Inhibition of lipid peroxidation by quercetin and quercetin derivatives: antioxidant and prooxidant effects

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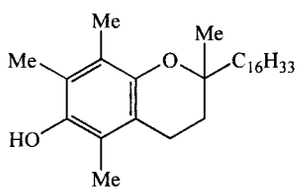
Flavonoids (polyphenols) are an important class of dietary antioxidants largely distributed in plants. In spite of their very fast reaction with lipid peroxyl radicals, flavonoids are relatively modest inhibitors of lipid peroxidation in solution. In addition, the absence of lag phase and the strong dependence of the antioxidant efficiency (the ratio of the rate constant of chain break to the rate constant of chain propagation) on the antioxidant concentration both point to a *nonclassic* behaviour. The inhibition of linoleic acid peroxidation by quercetin (3,3′,4′,5,7-pentahydroxyflavone) and its 3-*O*-glycoside rutin (two ubiquitous dietary flavonoids and potent antioxidants) has been investigated by UV–visible spectroscopy in pH 7.4 micellar solutions of SDS at 37 °C. The chain-breaking antioxidant α-tocopherol and two synthetic amphiphilic quercetin ethers are also considered for comparison purposes. From the general mathematical treatment developed in this work, flavonoids appear as strong chain-breaking antioxidants whose overall antiperoxidizing activity is, however, strongly restricted by the chain-initiating activity of the flavonoid radicals formed during inhibition (prooxidant effect).

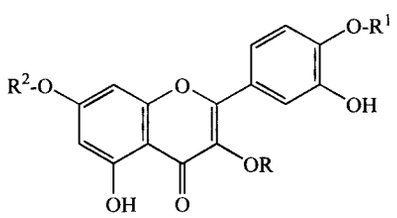
Introduction

Oxygen-centered free radicals (*e.g.*, hydroxyl, alkoxyl and alkylperoxyl radicals) may arise, for instance, from incomplete reduction of dioxygen in the electron transport chain or from the catalytic cycle of redox enzymes involved in purine and lipid metabolism or in antibacterial defense.[1] Degradation of biomolecules by such radicals is involved in various diseases including atherosclerosis, cancers, and neurodegenerescence, as well as the ageing process. In particular, peroxidation of polyunsaturated fatty acids (PUFA) present in plasma low-density lipoproteins (LDL) is known to trigger an immune response during which the modified LDL are internalized by macrophages. In this process, the macrophages become loaded with cholesteryl esters and turn into giant *spumous cells* which tend to deposit on the artery walls and initiate clots and lesions.[2] Being a radical chain mechanism, lipid peroxidation may proceed even in the presence of very low concentrations of initiating free radicals. It is thus a particularly pernicious phenomenon which must be fought as a priority by defense mechanisms including the action of antioxidants.[3]

Flavonoids[4] are a broad class of plant polyphenols generally endowed with *in vitro* antioxidant activity. Recent epidemiological studies strongly suggest that flavonoid-rich diets reduce the risk of death from coronary heart desease.[5] Surprisingly, whereas flavonoids rank among the antioxidants which react fastest with lipid peroxyl radicals (kinetics close to the diffusion-controlled limit),[6] they are relatively modest chain-breaking antioxidants, in any case much less potent than the endogenous membrane-bound antioxidant α-tocopherol (vitamin E).[7] This paradox has not so far found a completely satisfying explanation. This work is a UV–visible spectroscopic investigation of the inhibition of linoleic acid peroxidation by quercetin (3,3′,4′,5,7-pentahydroxyflavone), its 3-*O*-glycoside rutin and two amphiphilic quercetin ethers in pH 7.4 micellar solutions of sodium dodecyl sulfate (SDS) at 37 °C. A general mathematical treatment, including the initiation step of lipid peroxidation by flavonoid radicals (a *prooxidant* effect), allows us to propose a quantitative interpretation of the *flavonoid paradox* and to re-evaluate the efficiency of flavonoids as chain-breaking antioxidants.



α-tocopherol



$R = R^1 = R^2 = H$: *quercetin*
$R^1 = R^2 = H$, R = L-Rha-α-1,6-D-Glc-β: *rutin*
$R = R^2 = H$, $R_1 = n\text{-}C_{16}H_{33}$: **1**
$R = R^1 = H$, $R^2 = n\text{-}C_{12}H_{25}$: **2**

Results and discussion

Flavonoid antioxidants may act by a variety of ways including direct trapping of oxygen-centered radicals, inhibition of enzymes involved in their production, chelation of transition metal ions involved in radical-forming processes such as the Fenton reaction, and regeneration of membrane-bound antioxidants such as α-tocopherol (vitamin E).[4,6,8] Several

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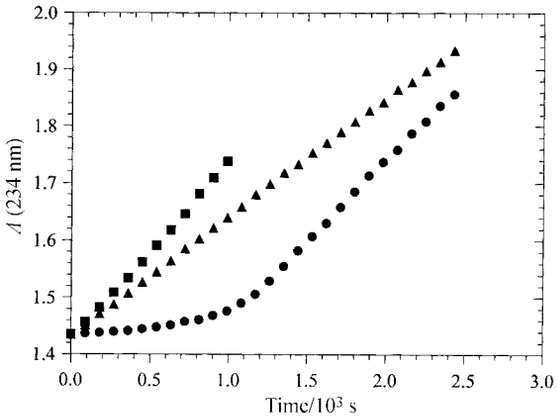

Fig. 1 Kinetic traces monitoring the formation of linoleic acid hydroperoxides during peroxidation inhibited by quercetin (4.8×10^{-7} mol dm^{-3}, *triangles*) or α-tocopherol (3.8×10^{-7} mol dm^{-3}, *circles*), *squares*: no antioxidant. Linoleic acid: 2.5×10^{-3} mol dm^{-3}. AAPH: 10^{-3} mol dm^{-3}. SDS: 0.1 mol dm^{-3}, pH 7.4, 37 °C.

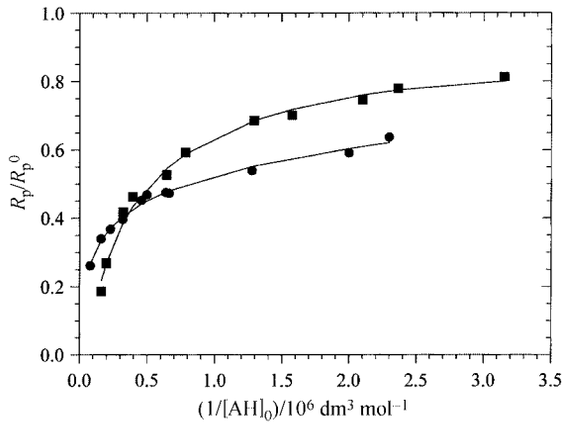

Fig. 2 Plots of the relative rate of inhibited peroxidation (R_p^0: peroxidation rate in the absence of antioxidant) as a function of the reciprocal of the initial antioxidant concentration for quercetin (*squares*) and rutin (*circles*) on a large range of antioxidant concentration. Linoleic acid: 2.5×10^{-3} mol dm^{-3}. AAPH: 10^{-3} mol dm^{-3}. SDS: 0.1 mol dm^{-3}, pH 7.4, 37 °C.

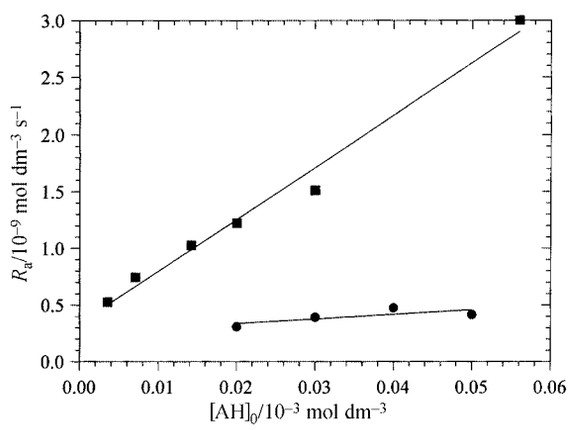

Fig. 3 Plot of the initial rate of flavonoid consumption as a function of the initial flavonoid concentration for quercetin (*squares*) and rutin (*circles*). AAPH: 10^{-3} mol dm^{-3}. SDS: 0.1 mol dm^{-3}, pH 7.4, 37 °C.

flavonoids including quercetin are known to efficiently inhibit lipid peroxidation (initiated by oxygen-centered radicals, metal ions or macrophages) in LDL,[9] in model solutions of linoleic acid[10] as well as in phospholipid bilayers.[11]

Quercetin and rutin have been selected because they rank among the most potent flavonoid antioxidants[9a,10a,12] and because they are ubiquitous in plants and relatively abundant in human diet.[13] In addition, conjugates (sulfoglucuronides) of quercetin and its 3′-methyl ether (which retain potent antioxidant properties) are recovered in significant concentrations (0.1–1 μmol dm^{-3}) in the plasma of humans after a meal rich in plant products.[14]

When quercetin and its derivatives are tested for their ability to inhibit the peroxidation of linoleic acid (2.5×10^{-3} mol dm^{-3}) initiated by AAPH (2,2′-azobis(2-amidinopropane) dihydrochloride, 10^{-3} mol dm^{-3}) in a pH 7.4 micellar solution of SDS (0.1 mol dm^{-3}) at 37 °C, their behaviour very significantly departs from that of typical chain-breaking antioxidants such as α-tocopherol.[15] For instance, when added to the peroxidizing mixture, α-tocopherol almost completely quenches the formation of lipid hydroperoxides (detected spectroscopically owing to the typical absorption of their conjugated diene moiety at 234 nm). A clearcut lag phase (period T) can be defined during which peroxidation is very slow. When α-tocopherol approaches consumption, peroxidation resumes and quickly reaches its rate before inhibition.[15] Unlike α-tocopherol, quercetin and its derivatives do not display a clearcut lag phase (Fig. 1) in agreement with previous observations with a variety of flavonoids.[7,16]

In the case of a typical chain-breaking antioxidant such as α-tocopherol, the initial rate of inhibited peroxidation R_p increases linearly as a function of the reciprocal of the initial antioxidant concentration $[AH]_0$ (*classic behaviour*).[15a] The slope of such plots is inversely proportional to the so-called antioxidant efficiency (AE_1) which is defined as the k_1/k_p ratio, k_1 and k_p being the rate constants of chain breaking and chain propagation, respectively (Scheme 1). In the case of quercetin and its derivatives, the R_p *vs.* $1/[AH]_0$ plots are linear at low antioxidant concentrations (lower than 1–2 × 10^{-6} mol dm^{-3}) only. For higher concentrations, strong deviations from linearity were observed, the rate of hydroperoxide formation becoming much lower than anticipated from the classic law (Fig. 2). Thus, everything happens as if the antioxidant efficiency (normally, a constant parameter) decreases with increasing antioxidant concentrations. This behaviour was observed with all flavonoids tested in this work. Similar abnormalities have been already pointed out during the investigation of the inhibition by flavonoids of methyl linoleate peroxidation in chlorobenzene at 50 °C with apparent AE_1 values for quercetin in the range 20–590 depending on the antioxidant concentration.[7] In pH 7.4 micellar solutions of SDS, AE_1 values of 110[7] and 340[15a] have been estimated at 37 °C.† Besides the discrepancies between these two values (which once more may be due to differences in the range of antioxidant concentration investigated), both are much lower than the AE_1 value of the order of 10^5 which can be calculated from the estimation of k_1 by pulse radiolysis experiments[6] ($k_1 = 18 \times 10^6$ dm^3 mol^{-1} s^{-1} at 25 °C in a pH 11.5 aqueous solution, k_p *ca.* 100 dm^3 mol^{-1} s^{-1}).

Inhibition by quercetin

In a pH 7.4 phosphate buffer (37 °C) containing AAPH (10^{-3} mol dm^{-3}) and SDS (0.1 mol dm^{-3}), the initial rate of quercetin consumption (R_a) displays a linear dependence on the concentration of quercetin (Fig. 3). Taking $\varepsilon = 19800$ dm^3 mol^{-1} cm^{-1} at λ_{max} (373 nm), R_a values in the range 5×10^{-10}–3×10^{-9} mol dm^{-3} s^{-1} were calculated for quercetin concentrations in the range 4×10^{-6}–6×10^{-5} mol dm^{-3}. The spectral changes (decrease of the quercetin absorption bands at 373 and 270 nm with the concomitant raising of a new band at 330 nm) are consistent with the general mechanism of H atom abstraction from quercetin followed by fast radical disproportionation and fast solvent addition on the quercetin quinone.[17] The rate of quercetin consumption is not significantly altered by the presence of linoleic acid (2.5×10^{-3} mol dm^{-3}).

† Under the same conditions, AE_1 values 3–6 times as large have been found in the case of α-tocopherol.[15a]

Table 1 H atom transfer reactions from flavonoids to DPPH at 25 °C. Stoichiometries (n) and average rate constants (k) are deduced from curve-fittings of the kinetic traces at λ_{max}(DPPH) (for details, see ref. 17). Typical time intervals: 60–100 s

Flavonoid	Quercetin[a]	Rutin[a]	**1**[b]	**2**[b]
DPPH/equiv.	4	4	6	6
k/dm^3 mol^{-1} s^{-1}	723 (±15) 3347 (±56)[c]	669 (±9) 1028 (±24)[c]	518 (±6)	1086 (±15)
n	3.19 (±0.02) 2.02 (±0.01)[c]	2.12 (±0.01) 1.91 (±0.01)[c]	1.30 (±0.01)	2.03 (±0.01)
DPPH/equiv.	8	8	8	8
k/dm^3 mol^{-1} s^{-1}	583 (±13)	718 (±7)	335 (±6)	423 (±6)
n	3.61 (±0.02)	2.33 (±0.01)	1.59 (±0.01)	2.84 (±0.01)
n_{tot}[d]	6.8	5.9	1.9	3.75

[a] From ref. 17, in MeOH unless otherwise specified. [b] In Bu^{n}OH. [c] This work, in MeOH–pH 7.4 phosphate buffer (1 : 1). [d] Determined over 10–30 min using $n_{tot} = c_0(1 - A_f/A_0)/c$ (A_f: visible absorbance at the end of the kinetic run, A_0: initial absorbance, c: initial antioxidant concentration, c_0: initial DPPH concentration). [e] For α-tocopherol in MeOH (DPPH–antioxidant molar ratio = 6), k = 367 (±5) dm^3 mol^{-1} s^{-1}, n = 2.70 (±0.01).

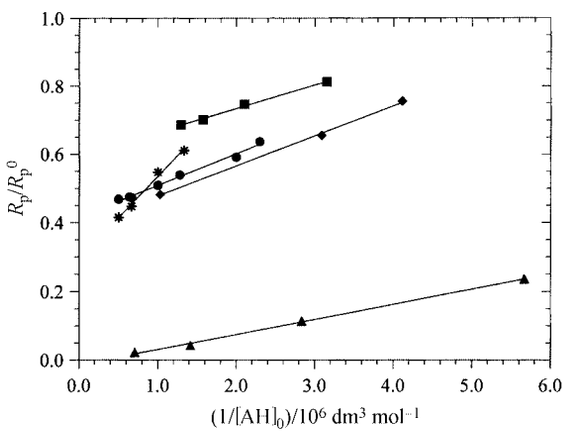

Fig. 4 Plots of the relative inhibited peroxidation rate (R_p^0: peroxidation rate in the absence of antioxidant) as a function of the reciprocal of the initial antioxidant concentration for quercetin (*squares*), rutin (*circles*), **1** (*diamonds*), **2** (*asterisks*) and α-tocopherol (*triangles*) in the range of low antioxidant concentrations. Linoleic acid: 2.5×10^{-3} mol dm^{-3} except for **2** (1.8×10^{-3} mol dm^{-3}). AAPH: 10^{-3} mol dm^{-3}. SDS: 0.1 mol dm^{-3}, pH 7.4, 37 °C.

Inhibition by rutin

Unlike quercetin, rutin is very slowly consumed in a pH 7.4 phosphate buffer (37 °C) containing AAPH (even in the absence of SDS) or in the peroxidizing mixture (AAPH, linoleic acid, SDS). Taking ε = 13500 dm^3 mol^{-1} cm^{-1} at λ_{max} (362 nm), R_a values in the range 3×10^{-10}–5×10^{-10} mol dm^{-3} s^{-1} were calculated for rutin concentrations in the range 2×10^{-5}–5×10^{-5} mol dm^{-3}. Surprisingly, the peroxidation rate in the presence of a constant (low) antioxidant concentration is slightly lower with rutin than with quercetin (Fig. 2), suggesting that rutin, in spite of its more hydrophilic character, is a better antioxidant than quercetin in this model. Similar observations were made in nonmicellar phosphate buffer (pH 7.4)–Bu^{t}OH (9 : 1) mixtures.

Inhibition by amphiphilic quercetin ethers

Linking a long hydrocarbon chain to the quercetin nucleus was aimed at facilitating the penetration of the antioxidant into the micelles. It was thus expected to favour a tocopherol-like antioxidant mechanism for the flavonoids. This is not the case: amphiphilic quercetin ethers qualitatively behave like quercetin. In particular, no lag phase is observed. When the peroxidation rates are compared at a constant antioxidant concentration, the amphiphilic ethers do not appear significantly more potent than quercetin or rutin (Fig. 4). In the whole concentration range investigated, quercetin and its hydrophilic or amphiphilic derivatives are all much less effective than α-tocopherol at reducing the rate of linoleic acid peroxidation.

DPPH test

DPPH (diphenylpicrylhydrazyl) is a highly coloured commercially available radical which can be used for estimating antioxidant stoichiometries (number of radicals trapped per antioxidant molecule).[17] When monitoring the H transfer reactions from a flavonoid antioxidant to DPPH by UV-vis spectroscopy, two steps can be typically distinguished: a first step during which the DPPH visible absorbance (λ_{max} = 516 nm in MeOH) quickly decays (typical time interval in MeOH: 60–90 s) and a second step during which the DPPH visible absorbance slowly decays to a final constant value (typical time interval in MeOH > 10 min). The fast step essentially refers to abstractions of the most labile H atoms (O3-H, O3′-H, O4′-H in the case of quercetin[17]) whereas the slow step reflects the remaining activity in the oxidation-degradation products. A total stoichiometry (n_{tot}) can be determined in a static way from the overall amplitude of the kinetic run. A *kinetic* stoichiometry n can also be estimated from the curve-fitting of the kinetic traces featuring the decay of the DPPH band during the fast step. To that purpose, an antioxidant of stoichiometry n is simply modeled as n independent *sub*-units AH (typically, flavonoid OH groups bearing most labile H atoms) which all transfer an H atom to DPPH with the same second-order rate constant k.[17] The k value provides a quantitative estimation of the overall reactivity of the antioxidant toward DPPH during the fast step of trapping. Whereas antioxidant efficiencies estimated from inhibition of lipid peroxidation in micelles may be governed by many factors including the hydrophilic–lipophilic balance of the antioxidants,[18] parameters k and n in the DPPH test afford a simple way to assess intrinsic antioxidant efficiencies. Table 1 clearly shows that 7-*n*-dodecylquercetin (**2**) is a more efficient antioxidant than 4′-*n*-hexadecylquercetin (**1**) from both viewpoints of reactivity and stoichiometry. This is consistent with the accepted view that a 1,2-dihydroxy substitution in the B ring is favourable to the antioxidant activity.[8b,12,19] Interestingly, whereas quercetin and rutin roughly display the same reactivity toward DPPH in MeOH, quercetin turns out to be significantly more reactive than rutin in MeOH–pH 7.4 phosphate buffer (1 : 1), probably because of partial dissociation of O3-H.

Kinetics of antioxidant consumption

The decay of an antioxidant (noted AH) of stoichiometry n during inhibited peroxidation must take place according to eqn. (1) which can be integrated as eqn. (2).[15] Eqns. (1) and (2)

$$R_a = -d[AH]/dt = R_i/n \quad (1)$$

$$[AH] = [AH]_0 - R_i t/n \quad (2)$$

are also valid in the absence of linoleic acid because of the reaction of the antioxidant with the initiator-derived peroxyl radicals (inhibition of initiation).

Using initial flavonoid concentrations large enough (higher than 5×10^{-6} mol dm^{-3}) to allow spectroscopic detection during inhibited peroxidation, the plot of the flavonoid concentration as a function of time must be a straight line with a slope equal to $-R_i/n$. However, the second term in eqn. (2) is much smaller than $[AH]_0$ even over periods as long as 1–2 hours. Hence, the flavonoid band should not significantly decrease during inhibited peroxidation. This is roughly verified for rutin. By contrast, the consumption of the corresponding aglycone quercetin is faster than accounted for by eqn. (1).

Autoxidation of quercetin typically occurs in alkaline aqueous or nonaqueous solutions[17] and happens by the transfer of one electron from the HOMO of quercetin anions to the half-occupied π* orbitals of dioxygen with subsequent formation of quercetin radical anions,[8g,20] superoxide and hydrogen peroxide.[21] The autoxidation kinetics are critically dependent on deprotonation of O3-H[17] which, from semi-empirical quantum mechanics, must be the third most acidic OH group of quercetin (after O7-H and O4′-H) with an experimental pK_a (assessed by potentiometric and spectrometric method) in the range 8.0–8.2 at 20 °C.[22] Hence, quercetin autoxidation could be significant even at physiological pH whereas autoxidation of rutin, in which O3-H is glycosylated, requires much more alkaline conditions.[17] Hence, it is proposed that quercetin autoxidation is actually responsible for the fast consumption of quercetin during inhibited peroxidation. Consequently, the rate of quercetin consumption must be expressed as eqn. (3) (k_a: apparent first-order rate constant of autoxidation).

$$R_a = -d[AH]/dt = R_i/n + k_a[AH] \quad (3)$$

Eqn. (3) accounts for the concentration dependence of the initial rate of quercetin consumption on the initial quercetin concentration. Plotting R_a as a function of $[AH]_0$ actually gives a straight line (Fig. 3) from which R_i/n and k_a can be estimated: $R_i/n = 3.4 \times 10^{-10}$ mol dm^{-3} s^{-1}, $k_a = 4.6 \times 10^{-5}$ s^{-1} (in comparison, R_a values for rutin are much lower and essentially independent of the antioxidant concentration). Similar k_a values were also found by simply monitoring the decay of the quercetin absorption band in the pH 7.4 SDS solution at 37 °C. From the R_i value calculated from the tocopherol lag phase,‡ a stoichiometry close to 2 was found for quercetin. This value is close to the stoichiometry estimated in the quantitative DPPH test at the end of the fast step (Table 1). This suggests that the oxidation-degradation products of quercetin do not appreciably take part in the inhibition of lipid peroxidation. In the case of rutin, lower and almost concentration-independent R_a values were measured in the range $3–5 \times 10^{-10}$ mol dm^{-3} s^{-1} (close to the above-reported R_i/n value for quercetin) as expected from the observation that rutin autoxidation is not significant in such conditions.

General mathematical treatment

The general mechanism of inhibited lipid peroxidation is reported in Scheme 1 (LH: linoleic acid, XN=NX: diazo initiator AAPH, AH: antioxidant, Q: flavonoid quinone).

The following assumptions were made:

$$X–N=N–X \longrightarrow 2eX^{\bullet} + (1 - e)X_2 + N_2$$
$$X^{\bullet} + O_2 \longrightarrow XOO^{\bullet}$$
$$XOO^{\bullet} + LH \xrightarrow{k_i} XOOH + L^{\bullet}$$
$$L^{\bullet} + O_2 \xrightarrow{k} LOO^{\bullet}$$
$$LOO^{\bullet} + LH \xrightarrow{k_p} LOOH + L^{\bullet}$$
$$LOO^{\bullet} + AH \xrightarrow{k_1} LOOH + A^{\bullet}$$
$$XOO^{\bullet} + AH \xrightarrow{k_2} XOOH + A^{\bullet}$$
$$LH + A^{\bullet} \xrightarrow{k'_i} AH + L^{\bullet}$$
$$2\,A^{\bullet} \xrightarrow{k_d} AH + Q \text{ and/or dimers}$$
$$AH \xrightarrow{k_a, O_2} A^{\bullet}$$

Scheme 1

(i) A steady state is achieved for all radicals.

(ii) The concentration of oxygen-sensitive flavonoids, such as quercetin, is low enough for autoxidation to be neglected. More precisely, the initial concentration of flavonoid must be kept much lower than $R_i/(nk_a)$ (see eqn. (3)) which is close to 10^{-5} mol dm^{-3} in the case of quercetin.

(iii) Flavonoid radicals essentially react through disproportionation or dimerization in agreement with pulse radiolysis investigations, which point to very fast second-order decays of flavonoid radicals in alkaline aqueous solutions.[23] Quinone intermediates that can be trapped as benzenesulfinate or solvent adducts have been evidenced in the reaction of quercetin and rutin with DPPH in methanol.[17] Alternatively, quercetin dimers are formed in high yield (56%) during the reaction of quercetin with the peroxyl radicals derived from 2,2′-azobis(2-methylpropionitrile) in ethyl acetate.[24] HPLC analysis suggests that such dimers (that have been completely characterized by NMR and mass) also formed in the course of the inhibited peroxidation of linoleic acid in similar conditions. In the micellar aqueous system used in this work, the spectral changes in the range 300–400 nm recorded at high antioxidant concentrations during inhibition by quercetin point to the formation of quinone–solvent adducts[17] and are thus more consistent with disproportionation.§ The overall process of H transfer from the flavonoids (noted QH_2 here for stoichiometry purposes) to the peroxyl radicals and subsequent disproportionation of the flavonoid radicals may be written as $QH_2 + 2LOO^{\bullet} \rightarrow Q + 2LOOH$ and is thus consistent with a stoichiometry of 2. Expressing the steady state conditions for radicals $L^{\bullet}$, $LOO^{\bullet}$, $XOO^{\bullet}$ and $A^{\bullet}$ yields eqns. (4)–(7).

$$k[L^{\bullet}][O_2] = k_i[LH][XOO^{\bullet}] + k_p[LOO^{\bullet}][LH] + k'_i[LH][A^{\bullet}] \quad (4)$$

$$k[L^{\bullet}][O_2] = k_1[LOO^{\bullet}][AH] + k_p[LOO^{\bullet}][LH] \quad (5)$$

$$R_i = k_2[AH][XOO^{\bullet}] + k_i[LH][XOO^{\bullet}] \quad (6)$$

$$k_1[LOO^{\bullet}][AH] + k_2[AH][XOO^{\bullet}] = 2k_d[A^{\bullet}]^2 + k'_i[LH][A^{\bullet}] \quad (7)$$

Combining eqns. (4) and (5) yields eqn. (8).

$$k_1[LOO^{\bullet}][AH] = k_i[LH][XOO^{\bullet}] + k'_i[LH][A^{\bullet}] \quad (8)$$

Combining eqns. (6), (7) and (8) yields eqn. (9).

$$R_i = 2k_d[A^{\bullet}]^2 \quad (9)$$

‡ Since the stoichiometry of α-tocopherol is 2, the radical flow R_i can be estimated from the lag phase T using the following relationship, which is a particular case of eqn. (1): $c_0/T = R_i/2$ (c_0: initial α-tocopherol concentration). A R_i value of 7×10^{-10} mol dm^{-3} s^{-1} was thus calculated and used throughout this work.

§ The radicals derived from 4′-*n*-hexadecylquercetin probably decay *via* dimerization since alkylation of the 4′-hydroxy group suppresses the possibility of *o*-quinone or *p*-quinonoid formation.

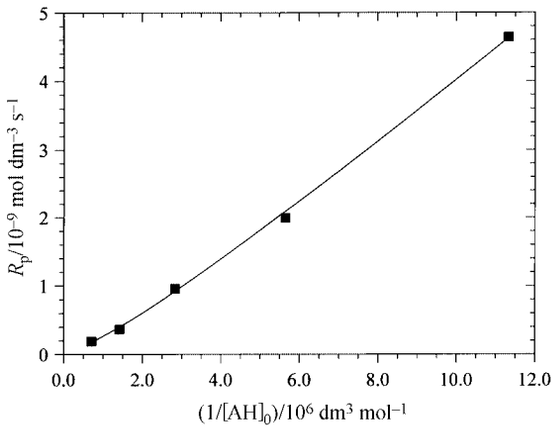

Fig. 5 Plots of the inhibited peroxidation rate as a function of the reciprocal of the initial concentration of α-tocopherol. Linoleic acid: 2.5×10^{-3} mol dm^{-3}. AAPH: 10^{-3} mol dm^{-3}. SDS: 0.1 mol dm^{-3}, pH 7.4, 37 °C. The solid line is the result of the curve-fitting procedure (*Scientist* program from *MicroMath*, Salt Lake City, USA) according to eqn. (13).

The rate of hydroperoxide formation can be expressed as eqn. (10).

$$R_p = d[LOOH]/dt = k_p[LOO^\bullet][LH] + k_1[LOO^\bullet][AH] \quad (10)$$

A combination of eqns. (6), (8)–(10) finally yields eqn. (11)

$$R_p = \left(1 + \frac{c}{AE_1[AH]}\right)\left(\frac{R_i}{1 + AE_2[AH]/c} + B\right) \quad (11)$$

(c: total linoleic acid concentration, $AE_1 = k_1/k_p$, $AE_2 = k_2/k_i$, $B = k'_i c(R_i/2k_d)^{1/2}$).

At low antioxidant concentrations ($[AH] \ll c/AE_2$), the inhibition of initiation can be neglected and eqn. (11) simplifies to eqn. (12).

$$R_p = \left(1 + \frac{c}{AE_1[AH]}\right)(R_i + B) \quad (12)$$

In the case of α-tocopherol, the mechanism displayed on Scheme 1 does not strictly hold, since the fate of the α-tocopheryl radical is reaction with a second peroxyl radical to give nonradical products rather than disproportionation. Assuming a stoichiometry of 2 consistent with this mechanism and no initiation of the propagation chain by the α-tocopheryl radical ($k'_i = 0$) in agreement with the occurrence of a lag phase in the beginning of inhibited peroxidation (*classic* behaviour), the mathematical treatment yields eqn. (13).

$$R_p = \frac{R_i}{1 + 2AE_2[AH]/c}\left(1 + \frac{c}{2AE_1[AH]}\right) \quad (13)$$

From eqn. (13), the peroxidation rate is expected to fall to zero at high tocopherol concentration and not R_i as would be expected if inhibition of initiation was neglected ($AE_2 = 0$). This is well verified since peroxidation rates lower than the initiation rate are actually reached with tocopherol concentrations higher than 5×10^{-7} mol dm^{-3}. Fitting the R_p *vs.* $1/[AH]_0$ plot against eqn. (13) for α-tocopherol (Fig. 5) gives AE_1 and AE_2 values of 1950 and 5750, respectively,¶ thus suggesting that inhibition of initiation is quite effective.

In the case of quercetin and its derivatives, the plots of the initial rate of inhibited peroxidation as a function of the reciprocal of the initial antioxidant concentration $[AH]_0$ (in the range 1×10^{-7}–1.4×10^{-6} mol dm^{-3}) give straight lines in agreement with eqn. (12) (Fig. 4). The intercepts are consistently much higher than the expected value of R_i for a classic behaviour (without inhibition of initiation). The difference between the observed and expected intercept values provides an estimate for parameter B from which the $k'^2_i/(2k_d)$ ratio can be easily calculated (Table 2). Moreover, the antioxidant efficiency is directly obtained from the intercept:slope ratio AE_1/c. Remarkably, the AE_1 value of quercetin (*ca.* 2×10^4) is only one order of magnitude lower than that calculated from absolute rate constants in nonmicellar (alkaline) solutions. In addition, using the k_d value estimated for quercetin from pulse radiolysis experiments in a pH 11.5 aqueous solution[23] ($2k_d = 3.4 \times 10^6$ dm^3 mol^{-1} s^{-1} at 25 °C), a k'_i value (*ca.* 140 dm^3 mol^{-1} s^{-1}) of the same order of magnitude as k_p can be calculated.

Table 2 Kinetic parameters deduced from the plot of the initial rate of hydroperoxide formation as a function of the reciprocal of the initial antioxidant concentration (1×10^{-7}–1.4×10^{-6} mol dm^{-3}) according to eqn. (12). AAPH: 10^{-3} mol dm^{-3}, linoleic acid: 2.5×10^{-3} mol dm^{-3}, SDS: 0.1 mol dm^{-3}, $R_i = 7 \times 10^{-10}$ mol dm^{-3} s^{-1}, pH 7.4, 37 °C. An average non inhibited peroxidation rate (R_p^0) of 9.6×10^{-9} mol dm^{-3} s^{-1} is used in the calculations

Anti-oxidant	AE_1	B/mol dm^{-3} s^{-1}	$k'^2_i(2k_d)^{-1}$ dm^3 mol^{-1} s^{-1}
Quercetin	$21.6\ (\pm1.3) \times 10^3$	$5.02\ (\pm0.07) \times 10^{-9}$	5.7×10^{-3}
Rutin	$11.3\ (\pm0.6) \times 10^3$	$3.30\ (\pm0.05) \times 10^{-9}$	2.5×10^{-3}
1	$11.1\ (\pm0.7) \times 10^3$	$3.04\ (\pm0.09) \times 10^{-9}$	2.1×10^{-3}
2[a]	$2.2\ (\pm0.3) \times 10^3$	$1.3\ (\pm0.1) \times 10^{-9}$	7.5×10^{-4}
α-Toco-pherol[b]	1950	—	—

[a] Linoleic acid: 1.8×10^{-3} mol dm^{-3}, $R_p^0 = 6.9 \times 10^{-9}$ mol dm^{-3} s^{-1}. [b] From eqn. (13).

If the flavonoid radicals were to react with the lipid peroxyl radicals to form quinone and hydroperoxide molecules (rate constant k'_1) rather than recombine through disproportionation or dimerization, eqns. (6), (8)–(10) should be changed into eqns (14)–(16) (assuming negligible inhibition of initiation).

$$k_1[LOO^\bullet][AH] + k'_1[LOO^\bullet][A^\bullet] = k_i[LH][XOO^\bullet] + k'_i[LH][A^\bullet] \quad (14)$$

$$R_i = k_i[LH][XOO^\bullet] = 2k'_1[A^\bullet][LOO^\bullet] \quad (15)$$

$$R_p = k_p[LOO^\bullet][LH] + k_1[LOO^\bullet][AH] + k'_1[LOO^\bullet][A^\bullet] \quad (16)$$

Combining these equations gives eqn. (17).

$$R_p = \frac{R_i}{2}\left\{1 + \frac{1}{2}\left(1 + \frac{c}{AE_1[AH]}\right)\left(1 + \sqrt{1 + 8c[AH]\frac{k_1k'_i}{k'_1R_i}}\right)\right\} \quad (17)$$

In the absence of chain initiation by flavonoid radicals ($k'_i = 0$), eqn. (17) simplifies to eqn. (18) which is equivalent to

$$R_p = R_i\left(1 + \frac{c}{2AE_1[AH]}\right) \quad (18)$$

eqn. (13) with the additional assumption $AE_2 = 0$ (no inhibition of initiation).

Unlike eqn. (12), eqn. (17) introduces a correction term which is dependent on the antioxidant concentration. It cannot account for the linearity of the R_p *vs.* $1/[AH]_0$ plots at low antioxidant concentrations (nor a more complicated version making allowance for inhibition of initiation). This is additional evidence that the fate of flavonoid radicals in the

¶ The AE_1 value is in very good agreement with that reported in the literature[15a] using a simplified version of eqn. (13): $R_p = cR_i/(2AE_1[AH])$.

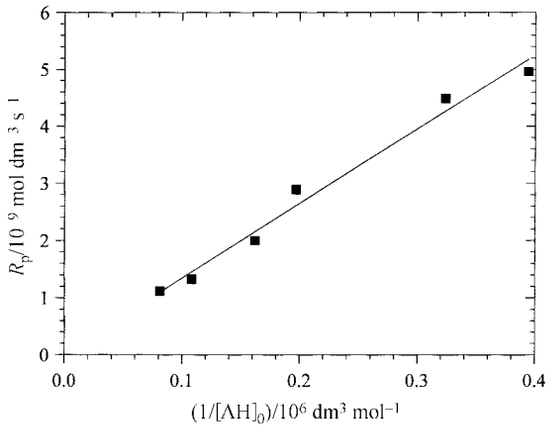

Fig. 6 Plots of the inhibited peroxidation rate as a function of the reciprocal of the initial concentration of quercetin in the range of high antioxidant concentrations. Linoleic acid: 2.5×10^{-3} mol dm^{-3}. AAPH: 10^{-3} mol dm^{-3}. SDS: 0.1 mol dm^{-3}, pH 7.4, 37 °C.

course of inhibited peroxidation essentially consists of radical recombination to give dimers and/or quinones (disproportionation).

Eqn. (11), or a more complicated version taking into account flavonoid autoxidation, failed to explain the strong deviations from linearity in the R_p *vs.* $1/[AH]_0$ plots observed at high antioxidant concentrations, especially in the case of quercetin. However, such deviations can be accounted for if one of the two following hypothesis are assumed:

(i) A side-reaction between the linoleic acid hydroperoxide and quercetin.

(ii) A reversibility of the prooxidant effect at high antioxidant concentration.

Since no evidence for reaction of quercetin with the cumylhydroperoxide could be gained, the latter hypothesis seems more probable. If H abstraction from linoleic acid by the quercetin radicals is actually reversible, it may be cancelled out by the reverse reaction at high quercetin concentrations. Since the term $c/(AE_1[AH]_0)$ is much lower than 1 for quercetin concentrations higher than 10^{-6} mol dm^{-3}, eqn. (11) simplifies to eqn. (19), which reasonably accounts for the linear portion of

$$R_p = \frac{cR_i}{AE_2[AH]} \qquad (19)$$

the R_p *vs.* $1/[AH]_0$ plot (with *ca.* zero intercept) at high antioxidant concentrations.

From the slope of this linear portion (Fig. 6), a AE_2 value of *ca.* 130 could be estimated, which is very close to that previously estimated, for quercetin in similar conditions.[7] Hence, at high antioxidant concentrations, flavonoids may exert their antiperoxidizing activity essentially *via* inhibition of initiation.

In spite of its lower intrinsic antioxidant activity due to alkylation of the reactive catechol group, 4′-*n*-hexadecylquercetin (**1**) is a better chain breaker (higher AE_1 value) than 7-*n*-dodecylquercetin (**2**). This suggests that the location of flavonoids within the micelles is an important factor. The hydrocarbon tail of **1** confers a hydrophobic character on the antioxidant moiety (ring B) and probably buries it in the micelles, thereby favouring H transfer to the lipid peroxyl radicals. On the contrary, the hydrocarbon tail of **2** would favour the location of ring A in the micelles, the relatively hydrophilic antioxidant moiety now protruding outside. In spite of its favourable positioning in the micelles, **1** remains a weaker chain-breaking antioxidant than quercetin.

Although quercetin is the most efficient chain-breaking antioxidant in the series investigated (*ca.* 10 times as potent as α-tocopherol as judged from the AE_1 values), the inhibited peroxidation rate at a fixed antioxidant concentration is much lower in the case of α-tocopherol. This is because the chain-breaking antioxidant activity of quercetin is partially counterbalanced by the prooxidant activity of the quercetin radicals, which are reactive enough to initiate a chain *via* H abstraction from linoleic acid. Such opposing effects must account for the differences observed between quercetin and its derivatives. Since both the disproportionation and chain initiation rate constants contribute to the experimentally determined $k'_i{}^2/(2k_d)$ ratio, it is not possible to rigorously discuss the magnitude of the prooxidant effect in the series of flavonoids investigated. In spite of its more hydrophilic character and lower intrinsic antioxidant efficiency (slower reaction with DPPH in MeOH–pH 7.4 buffer (1 : 1), lower AE_1 value), rutin is slightly more effective than quercetin at reducing the peroxidation rate. This is due to a lower $k'_i{}^2/(2k_d)$ ratio which points to a faster disproportionation of the rutin radicals and/or a slower chain initiation. Since the rutin radicals are probably less embedded in micelles than the smaller less hydrophilic quercetin radicals, both hypotheses seem reasonable. H abstraction from linoleic acid (or methyl linoleate) by flavonoid radicals has already been proposed to account for the moderate antiperoxidizing activity of flavonoids in organic or aqueous micellar solutions and the strong dependence of the antioxidant efficiency on the flavonoid concentration.[7,25] Such a phenomenon can be assessed quantitatively using the simple mathematical treatment developed in this work.

Conclusion

Besides their well-known ability to act as antioxidants by trapping reactive oxygen species and eventually inhibiting enzymes involved in their production, flavonoids can display prooxidant effects such as production of superoxide and hydrogen peroxide during their autoxidation[21] and reduction of transition metal ions into low-valent species liable to be involved in the Fenton reaction.[26] This work emphasizes a third possible prooxidant effect of flavonoids: the ability of flavonoid radicals produced during inhibited peroxidation to initiate propagation chains upon H abstraction from PUFA. Taking into account this prooxidant effect allows us to propose an explanation of the *flavonoid paradox*: flavonoids are strong chain-breaking antioxidants (high AE_1 values) in agreement with their fast reaction with lipid peroxyl radicals. However, the overall antiperoxidizing activity of flavonoids is severely restricted by the chain-initiating activity of their radicals. The prooxidant effect, best evidenced at low antioxidant concentrations (<1–2 × 10^{-6} mol dm^{-3}), may be reversible at higher concentrations and masked by dominant inhibition of initiation.

The moderate chain-breaking activity of flavonoids during PUFA peroxidation and the very weak (if any) affinity of flavonoids for LDL[27] suggest that the dominant antioxidant mechanism of flavonoids is not to directly trap PUFA peroxyl radicals in LDL. Flavonoids more likely exert their protective effect toward LDL by transferring their labile H atoms to the α-tocopheryl radical, thereby regenerating the potent chain breaker α-tocopherol.[9c,9d] This vitamin C-like antioxidant activity could be all the more important since the α-tocopheryl radical itself is chain-initiating in LDL peroxidation.[28]

Experimental

Materials

Quercetin, rutin, DPPH, AAPH, linoleic acid and SDS (Sigma-Aldrich) of the highest quality available (95–99%) were used without purification.

Absorption spectra

Spectra were recorded on a Hewlett-Packard 8453 diode-array

spectrometer equipped with a magnetically stirred quartz cell (optical pathlength: 1 cm). The temperature in the cell was kept constant by means of a thermostatted water bath.

NMR experiments

NMR experiments were carried out on Bruker spectrometers (300 and 500 MHz, 27 °C, δ in ppm, J in Hz). The assignment of the ^{13}C signals and the position of the alkyl chain in the amphiphilic quercetin ethers were deduced from ^{1}H–^{13}C correlation experiments (HMBC).

Kinetic experiments

Inhibition of linoleic acid peroxidation. 2 cm^3 of a freshly prepared solution of linoleic acid (2.5×10^{-3} mol dm^{-3}) and SDS (0.1 mol dm^{-3}) in a pH 7.4 buffer (0.05 mol dm^{-3} NaH_2PO_4, pH adjusted to 7.4 by addition of conc. NaOH without dilution) were placed in the spectrometer cell thermostated at 37 °C. 25 mm^3 of a freshly prepared 0.08 mol dm^{-3} solution of AAPH in the pH 7.4 buffer were added and the absorbance at 234 nm was monitored. After the peroxidation rate had reached a constant value (5–10 min), 25 mm^3 of a solution of flavonoid in MeOH or Bu^{n}OH (typical concentration range 3×10^{-5}–10^{-3} mol dm^{-3}) was added. From the slope of the linear absorbance *vs*. time plots (recorded over *ca*. 10 min), the inhibited peroxidation rate was calculated using 26 100 dm^3 mol^{-1} cm^{-1} as the molar absorption coefficient of the linoleic acid hydroperoxides at 234 nm.[15a]

H atom transfer to DPPH. To 2 cm^3 of a freshly prepared solution of DPPH in Bu^{n}OH (2×10^{-4} mol dm^{-3}) or in MeOH–pH 7.4 phosphate buffer (1:1) (10^{-4} mol dm^{-3}) placed in the spectrometer cell were added small volumes of a 10^{-3} mol dm^{-3} solution of flavonoid in Bu^{n}OH or MeOH. The curve-fittings of absorbance *vs*. time plots were carried out as previously reported.[17]

Amphiphilic quercetin ethers

4′-*O*-*n*-Hexadecyl-3,3′,4′,5,7-pentahydroxyflavone (1). A solution of quercetin (676 mg, 2 mmol) in DMF (10 cm^3) was dried upon co-evaporation with toluene and placed under N_2. Bu^{t}OK (224 mg, 2 mmol) was added and the mixture was brought to 60 °C. After complete dissolution of Bu^{t}OK, iodohexadecane (625 mm^3, 2 mmol) was added and the solution stirred for 3 days at 60 °C. After cooling, the reaction mixture was diluted with ethyl acetate (50 cm^3) and washed repeatedly with 1 mol dm^{-3} HCl, dried over $MgSO_4$ and concentrated. The mixture of mono- and diethers thus obtained was submitted to chromatography on silica gel (petroleum ether–ethyl acetate (85:15)) which yielded a fraction of pure **1** (30 mg). TLC (silica plate, petroleum ether–ethyl acetate (80:20)): $R_f = 0.24$. λ_{max} (Bu^{n}OH) = 371 nm. ^{1}H-NMR (300 MHz, DMSO-d_6): 7.68 (1H, d, J 2.2, H-2′), 7.62 (1H, dd, J 8.8, 2.2, H-6′), 7.05 (1H, d, J 8.8, H-5′), 6.41 (1H, d, J 2.2, H-8), 6.19 (1H, d, J 2.2, H-6), 4.02 (2H, t, J 6.6, Ar-O-CH_2-R), 1.73 (2H, quint., J 6.6, Ar-O-CH_2-CH_2-R′), 1.22 (26H, m, $(CH_2)_{13}$), 0.84 (3H, t, J 7.3, CH_3). ^{13}C-NMR (125 MHz, DMSO-d_6): 176.8 (C-4), 164.8 (C-7), 161.6 (C-5), 157.0 (C-9), 149.5 (C-4′), 147.2 (C-2), 147.1 (C-3′), 137.0 (C-3), 124.1 (C-1′), 120.5 (C-6′), 115.5 (C-2′), 113.6 (C-5′), 103.9 (C-10), 99.0 (C-6), 94.2 (C-8), 69.0 (Ar-O-*C*H_2-R), 32.2–14.8 (other C atoms of the alkyl chain). Mass (FAB, positive mode): m/z = 527.4.

7-*O*-*n*-Dodecyl-3,3′,4′,5,7-pentahydroxyflavone (2). The same procedure with iododododecane as the alkylating agent yielded a fraction of pure **2** (25 mg). TLC (silica plate, petroleum ether–ethyl acetate (60:40)): $R_f = 0.52$. λ_{max} (Bu^{n}OH) = 375 nm. ^{1}H-NMR (300 MHz, DMSO-d_6): 7.73 (1H, br s, H-2′), 7.57 (1H, br d, J 8.1, H-6′), 6.89 (1H, d, J 8.1, H-5′), 6.69 (1H, br s, H-8), 6.32 (1H, br s, H-6), 4.08 (2H, t, J 6.6, Ar-O-CH_2-R), 1.73 (2H, quint., J 6.6, Ar-O-CH_2-CH_2-R′), 1.23 (18H, m, $(CH_2)_9$), 0.85 (3H, t, J 7.3, CH_3). ^{13}C-NMR (125 MHz, DMSO-d_6): 176.8 (C-4), 165.2 (C-7), 161.2 (C-5), 156.9 (C-9), 148.7 (C-4′), 148.1 (C-2), 145.9 (C-3′), 136.9 (C-3), 122.8 (C-1′), 120.6 (C-6′), 116.4 (C-5′), 116.2 (C-2′), 104.8 (C-10), 98.6 (C-6), 93.1 (C-8), 69.3 (Ar-O-*C*H_2-R), 32.2–14.8 (other C atoms of the alkyl chain). Mass (FAB, positive mode): m/z = 471.3.

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