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THE RELATIVE ANTIOXIDANT ACTIVITIES OF PLANT-DERIVED POLYPHENOLIC FLAVONOIDS

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The relative antioxidant activities, against radicals generated in the aqueous phase, of a range of plant-derived polyphenolic flavonoids, constituents of fruit, vegetables, tea and wine, have been assessed. The results show that compounds such as quercetin and cyanidin, with 3',4' dihydroxy substituents in the B ring and conjugation between the A and B rings, have antioxidant potentials four times that of Trolox, the vitamin E analogue. Removing the ortho-dihydroxy substitution, as in kaempferol, or the potential for electron delocalisation by reducing the 2,3 double bond in the C ring, as in catechin and epicatechin, decreases the antioxidant activity by more than 50%, but these structures are still more effective than α-tocopherol or ascorbate. The relative significance of the positions and extents of hydroxylation of the A and B rings to the total antioxidant activity of these plant polyphenolics is demonstrated.

KEY WORDS: flavonoid, polyphenol, antioxidant activity, anthocyanidin.

INTRODUCTION

Many studies are demonstrating the protective properties of the polyphenolic flavonoids, plant-derived constituents of the human diet. The antimutagenic and anticarcinogenic properties of the bioflavonoids have all been reported as well as their immune-stimulating, anti-allergic and anti-viral effects, and oestrogenic activities.[1-3] It has been suggested that many of these components play a role as antioxidants[4] inhibiting lipid peroxidation,[5-7] low density lipoprotein (LDL) oxidation[8-12] and scavenging oxygen radicals.[13-18]

The polyphenols are able to act as antioxidants by virtue of the hydrogen-donating capacity of their phenolic groups; another property is their metal-chelating potential which may also play a role in the protection against iron- and copper-induced free radical reactions.[19,20] For example, (+)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,[7] although the electron-donating ability of the catechins are lower than that of quercetin. On the other hand, quercetin is more effective than catechin as an antioxidant in protecting low density lipoproteins from oxidation in copper-mediated peroxidation systems.[12] Furthermore, these flavonoids have been shown to conserve endogenous α-tocopherol in LDL and quercetin is the most effective of the compounds studied.[8,12,21] It has been proposed that

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TABLE I
Sources of polyphenolic flavonoids[22-24]

Flavan-3-ols	
(+)-catechin	tea (camellia)
(−)-epicatechin	tea (camellia)
Flavanones	
naringin	peel of citrus fruits, esp. grapefruit.
naringenin	eucalyptus
taxifolin	citrus fruits
Flavones	
chrysin	fruit skins, conifers
apigenin	parsley, celery
Flavonols	
kaempferol	endives, leek, broccoli, radish, black tea, grapefruit,
quercetin	onion, lettuce, broccoli, tomato, cranberry, apple skin, olive oil, red wine, tea, berries,
Anthocyanidins	
apigenidin	coloured fruits and peels
perlargonidin	flowers of perlargonium, scarlet roses
cyanidin	cherry, raspberry, strawberry
malvidin	blue grapes

flavonoids located near the surface of phospholipid structures are ideally located for scavenging oxygen radicals generated in the aqueous phase.

In this study we have assessed the relative activities of a range of higher plant flavonoids as scavengers of radicals generated in the aqueous phase. The dietary sources of the substances studied are shown in Table 1.[22-24] Their structures are characterised by varying degrees of ring unsaturation and substitution with hydroxyl groups (Figure 1).

The relative antioxidant activities were assessed by monitoring their abilities to quench the chromogenic radical cation, $ABTS^{\cdot+}$, generated from interaction between 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonate) (ABTS) and myoglobin activated with hydrogen peroxide. The hierarchy of antioxidant potentials shows that quercetin and cyanidin are more effective than (+)-catechin, (−)-epicatechin, peonidin, apigenidin and taxifolin, while narigenin, apigenin and kaempferol are among the least efficacious but still have antioxidant potentials against aqueous phase radicals which are approximately 150% that of α-tocopherol or ascorbate.

MATERIALS AND METHODS

2.5 mM Trolox (6-hydroxy-2,5,8-tetra methyl chroman-2-carboxylic acid, Hoffman-La Roche) was prepared in phosphate buffered saline (PBS), pH 7.4 for use as an antioxidant standard. At this pH and concentration, the upper limit of the solubility of Trolox is approached and gentle ultrasonication is required to dissolve the crystals. A fresh standard was prepared weekly, until experiments confirmed that frozen Trolox (−20°C) at this concentration was stable for more than 6 months. Fresh working standards (0.5, 1.0, 1.5, 2.0 mM initial concentrations) were prepared daily by mixing 2.5 mM Trolox with PBS.

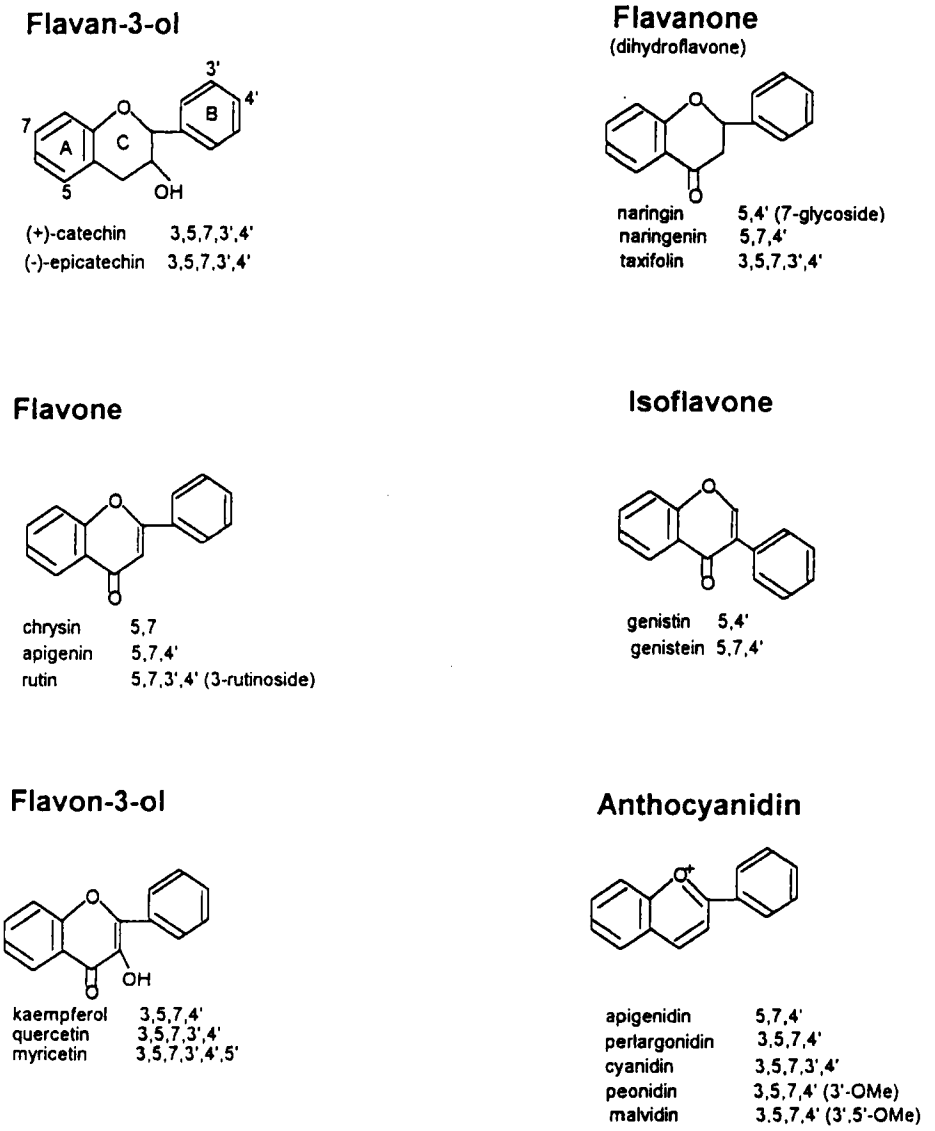

FIGURE 1 The structures of the flavanols, flavanones, flavones and anthocyanidins

Metmyoglobin was prepared and purified as previously described[25] from equine metmyoglobin (Sigma). Working solutions of hydrogen peroxide were prepared from stock Aristar grade 30% solution (BDH), with a specific gravity of 1.10 (1.099–1.103) after dilution with PBS to an initial concentration of 500 mM. For the automated antioxidant assay, a concentration of hydrogen peroxide working solution of 1.08 mM was used, giving a final concentration of 75 μM in the reaction cuvette. 5 mM ABTS (Aldrich) was prepared in phosphate-buffered saline.

Polyphenols were purchased from Sigma Chemical Co (Fancy Road, Poole, Dorset) with the exception of naringenin, apigenidin, cyanidin, malvidin and peonidin, which

were obtained from Extrasynthese (BP 62, Z1 Lyon Nord, 69730 Genay, France). All compounds were dissolved in HPLC grade ethanol (Rathburn Chemicals, Walkerburn Scotland) for Trolox equivalent antioxidant activity (TEAC) estimation, except for catechin and epicatechin which were dissolved in HPLC grade (18 mΩ) water and for quercetin and chrysin which were solubilised in 70% aqueous dimethyl sulphoxide (Aldrich Chemical Co.).

Measurement of total antioxidant activity

The ferryl myoglobin/ABTS assay was used to measure the antioxidant activity of compounds.[25] This technique measures the relative ability of antioxidant substances to scavenge the ABTS radical cation ($ABTS^{\cdot+}$) generated in the aqueous phase, compared with standard amounts of the synthetic antioxidant Trolox, the water-soluble vitamin E analogue. This spectrophotometric method is based on the reduction of the blue-green $ABTS^{\cdot+}$ radical by hydrogen-donating antioxidants, which is measured by the suppression of its characteristic long wave absorption spectrum with maxima at 645 nm, 734 nm and 815 nm. $ABTS^{\cdot+}$ is generated by the interaction of ABTS with the ferrylmyoglobin radical species, using metmyoglobin (2.5 μM), H_2O_2 (75μM) and ABTS (150 μM) (final concentrations). The linearity of the dose response curve of ascending Trolox concentration against declining absorbance at 734nm[25] demonstrates that there is no interference between reduction of $ABTS^{\cdot+}$ by the antioxidant and direct interaction with ferrylmyoglobin on this timescale of measurement (370 seconds). In addition the assay may be performed by generating $ABTS^{\cdot+}$ to an equilibrium concentration and then allowing the antioxidant to decolonise the solution: analogous results are obtained by this technique (Miller and Rice-Evans, submitted for publication, 1994). Antioxidant compounds reduce $ABTS^{\cdot+}$, as detected by the suppression of the absorption of $ABTS^{\cdot+}$, to an extent and on a timescale dependent on the antioxidant capacity of the substance under investigation. The method estimates antioxidant activity, as opposed to antioxidant concentration (which might include a proportion of biologically inactive antioxidant). With automated timing and reagent additions, this precise quantitative assay is applied to the measurement of the antioxidant activity of pure solutions, relating them to Trolox and to one another by deriving the "Trolox Equivalent Antioxidant Capacity" (TEAC): the TEAC is equal to the millimolar concentration of a Trolox solution having the antioxidant capacity equivalent to a 1.0 mM solution of the substance under investigation.

Antioxidant activity measurement using the Cobas Bio centrifugal analyser

300 μl of ABTS/myoglobin reagent is mixed with sample (3 μl), the probe flushed with 30 μl of water, and then hydrogen peroxide (25 μl of 1.08 mM) is added to start the reaction. Reagents are prepared at concentrations at which the subsequent dilution into this incubation volume leads to the following final concentrations: 2.5 μM metmyoglobin; 150 μM ABTS; 75 μM H_2O_2; 0.84% sample fraction. This is performed by mixing 2.143 ml of 140 μM metmyoglobin with 3.6 ml of 5 mM ABTS and diluting the mixture to 100 ml with PBS. The instrument settings used for the ABTS antioxidant assay on the Cobas Bio centrifugal analyser are shown below:

Transfer	3.0 µl sample	Trolox standard or flavanoid sample
	30 µl water	flushes transfer probe
	300 µl reagent	ABTS 150 µM + Metmyoglobin 2.5 µM
Mix		
Read	Initial absorbance	
Transfer	25 µl H_2O_2	final concentration 75 µM
Mix	H_2O_2 starts reaction	
Incubation	at 30°C	absorbance readings every 10 seconds
Read	all cuvettes	final absorbance at 370 seconds
Plot	ΔA_{734nm} vs. Trolox concn.	Logit/log 4 curve fit

Using these reagent concentrations the end of the lag phase (i.e. the point at which the absorbance at 734 nm started to increase) for the 2.5 mM Trolox standard was at 370 seconds, which was taken as the measuring time for ΔAbs_{734nm}. Analyticals were added to the incubation mixture as an aliquot of an aqueous or an ethanolic solution. Ethanol does not react with the $ABTS^{\cdot+}$ radical cation.[25]

Estimation of Trolox equivalent antioxidant activity (TEAC)

The procedure for the estimation of a TEAC value was to prepare 3 working solutions of differing concentrations equivalent to Trolox solutions of 1.0 to 2.0 mM (initial concentrations). These solutions were then each included in triplicate as analyticals in the total antioxidant assay and the TEAC value for each solution determined. The mean TEAC value (of the 9 separate determinations in the assay) was calculated. Derivation of the TEAC was then repeated with solutions on at least 2 separate days (for n=3) and the mean value (± the standard deviation) calculated.

Due to their highly non-polar nature quercetin and chrysin dissolved in ethanol at these concentrations did not prove to be fully miscible with the reaction mixture. This was reflected in poor agreement between triplicates obtained when deriving the TEAC. Quercetin and chrysin were therefore dissolved in 70% aqueous DMSO at a concentration of 5 mM and also diluted in DMSO to working initial concentrations for assay (0.2, 0.3 and 0.4 mM). Dilutions of Trolox in 70% DMSO were also run as samples (in additions to Trolox standards in PBS) and the TEAC figure derived for quercetin corrected for the value obtained for Trolox in DMSO: this corrected figure is quoted in Table 2 as the TEAC for quercetin.

RESULTS AND DISCUSSION

The relative total antioxidant potentials against aqueous phase radicals (the TEAC values) of the range of polyphenols studied here are tabulated in Table 2. The values range from 4.7 to 0.24. The two most effective antioxidants (quercetin and cyanidin) have the same hydroxyl configurations, with conjugation between rings A and B via a planar C ring. Evidence for the requirement for conjugation between the A and B rings is seen in the effects on the antioxidant activity of specific structural changes. For example, the antioxidant activity is lowered when the flavonoids quercetin (4.7) or cyanidin (4.2) lose conjugation in the C ring (connecting the A and B rings) as in taxifolin (1.9). The consequent change in shape might also have its own effects here.

TABLE 2
Hierarchy of Trolox equivalent antioxidant activities of polyphenols compared with plasma antioxidants

Compound	*Free OH-substituents*	*TEAC*	*Family*
quercetin	3,5,7,3′,4′	4.7±0.1 [6]	flavonol
cyanidin	3,5,7,3′,4′	4.2±0.12 [5]	anthocyanidin
myricetin	3,5,7,3′,4′,5′	3.1±0.30 [6]	flavonol
epicatechin	3,5,7,3′,4′	2.5±0.02 [6]	flavanol
catechin	3,5,7,3′,4′	2.2±0.05 [9]	flavanol
rutin	5,7,3′,4′	2.42±0.06 [7]	flavonol
apigenidin	5,7,4′	2.35±0.2 [4]	anthocyanidin
peonidin	3,5,7,4′ (3′-OMe)	2.22±0.2 [4]	anthocyanidin
malvidin	3,5,7,4′ (3′,5′-di-OMe)	2.06±0.1 [4]	anthocyanidin
taxifolin	3,5,7,3′,4′	1.9±0.03 [6]	flavanone
naringenin	5,7,4′	1.53±0.05 [4]	flavanone
apigenin	5,7,4′	1.45±0.08 [6]	flavone
chrysin	5,7	1.43±0.07 [6]	flavone
kaempferol	3,5,7,4′	1.34±0.08 [6]	flavonol
perlargonidin	3,5,7,4′	1.30±0.1 [6]	anthocyanidin
genistein	5,7,4′	1.00±0.08 [6]	isoflavone
genistin	5,4′	0.79±0.04 [3]	isoflavone
naringin	5,4′	0.24±0.02 [7]	flavanone
α-tocopherol		0.97±0.01 [3]	
ascorbate		0.99±0.04 [5]	
urate		1.02±0.06 [5]	
glutathione		0.90±0.03 [3]	
bilirubin		1.5±0.12 [3]	
albumin		0.69±0.02 [3]	

[TEAC values are depicted as mean ± s.d. with the number of separate experiments in brackets]

The contribution of the 3′,4′ di-OH substitution in the B ring is highly significant, as demonstrated in the flavonols and the anthocyanidins. Insertion of the additional 3′-OH group into kaempferol (1.3) produces the quercetin configuration (4.7). It is interesting to note that an additional OH group at C5′, as in myricetin (3.1), reduces the TEAC. Incorporation of the 3′-OH group into pelargonidin (1.3) produces the cyanidin structure with a TEAC of 4.2. Blocking the 3′-OH group in the B ring by replacement with -OMe (as in peonidin) lowers the TEAC to 2.2. The 3′,4′-di-OH effect is strongly boosted by conjugation between the A and B rings, comparing again quercetin with taxifolin.

The evidence for the C3-OH requirement from the structures studied here is doubtful. For example, for the anthocyanidins, the introduction of the 3-OH into apigenidin (2.4) substantially lowers the TEAC, as in pelargonidin. In the case of the flavone to flavon-3-ol conversion of apigenin (1.45) to kaempferol (1.3) there is clearly very little change. However, interestingly, blocking the C3-OH in flavonols reduces the TEAC from 4.7 (quercetin) to 2.4 (rutin).

The importance of the C7-OH requirement seems to be dependent on other structural considerations. A large change is seen in the flavanone naringenin (1.5) when this group is blocked, producing naringin (0.24) with a 7-O-glycosidic structure. However, the isoflavone genistein (1.0) does not decrease so dramatically on insertion of the 7-O-glycoside, as in genistin (0.79).

The proposals of Bors et al[18] concerning the chemical criteria for determining the radical scavenging functions of the flavonoids are (i) the presence of the 3′,4′-dihydroxy structure in the B ring; (ii) the presence of the 2,3-double bond in conjunction with the 4-oxo group in the C ring, which allows conjugation between the A and B ring, or electron delocalisation; (iii) the presence of a 3-hydroxyl group in the C ring and a 5-hydroxyl group in the A ring for maximal radical scavenging potentials. The antioxidant activities determined here are consistent with proposals (i) and (ii) but the definition of proposal (iii) does not emerge unequivocally from the flavonoids selected for this study.

The antioxidant hierarchy (from the Trolox equivalent antioxidant activity) of the polyphenols studied here is shown with the values for some plasma antioxidants for reference (Table 2). α-Tocopherol, urate and glutathione all have the same Trolox equivalent antioxidant value of unity.[25] Bilirubin is more effective against radicals generated in the aqueous phase and indeed has been shown to contribute significantly to the total antioxidant capacity in neonatal plasma.[26] The findings for the polyphenols reveal the strong evidence for the C 3′,4′ dihydroxy structure in the B ring as a contributor to the TEAC which is enhanced with conjugation between the A and B rings. Thus these criteria allow enhanced hydrogen-donating potential and antioxidant activities which are more favourable than that of α-tocopherol, the greatly enhanced antioxidant potentials of polyphenols with structures such as those of quercetin and cyanidin being dependent not only on extensive electron delocalisation between the A and B rings as a prerequisite for radical stabilisation, as promulgated by Bors et al,[18] but also on the presence of both the 3′,4′-dihydroxy substituents in the B ring. For these reasons, of the polyphenols studied, quercetin and cyanidin are much more effective as scavengers of aqueous phase radicals than catechin and epicatechin which lack the conjugation between the A and B rings. This is in contrast to the proposals of others that the catechins are powerful antioxidants with an effectiveness comparable to that of quercetin against lipid peroxidation in phospholipid bilayers induced by aqueous oxygen radicals.[7]

The Zutphen Elderly study[27] showed that dietary flavonoid intake is correlated inversely with coronary heart disease mortality, particular sources being tea, onions and apples. Yet, previous studies of the distribution of radiochemically labelled compounds reveal that the major portion of ingested flavonoids is present in the gastrointestinal tract (eg. 44% ingested quercetin) before excretion into the bile.[28] The absorption and metabolism of the polyphenolic bioflavonoids in humans still needs much further study. It is of interest to note that as early as 1936, Szent-Gyorgi showed that flavonoid preparations from citrus peel and paprika could heal scorbutic pigs where ascorbate alone was ineffective.[29] Indeed a number of studies have suggested that the flavonoids are able to enhance the antioxidant capacity of ascorbate.

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