe^{II} + H^+$$

$$LOOH + HX - Fe^{II} \rightarrow LO^\bullet + HX\text{-}\ Fe^{III} + OH^-$$

$$LOOH + Cu^{II} \quad \rightarrow \quad LOO^\bullet + Cu^{I} + H^+$$

$$LOOH + Cu^{I} \quad \rightarrow \quad LO^\bullet + Cu^{II} + OH^-$$

Scheme 3. The oxidative and reductive decomposition of lipid hydroperoxides mediated by haem proteins or transition metal ions.

propagate the decomposition of the minimal levels of endogenous pre-formed lipid hydroperoxides. Copper ions were avoided in order to eliminate the confounding effects of the polyphenols as metal-chelators.

In LDL, on oxidation, the aldehydic decomposition products of peroxidation can be assessed as markers of the oxidation of the polyunsaturated fatty acids. They can bind to the apolipoprotein B_{100} on the surface of the LDL, specifically the amino groups, altering the charge and recognition properties, and these modifications can be monitored as changes in electrophoretic mobility as a further indication of the oxidative modification of the LDL. Thus the extent of inhibition of LDL oxidation by the polyphenols can be assessed. The relative effectiveness of the catechin/gallate polyphenols in inhibiting LDL oxidation is shown in Table 4. The data show that gallic acid is the least effective, requiring about 1.2 μM for

LDL ———— initiating ————→ LDLOOH
                species

LDLOOH   ———— decomposition   ————→⟨ → LDLO·
                        haem protein/   ↘LDLOO·
                        transition metal

                                        ⌠   ⌡
                                       LDL   H-DONATING
                                                    ANTIOXIDANT

                                        ↓   ↓

                              LDLOOH/LDLOH   LDLOOH/
                                    +   LDLOH
                                              more LDL
                              LDL· → oxidation

Scheme 4. A schematic representation of the fate of LDL hydroperoxides.

Table 4. The abilities of the catechin/gallate polyphenols as chain-breaking antioxidants against propagating lipid peroxides. [LDL] = 125 μg/ml; metmyoglobin 5 μM; incubation time 5 h.

	Concentration for 50% inhibition of peroxidation (μM)
Epicatechin gallate	0.25
Epicatechin	0.30
Catechin	0.30
Epigallocatechin gallate	0.38
Epigallocatechin	0.75
Gallic acid	1.2

50% inhibition of maximal oxidation, epigallocatechin 0.75 μM, whereas catechin, epicatechin, epicatechin gallate (ECG) and epigallocatechin gallate (EGCG) were all very similar with values ranging from 0.25 to 0.38 μM. A similar sequence is seen in the inhibition of altered relative electrophoretic mobility, as expected.

The catechin/gallate family of compounds was also studied for their ability to spare vitamin E and protect it from oxidation. LDL contains a number of endogenous antioxidants including α- and γ-tocopherols, β-carotene, lycopene, etc. The reduction potentials of flavonoid radicals are higher than that of Trolox which means that their reaction with vitamin E is thermodynamically feasible [48]. Monitoring the consumption of vitamin E in LDL when challenged with a pro-oxidant in the form of metmyoglobin in the presence of the polyphenols (2 μM)

Table 5. Effects of catechin and catechin-gallate esters (2 μM) on the time to consumption of α-tocopherol in LDL on pro-oxidant challenge with 5 μM metmyoglobin. [LDL] = 125 μg/ml.

Additions	Time (h)
Control (no catechin)	1
Gallic acid	1.5
Epigallocatechin	1.5
Epicatechin	2
Epicatechin gallate	4
Epigallocatechin gallate	4

demonstrates (Table 5) that gallic acid and epigallocatechin are indeed the least effective in sparing the vitamin E, reflecting their lesser contribution to increasing the resistance of LDL to oxidation, whereas the delay in consumption of the LDL-vitamin E was prolonged by EGCG and ECG. Flavonoid aglycones are rather lipophilic antioxidants although generally more hydrophilic than α-tocopherol. It has been hypothesized that catechins might be localized near the membrane surface scavenging aqueous radicals and preventing the consumption of tocopherol, whereas α-tocopherol mainly acts as a chain-breaking lipid peroxyl radical scavenger within the LDL [31].

The study of the catechins is particularly important for the understanding of the antioxidant properties of tea, since the total polyphenolic content of green and black teas is 44–45%, of which the catechins constitute 26.7% of the tea solid content of freeze-dried green tea, i.e. approx. 60% of the total polyphenolic composition of green tea [49].

Conclusions

These studies along with those from other laboratories help to identify the active ingredients in beverages, vegetables and fruit that may protect against radical damage and LDL oxidation, implicated in the pathogenesis of coronary heart disease, platelet aggregation and endothelium-dependent vasodilatation of the arteries. This might be useful information not only from the point of view of identifying appropriate foods that are rich in these protective components for the consumer but also presents opportunities for the development of safe food products and additives with appropriate antioxidant properties. Evidence exists that catechin is absorbed by the human gut [42] and that quercetin might reach levels of up to 1 μM in human plasma [50] although the findings are conflicting. Others studies have reported that quercetin after oral administration in humans is not detected in plasma or urine but $\sim 50\%$ is recovered in the faeces suggestive of extensive degradation [51]. Dietary studies in rats have shown a reduction in

mammary tumours [52] and suggested only 20% is absorbed from the gastro-intestinal tract [53]. Others report that the major portion of ingested flavonoids (44%) is present in the gastrointestinal tract before excretion in the bile. However, there is a paucity of information on the absorption, metabolism and excretion of these compounds in man and much work needs to be done in this area.

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