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Luteolin-Rich Artichoke Extract Protects Low Density Lipoprotein from Oxidation *In Vitro*

JONATHAN E. BROWN and CATHERINE A. RICE-EVANS*

International Antioxidant Research Centre, UMDS-Guy's Hospital, St. Thomas' Street, London, SE1 9RT, UK

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Flavonoids represent a diverse group of phytochemicals which possess the capacity to act as antioxidants *in vitro*. This study examined the free radical scavenging properties of a luteolin-rich artichoke extract and some of its pure flavonoid constituents by assessing their ability to prevent Cu^{2+}-mediated LDL oxidation. Artichoke extract retarded LDL oxidation in a dose-dependent manner as measured by a prolongation of the lag phase to conjugated diene formation, a decrease in the rate of propagation and a sparing of endogenous LDL α-tocopherol during oxidation. The pure aglycone, luteolin (1 μM), demonstrated an efficacy similar to that of 20 μg/ml artichoke extract in inhibiting lipid peroxidation. Luteolin-7-O-glucoside, one of the glycosylated forms in the diet, also demonstrated a dose-dependent reduction of LDL oxidation that was less effective than that of luteolin. Studies of the copper-chelating properties of luteolin-7-O-glucoside and luteolin suggest a potential role for chelation in the antioxidative effects of artichoke extract. Overall, the results demonstrate that the antioxidant activity of the artichoke extract relates in part to its constituent flavonoids which act as hydrogen donors and metal ion chelators, and the effectiveness is further influenced by their partitioning between aqueous and lipophilic phases.

Keywords: Artichoke extract, flavonoid, luteolin, luteolin-7-O-glucoside, partition coefficient, oxidised LDL

Abbreviations: BHT, butylated hydroxytoluene; LDL, low density lipoprotein; PBS, phosphate buffered saline; REM, relative electrophoretic mobility

INTRODUCTION

There is considerable interest in the potential health beneficial properties of the constituents of fruit, vegetables, beverages and grains. In particular, the antioxidant components such as vitamin C, the flavonoids and related phenolics, the carotenoids and tocopherols are well-recognised, together with the potential anticarcinogenic and cardioprotective properties of some non-antioxidant constituents. The proposed role of dietary flavonoids in protecting against coronary heart disease and, hence, in decreasing risk of myocardial infarction, was initially indicated in the Zutphen study[1] and supported by the Seven Countries[2] and the Finnish Study[3] although the outcome of the Caerphilly study disputed this contention.[4]

*Corresponding author. Tel.: +44-171 955 4240. Fax: +44-171 955 4983. E-mail: c.rice-evans@umds.ac.uk.

The potential protective effects of consuming flavonoid-rich foods have been proposed from studies with broccoli[5] and also with the consumption of Brussels sprouts which showed decreased oxidative DNA damage.[6] Flavonoids are antioxidants *in vitro* in part by virtue of the activity of the phenolic hydroxyl groups as hydrogen donors[7] and as metal chelators.[8] Their ability to scavenge lipid peroxyl radicals in oxidising low density lipoprotein (LDL) and pure lipid systems[8–11] as well as their activities in the inhibition of peroxynitrite-mediated nitration of tyrosine[12] are well-documented. There is still a paucity of information on the bioavailability and bioactivity of phenolics from natural or supplemental sources.[13–16] We are interested in the screening of antioxidant supplements for their free radical scavenging properties. Artichoke is rich in flavonoids and marked antioxidant properties have been demonstrated for extracts of this vegetable in reducing reactive oxygen species from stimulated human neutrophils[17] and in the protection of hepatocytes from *t*-butyl hydroperoxide-induced cytotoxicity.[18] Artichoke contains flavonoids and hydroxycinnamic acids which have been shown to be effective hydrogen donors.[7,19]

The purpose of this study was to compare the antioxidant properties of artichoke extract with its major constituent flavonoid luteolin-7-O-glucoside and the aglycone luteolin (Figure 1) against free radicals generated in the lipophilic phase by studying their abilities to prevent the oxidation of LDL and to spare endogenous α-tocopherol in the process.

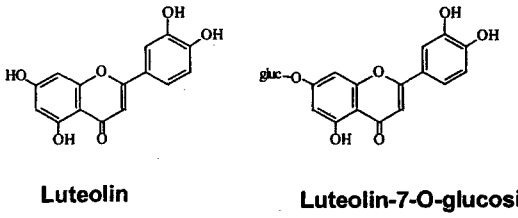

FIGURE 1 Structural features of luteolin-7-O-glucoside and luteolin.

MATERIALS AND METHODS

Materials

The artichoke leaf extract (Extr. *Cynarae fol.*) preparation 'Supra-Sern®' with 80% (w/w) genuine extract (extractant: water) and 20% (w/w) inert constituents, was a gift from Sertürner Arzneimittel GmbH (Gütersloh, Germany). The content of flavonoids was *ca.* 0.4% (w/w), referring to the extract preparation. Luteolin-7-O-glucoside was purchased from Extrasynthèse S.A. (Genay, France) and luteolin was obtained from Carl Roth GmbH (Karlsruhe, Germany). All other chemicals used were of analytical grade.

Experimental

Stock solutions were freshly prepared for each set of experiments. For the artichoke leaf extract 40 mg was dispersed in 4 ml Milli-Q water. The solution was then vortexed (1 min) and filtered (0.2 µm). Further dilutions were prepared in Milli-Q water for the assessment of the antioxidant activity of the filtrate. For luteolin and luteolin-7-O-glucoside stock solutions and subsequent dilutions were prepared in methanol.

The approach is to expose LDL to pro-oxidant conditions such as Cu^{2+}, leading to the increased formation of conjugated dienes as enhanced lipid peroxide formation occurs.[20,21] The ability of the artichoke extract and some of its phenolic constituents to inhibit the surface charge modification on the LDL particle and to spare α-tocopherol were also examined. LDL oxidations were terminated with butylated hydroxytoluene (BHT) which was dissolved in methanol (stock concentration 4 mM) and used at a final concentration of 20 µM.

LDL Isolation and Preparation

Blood was collected from healthy volunteers by venepuncture into vials containing acid–citrate–dextrose and 100 µM EDTA. LDL was isolated

using a modified discontinuous ultracentrifugation method.[22] Isolated LDL was then sterilised by passing it through a 0.2 μm filter (Flowpore, ICN Pharmaceuticals, USA). For each set of oxidation experiments, LDL was dialysed for six hours at 4°C with 10 mM phosphate buffered saline (PBS, 10 mM phosphate/154 mM NaCl; pH 7.4; containing 10 μM EDTA). LDL protein concentrations were estimated using a modified Lowry method.[23]

LDL Oxidation

The relative efficacies of the artichoke extract and that of luteolin and luteolin-7-O-glucoside at delaying lipid peroxidation were evaluated by examining their inhibitory effects on the oxidative modification of LDL *in vitro* over a range of concentrations of artichoke extract (1–20 μg/ml) and constituent flavonoids (0.1–1.0 μM).

LDL oxidation was performed using a modification of the method described.[20] Incubations were carried out with 62.5 μg/ml LDL protein pre-treated with either aqueous solutions of the artichoke extract or methanolic solutions of the pure flavonoids. Additional methanol or water was added so that all incubations were matching. Control incubations contained both methanol and water at levels equal to those present in experimental incubations. Prior to oxidation a blank reading was taken from the control and match correction used to correct for differences between experimental samples at 234 nm. Oxidation was initiated by the addition of Cu^{2+} at a final concentration of 1.66 μM as copper sulphate (over and above the final EDTA concentration of 0.6 μM). All incubations were carried out at 30°C.

The kinetics of the oxidation (assessed by conjugated diene formation) were determined by continuously monitoring absorbance at 234 nm on a Beckman DU 7500 photo-diode array spectrophotometer equipped with Peltier control. Results from the conjugated diene measurements are expressed in terms of lag phase (defined as the intercept at the *abcissa* in the diene *versus* time plot[20]) and rate of oxidation during the propagation phase.

The extent to which the artichoke extract and each flavonoid inhibited an increase in the net negative surface charge of the apolipoprotein B_{100} was also monitored. For these measurements, oxidation of the LDL was terminated at four hours with the addition of 20 μM (final concentration) BHT. Control and oxidised LDL samples (10 μl) were applied to Beckman pre-cast agarose gels. Electrophoresis was conducted at pH 8.6 for 45 min (100 V). Lipoprotein bands were visualised by staining the gel with a lipid specific stain (Paragon® Lipo Stain). Relative electrophoretic mobility (REM) was calculated by determining the ratio of the distance from the origin to the mid-point of the oxidised LDL band in relation to that for the native, untreated LDL. These values were compared with the REM of LDL incubated with Cu^{2+} but in the absence of flavonoid.

In investigating the ability of added antioxidant phytochemical components to prevent oxidative damage to the LDL particle and enhance its antioxidant status, it is of interest to determine whether the antioxidant constituents of the added artichoke extract protect the endogenous α-tocopherol from consumption. Thus the influence of the artichoke extract and luteolin on the rate of consumption of vitamin E in LDL during oxidation was also assessed.

At specific time points during the Cu^{2+}-mediated LDL oxidation, aliquots were withdrawn from the incubation medium, the reaction terminated with the chain breaking antioxidant BHT (20 μM final concentration) and HPLC analysis performed.[24] The internal standard used was δ-tocopherol and samples were extracted with 1 ml hexane following deproteinisation with methanol (1 ml). Tocopherols (α and δ) were measured by normal phase HPLC (Nova-Pak® silica: 4 μm, 3.9 × 156 mm). The isocratic mobile phase (1 ml/min flow rate) was hexane (92%) and *t*-butyl ether (8%). Detection was performed fluorimetrically (excitation 295 nm, emission 340 nm).

Copper Interaction Studies

Luteolin and luteolin-7-O-glucoside (stock concentration 1 mM) were prepared in methanol. From these stock solutions, 25 μM solutions were prepared by dilution into PBS (pH 7.4) and the absorption spectra recorded between 200 and 600 nm. Flavonoids were incubated with Cu^{2+} (as copper sulphate), final concentration 50 μM, and spectra obtained 10 s after interaction. The effect of the copper chelator, EDTA (final concentration 125 μM) on the flavonoid–copper complex was examined for up to 60 min.

Statistical Methods

Results are expressed as the mean ± one standard deviation. Significant differences ($p < 0.05$) were determined by either paired or unpaired *t*-test after one-way analysis of variance.

RESULTS

The extent to which the artichoke extract, luteolin-7-O-glucoside and its aglycone prevented LDL oxidation was assessed by investigating the suppression of lipid peroxidation, the inhibition of apolipoprotein B_{100} modification and the extent to which α-tocopherol was spared during the incubation with the pro-oxidant Cu^{2+}. A concentration-dependent enhancement of the lag phase to oxidation was observed on measuring increased formation of conjugated dienes, with approximately two- and three-fold enhancement in relation to control with 10 and 20 μg/ml of artichoke extract, respectively (Figure 2). In comparison (Table I), the aglycone, luteolin, in the concentration range 0.1–1.0 μM extended the lag time to oxidation in a dose-dependent manner, similar to the artichoke extract.

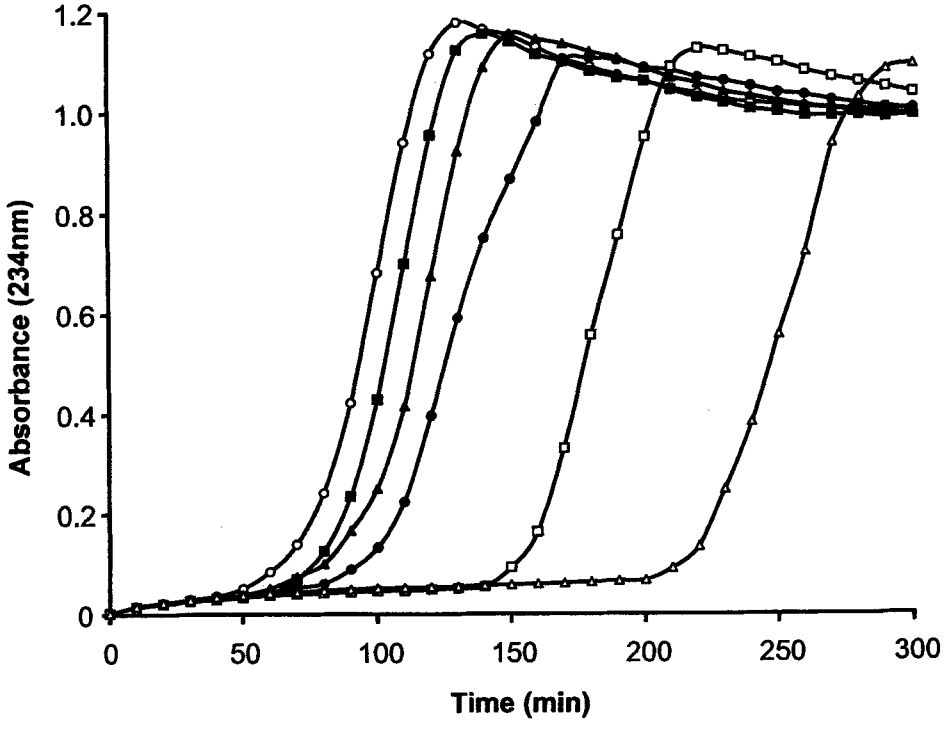

FIGURE 2 Time course of conjugated diene formation on oxidation of LDL mediated by Cu^{2+} and the dose-dependent effect of increasing concentrations of artichoke extract in prolonging the time to propagation of oxidation at 30°C. Concentration of artichoke: 1 μg/ml (■), 2 μg/ml (▲), 5 μg/ml (●), 10 μg/ml (□) and 20 μg/ml (△). Control incubation (○).

TABLE I Effect of artichoke extract, luteolin and luteolin-7-O-glucoside on lag phase extension

Conc. (μg/ml)	Artichoke extract		Conc. (μM)	Luteolin		Luteolin-7-O-glucoside	
	Lag phase (min)	Increase (%)		Lag phase (min)	Increase (%)	Lag phase (min)	Increase (%)
0	73 ± 17	0	0	67 ± 20	0	79 ± 12	0
1	86 ± 15[a]	20 ± 11	0.1	80 ± 19[a]	21 ± 15	86 ± 19	11 ± 4
2	98 ± 17[a]	37 ± 17	0.25	93 ± 21[a]	41 ± 17	92 ± 19[a]	19 ± 11
5	107 ± 15[a]	52 ± 24	0.50	119 ± 29[a]	81 ± 28	105 ± 15[a]	37 ± 11
10	145 ± 39[a]	99 ± 27	1.00	189 ± 36[a]	194 ± 70	127 ± 19[a]	64 ± 33
20	210 ± 52[a]	192 ± 45					

Results expressed as mean ± SD. $n = 5$–7 individual experiments with separately isolated LDL. [a]Significantly different from respective control lag phase ($p < 0.05$ level) after paired t-test.

TABLE II Effect of artichoke extract, luteolin and luteolin-7-O-glucoside in inhibiting the increase in surface charge on the apolipoprotein B_{100}

Conc. (μg/ml)	Artichoke extract (Mean ± SD)	Conc. (μM)	Luteolin (Mean ± SD)	Luteolin-7-O-glucoside (Mean ± SD)
1	2.4 ± 3.3	0.1	5.0 ± 10.8	−7.0 ± 7.9
2	5.0 ± 4.5	0.25	9.4 ± 6.3	1.2 ± 5.4
5	29.6 ± 17.3[a]	0.50	11.8 ± 4.8[a]	13.2 ± 2.9[a]
10	68.2 ± 6.0[a]	1.00	45.0 ± 18.9[b]	18.6 ± 7.8[b]
20	65.8 ± 1.6[a]			

Results (mean ± SD) expressed as percentage inhibition of REM increase compared with control (LDL incubated with Cu^{2+} alone). $n = 3$–4 individual experiments with separately isolated LDL. [a]Significantly different from respective control ($p < 0.05$ level) after paired t-test. [b]Trend towards statistical significance $0.05 < p < 0.1$ level.

Luteolin-7-O-glucoside (0.1–1.0 μM) was less effective at the higher end of the range with only a 64% enhancement of lag phase at 1.0 μM (Table I).

The ability of the artichoke extract and its constituent flavonoids to inhibit the alteration in surface charge of the apolipoprotein B_{100} when LDL was incubated with Cu^{2+} was monitored by agarose gel electrophoresis (Table II). At concentrations of up to 2 μg/ml artichoke extract and up to 0.25 μM flavonoids, no significant inhibition of the increased relative electrophoretic mobility was observed. At 5 μg/ml extract and 0.5 μM pure constituents, inhibition was observed with approximately a doubling of the effect as the concentration increased to 10 μg/ml and 1.0 μM, respectively. No significant differences were observed between luteolin and luteolin-7-O-glucoside at 1.0 μM ($p = 0.065$).

Studying the kinetics of LDL peroxidation, and rates of conjugated diene formation, show that luteolin, luteolin-7-O-glucoside (1 μM) and artichoke extract (1 μg/ml) all decrease the rate of peroxidation relative to control (Table III).

In order to determine whether the artichoke extract or its constituents exerted a sparing effect on the α-tocopherol within the LDL, the time-dependent consumption of α-tocopherol was measured. Figure 3 demonstrates the residual α-tocopherol as a percentage of the original level as a function of incubation time with 10 and 20 μg/ml artichoke extract, in comparison with luteolin (1 and 2 μM). The lower rate of consumption of α-tocopherol under the influence of artichoke extract (10 μg/ml) is shown. The higher concentration of artichoke extract further prolonged the effects, although not proportionately. Luteolin (1 μM) was also effective in slowing the rate of α-tocopherol depletion under the conditions of this study. Increasing the luteolin concentration to 2 μM resulted in a further

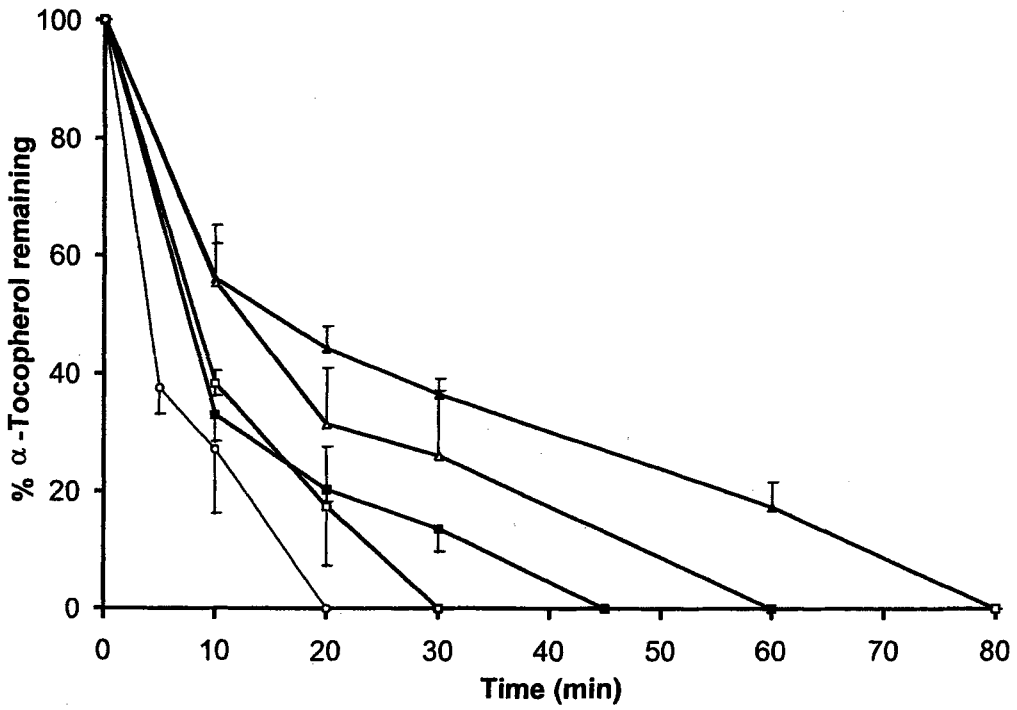

FIGURE 3 Effect of artichoke extract and luteolin on the sparing of endogenous α-tocopherol during Cu^{2+} mediated LDL oxidation. Final concentrations of artichoke extract were 10 μg/ml (△) and 20 μg/ml (▲). For luteolin, concentrations were 1 μM (□) and 2 μM (■). Control incubation (○).

TABLE III Effect of artichoke extract, luteolin and luteolin-7-O-glucoside on the rate of propagation during LDL oxidation

Extract/compound	Control rate	Experimental rate	Reduction (%)
Artichoke (20 μg/ml)	0.021 ± 0.004	0.014 ± 0.002[a]	32
Luteolin (1 μM)	0.021 ± 0.004	0.017 ± 0.004[a]	17
Luteolin-7-O-glucoside (1 μM)	0.022 ± 0.002	0.018 ± 0.004[a]	17

Results expressed as mean ± SD. Propagation rate expressed in terms of ΔA_{234}/min. $n = 5$–7 individual experiments with separately isolated LDL. [a]Significantly different from respective control ($p < 0.05$ level) after paired t-test.

reduction in the rate of α-tocopherol depletion. The starting α-tocopherol concentration was 12 nmol/mg LDL protein in this experiment.

In order to investigate whether copper chelation could constitute part of the protective effect of these compounds on LDL oxidation, the interaction of Cu^{2+} ions with luteolin-7-O-glucoside at pH 7.4 was studied, in comparison with that of luteolin, in order to ascertain the influence of 7-glycosylation. The results can be described in terms of shifts in band I (320–385 nm) and band II (250–285 nm), which relate to the B and A ring absorptions of the flavonoids, respectively.

Interactions of Cu^{2+} ions with luteolin-7-O-glucoside at a 2:1 ratio produced a bathochromic shift of 65 ± 3 nm in band I from 352 nm (Figure 4(a)). No shifts in band II were observed with this concentration of Cu^{2+} ions. The addition of a 2.5 molar excess of EDTA returned the spectrum to its original position and absorbance,

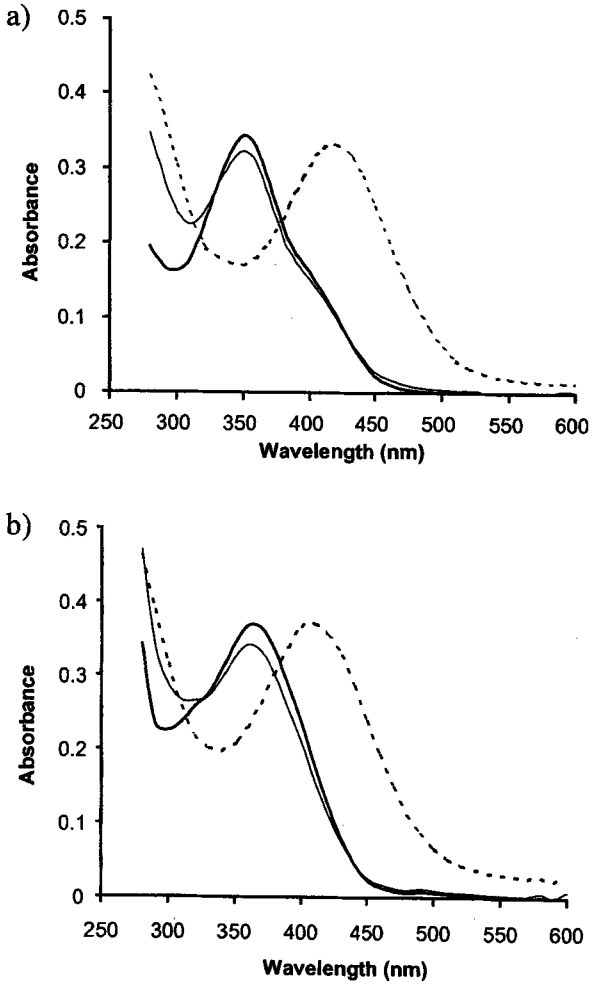

FIGURE 4 Effect of EDTA on copper chelate formation with (a) luteolin, and (b) luteolin-7-O-glucoside. Flavonoid (25 μM) —; Flavonoid (25 μM) plus copper (50 μM) --; Flavonoid (25 μM) plus copper (50 μM) after addition of EDTA (125 μM) —.

except at the shorter wavelengths, due to the added absorbance of the underlying spectrum of Cu^{2+}-EDTA.

With luteolin, as previously shown,[8] the presence of the two-fold concentration of Cu^{2+} ions caused a smaller bathochromic shift (42 nm) of band I than that observed for the 7-O-glucoside (Figure 4(b)). The addition of EDTA (125 μM) resulted in the original spectrum being restored.

DISCUSSION

There are several criteria to consider when investigating the efficacy of dietary antioxidants as free radical scavengers,[25] namely, (i) their propensities for hydrogen or electron donation, or their redox properties, (ii) their partition coefficients or relative abilities to localise in the lipid phase and scavenge lipophilic radicals and (iii) their metal-chelating properties.

Measurement of their abilities to scavenge free radicals generated in the aqueous phase as hydrogen donors[19,26] has shown that the antioxidant activity relative to Trolox is 2.1 ± 0.05 mM for luteolin, the blocking of the 7-hydroxyl group reducing this value by *ca.* 10–20%. This is consistent with the reduction potentials of these compounds which are in the main, dependent on the B ring dihydroxy structure[27] which is the same for both compounds.

The ability to scavenge free radicals in the lipophilic phase depends on the accessibility of the antioxidant constituents of the extract to the lipid peroxyl radicals and the varying partition coefficients of the different components. Our recent studies have demonstrated a relationship between partition coefficients and inhibition of the rate of propagation during lipid oxidation in LDL.[8] The partition coefficient of luteolin in octan-1-ol : water mixtures is 22.2[8] and the value for luteolin-7-O-glucoside will be considerably lower as glycosylation enhances the water solubility. This is consistent with the greater effectiveness for luteolin than the 7-glucoside in inhibiting LDL oxidation because of the hydrophobic partitioning properties of the former; the less lipophilic glucoside presumably has less access to the radicals in the lipophilic phase. Studies on the catechin/gallate constituents of tea have demonstrated the relationship between ability to inhibit oxidation of LDL and partition coefficients.[11] Chlorogenic acid has a partition coefficient of 0.02 and it has been suggested that it exerts its effects in protecting LDL from oxidation from the aqueous phase.[28] The propensity

to interact within the lipophilic compartment was concluded to be a major factor in differentiating the antioxidant activities of quercetin and rutin in iron-induced peroxidation of linoleic acid.[10]

The role of metal ion chelation in the antioxidant properties of flavonoids is also of relevance. In the case of luteolin, the ortho-3′, 4′-dihydroxy substitution in the B ring has been shown to be important for copper chelation,[8] thereby influencing antioxidant activity. For luteolin-7-O-glucoside, the sites for transition metal ion chelation would probably be the same as for the aglycone in that glycosylation of the 7-OH group would not influence the reactivity of the catechol structure in the B ring. This is indeed evident from the chelation studies with Cu^{2+} ions in that both luteolin and its 7-O-glucoside are capable of chelating this transition metal ion in a similar way, as judged by the spectral changes. However, glycosylation at the 7-position would render the flavonoid more soluble in the aqueous environment thus potentially increasing access to Cu^{2+} ions and enhancing the extent of chelation. Chelation of metal ions over and above the scavenging of lipid peroxyl radicals is indicated by the change in the propagation rate during LDL oxidation in the presence of both luteolin and luteolin-7-O-glucoside, and suggests that both compounds interact with Cu^{2+} to the same extent under these conditions.

Artichoke extract has a dose-dependent sparing effect on α-tocopherol, the major antioxidant in LDL. Luteolin also spared α-tocopherol but not as effectively. This may appear to be an anomaly as both 20 μg/ml artichoke extract and 1.0 μM luteolin extended lag phase to oxidation to a similar extent. However, other constituents present within the artichoke extract may contribute to the sparing of α-tocopherol. This combination of constituents would interact at different levels of the free radical scavenging process thus extending the life of the α-tocopherol.

Taking into account that other components within the artichoke extract may be acting as antioxidants, it is useful to calculate the contribution of the luteolin-7-O-glucoside to the total antioxidant capacity of the extract as measured by the extension of lag phase to oxidation. The amount of luteolin-7-O-glucoside present when 20 μg/ml artichoke extract is used is equivalent to *ca.* 0.2 μM. This is calculated to increase lag phase by ~20%. It is therefore evident that other components contribute to the antioxidant properties of the extract, a major one being caffeoylquinic acid. The calculated content of caffeoylquinic acid present in 20 μg/ml artichoke extract is *ca.* 0.7 μM. This hydroxycinnamic acid is an effective antioxidant capable of scavenging peroxyl radicals *in vitro*[29] and as such a contribution of the antioxidant potential may be due to this compound.

The finding that artichoke extract has considerable antioxidant properties against oxidation of LDL *in vitro* has potential importance, along with the flavonoid constituents of other vegetables and fruit, in antioxidant protection *in vivo*, although the bioavailability and bioactivity need to be ascertained. It has been reported that artichoke extract inhibits cholesterol biosynthesis,[30] reduces reactive oxygen species generation from neutrophils[17] and lowers serum cholesterol levels in patients,[31] the latter indicating the bioavailability of constituents of artichoke.

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