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Research Article

Antioxidant Capacity of Flavonoids in Hepatic Microsomes Is not Reflected by Antioxidant Effects *In Vivo*

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Flavonoids are polyphenolic compounds with potential antioxidant activity via multiple reduction capacities. Oxidation of cellular lipids has been implicated in many diseases. Consequently, this study has assessed the ability of several dietary flavonoid aglycones to suppress lipid peroxidation of hepatic microsomes derived from rats deficient in the major lipid soluble antioxidant, dα-tocopherol. Antioxidant effectiveness was galangin > quercetin > kaempferol > fisetin > myricetin > morin > catechin > apigenin. However, none of the flavonoids were as effective as dα-tocopherol, particularly at the lowest concentrations used. In addition, there appears to be an important distinction between the *in vitro* antioxidant effectiveness of flavonoids and their ability to suppress indices of oxidation *in vivo*. Compared with dα-tocopherol, repletion of vitamin E deficient rats with quercetin, kaempferol, or myricetin did not significantly affect indices of lipid peroxidation and tissue damage. Direct antioxidant effect of flavonoids *in vivo* was not apparent probably due to low bioavailability although indirect redox effects through stimulation of the antioxidant response element cannot be excluded.

1. Introduction

Many polyphenols can act as antioxidants in chemical systems and food matrices as their extensive conjugated π-electron systems facilitate the donation of electrons from the hydroxyl moieties to oxidising radical species. For example, flavonoids which are formed via the plant phenylpropanoid pathway can have multiple reduction capacities. Reactivity, which is largely determined by the O–H bond dissociation energies, is highly dependent on the configuration of the O–H groups of the B and C rings (Figure 1). Thus reaction stoichiometries for many common dietary-derived flavonoids exceed that of vitamin E (dα-tocopherol), a major recognised antioxidant in biological systems [1]. The effectiveness of flavonoids as free radical scavengers in a wide range of chemical oxidation systems has resulted in suggestions that they may also have a role as dietary antioxidants which benefit health [2]. This is because the free radical mediated oxidation of proteins, lipids, and DNA is implicated in the pathogenesis of many diseases including heart disease and cancers [3]. However, many redox effects of flavonoids and other polyphenols observed in experimental systems may not be of biological relevance unless they gain access to tissues where they can exert hydrogen donating activity [4].

Whether flavonoids are redox active *in vivo* is a matter of debate. For example, several reviewed epidemiological studies have found inverse relationships between intake and diseases which involve oxidative stress [2]. However, a recent review concluded that there is little convincing evidence from human studies for a direct antioxidant effect of flavonoids or other polyphenols [5]. Such disparate views may reflect the diverse molecular structures of flavonoids within the food matrix. Commonly, they are glycosylated, methylated or acetylated at hydroxyl sites on their structure primarily at positions 3, 5, and 7. Such conjugation may not only decrease antioxidant effectiveness but may also impact on bioavailability so that ingested flavonoids pass into the colon relatively unabsorbed [6]. In contrast, lipophilic flavonoid aglycones in digested foods may enter cell membranes by simple diffusion potentially allowing hydrogen donation in oxidatively active cellular sites. For example, quercetin appears to be more bioavailable as an aglycone than as a glycoside when provided from dietary sources [7].

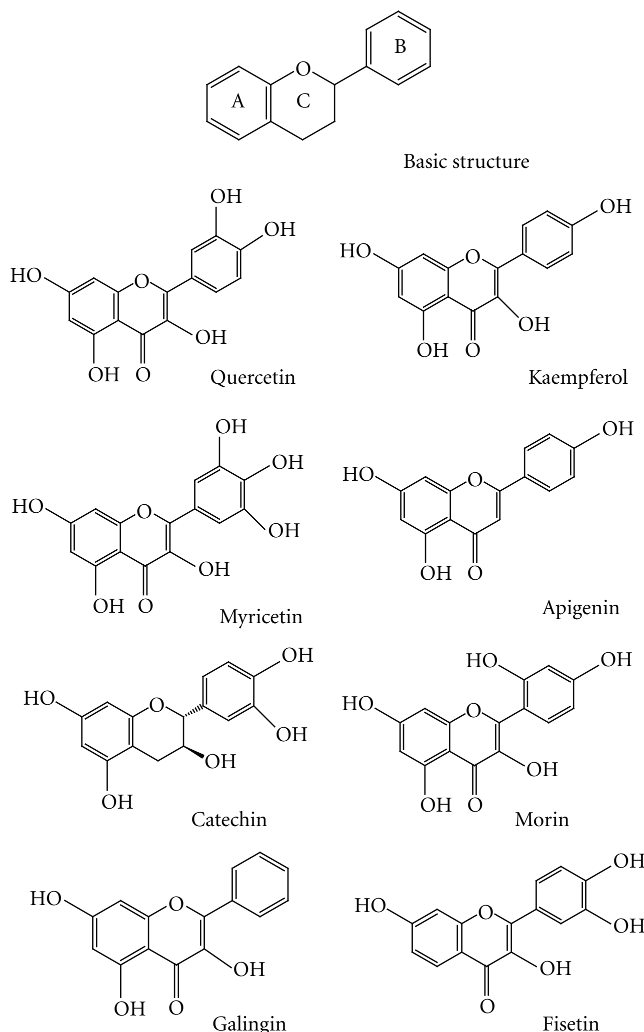

Figure 1: Basic flavonoid structure and structures of compounds used in the present study.

Aims. Increased indices of oxidation of cellular lipids are a characteristic of several diseases and nutritional antioxidant deficiencies [3]. Consequently, we have compared the ability of flavonoid aglycones and dα-tocopherol to inhibit lipid peroxidation of hepatic microsomes derived from rats deficient in vitamin E. In addition, we have determined the effects of consumption of three common dietary flavonoids in an oxidatively stressed rat model. Oxidative stress was induced by maintaining the rats for several weeks on diets deficient in vitamin E. The effects *in vivo* of repletion with quercetin, myricetin, and kaempferol on indices of lipid peroxidation and tissue damage were compared with that of vitamin E and contrasted with antioxidant ability *in vitro*.

2. Materials and Methods

2.1. Microsomal Incubations. Vitamin E deficient microsomal incubations were as previously described [8]. In brief, livers were removed under terminal anaesthesia from male hooded Lister rats maintained for 12 weeks on a semisynthetic diet containing less than 0.5 mg vitamin E/kg. Microsomes were prepared and the protein concentration was adjusted to 10 mg/mL with 0.05 M potassium phosphate buffer (pH 7.4). Ethanolic solutions (40 μL) of eight flavonoid aglycones or dα-tocopherol (0.1, 0.25, and 0.5 mM final concentrations) purchased from Fluka/Riedel-de Haën (Schwei Buchs, Switzerland) were incubated with 1 mL microsomal suspensions for 20 min at 25°C on an asymmetric roller. Peroxidation was then initiated with an ascorbate/Fe^{2+}/ADP complex [8], and aliquots removed for determination of thiobarbituric acid reactive substances (TBARS) by HPLC [9]. From the oxidation curves, the relative abilities of the flavonoids to delay oxidation (lag phase) and suppress maximum peroxidation was calculated relative to the vitamin E deficient microsomes [8].

2.2. In Vivo Model. The study protocol was conducted in compliance with the Animals (Scientific Procedures) Act, 1986, and the format is previously described [9, 10]. Weanling male rats were randomly allocated to 5 intervention groups of 6 animals each. Four intervention groups were offered, *ad libitum*, the vitamin E deficient semisynthetic

diet for 10 weeks. Rats were then offered this diet supplemented with either dα-tocopherol, quercetin, myricetin, or kaempferol at a concentration of 100 mg/kg diet for a further 2 weeks. These compounds were selected as current food compositional databases [11] suggest that they are the most commonly consumed flavonols in the N. European diet. The amount incorporated into the rat chow was selected as reflecting that which is nutritionally achievable by humans by dietary means [11] as the current study was not aimed at establishing responses to pharmaceutical concentrations. The compounds were dissolved in chloroform and mixed to an even distribution in the diet. The remaining rats were maintained on the vitamin E deficient ration. After 12 weeks, (when 16 weeks old and weighing approximately 400 g, see Table 3) the rats were terminally anaesthetized and blood samples were collected by cardiac puncture into heparinized tubes. Plasma was separated by centrifugation for 15 min at 1750 g, 4°C, and red cells were isolated and washed twice prior to being re-suspended to the original volume in phosphate buffered saline. The liver was perfused *in situ* with chilled isotonic KCl (0.154 M) via the hepatic portal vein and snap frozen in liquid nitrogen before storage at −80°C until analysis. Plasma concentrations of vitamin E, quercetin, myricetin, and kaempferol were determined by HPLC using previously described procedures [12, 13]. Plasma levels of TBARS were determined by HPLC with fluorimetric detection [9]. Functional antioxidant status was estimated by the susceptibility of washed erythrocytes to hydrogen peroxide-induced peroxidation [14] and by an electron paramagnetic resonance procedure with spin trapping of lipid radicals with 4-POBN in liver homogenates [15]. Plasma pyruvate kinase (PK) activities were measured as indices of tissue damage by the method of Chow [16].

2.3. Statistical Analysis. Results are presented as mean ± SEM. Data were initially analysed by one-way analysis of variance and comparison of groups of rats was made using the Bonferroni multiple comparison test. A P value of less than 0.05 was considered significant.

3. Results

In general, an observed time-dependent increase in lipid peroxidation of the hepatic microsomal preparations derived from vitamin E deficient rats was moderated by preincubation with ethanolic solutions of flavonoids. Protection against peroxidation increased with increased concentration of flavonoid as shown for quercetin (Figure 2).

Interpolation of the incubation curves to quantify the data as the ability to delay the onset of peroxidation (Table 1) and the suppress maximum peroxidation (Table 2) compared with that of microsomes without flavonoid addition indicated antioxidant effectiveness as galingen > quercetin > kaempferol > fisetin > myricetin > morin > catechin > apigenin. However, none of the flavonoids were as effective as dα-tocopherol, particularly at the lowest concentration of 0.1 mM (Tables 1 and 2).

In the animal model, repletion with diets containing quercetin and kaempferol was associated with the presence of the flavonoids in plasma of vitamin E deficient rats. However, no myricetin was detected in any of the rats even when provided with the flavonoid in the diet for 2 weeks. However, repletion with a similar concentration of dietary dα-tocopherol resulted in a marked increase of the compound in plasma which was an order of magnitude greater than any of the detected flavonoids (Table 3).

Repletion of the vitamin E deficient rats with 100 mg dα-tocopherol/kg diet resulted in a significant decrease in concentrations of TBARS in both plasma ($P < 0.05$) and liver ($P < 0.001$). In contrast, concentrations were unaffected by repletion with similar amounts of either quercetin, kaempferol, and myricetin (Table 4). Repletion with dα-tocopherol was also associated with a marked decrease in membrane damage as indicated by lower activities of pyruvate kinase in plasma and by decreases in the functional markers of oxidative susceptibility, namely, erythrocyte lipid peroxidation, and hepatic tissue EPR signal height. However,

Table 1: Abilities of polyphenol aglycones and dα-tocopherol to delay the onset of peroxidation (lag phase) of hepatic microsomal preparations from vitamin E deficient rats.

Compound	Lag phase (min)		
	0.1 mM	0.25 mM	0.5 mM
Quercetin	8.7	>20	>20
Kaempferol	7.8	19.8	>20
Myricetin	7.0	12.6	14.3
Apigenin	4.7	4.7	4.8
Catechin	5.8	5.9	6.2
Morin	4.1	7.4	9.6
Galingin	3.3	>20	>20
Fisetin	4.9	15.0	>20
dα-tocopherol	>20	>20	>20

Peroxidation was initiated with an ascorbate/Fe^{2+}/ADP complex and estimated by the formation of thiobarbituric acid substances (TBARS) as described in Section 2. Values are means of duplicate determinations.

Table 2: Abilities of polyphenol aglycones and dα-tocopherol to inhibit maximum peroxidation of hepatic microsomal preparations from vitamin E deficient rats.

Compound	Inhibition (%)		
	0.1 mM	0.25 mM	0.5 mM
Quercetin	38	87	95
Kaempferol	21	88	95
Myricetin	11	67	60
Apigenin	1	1	0
Catechin	1	1	1
Morin	7	13	44
Galingin	13	88	94
Fisetin	10	68	90
dα-tocopherol	93	97	95

Peroxidation was initiated with an ascorbate/Fe^{2+}/ADP complex and estimated by the formation of thiobarbituric acid substances (TBARS) as described in Section 2. Values are means of duplicate determinations.

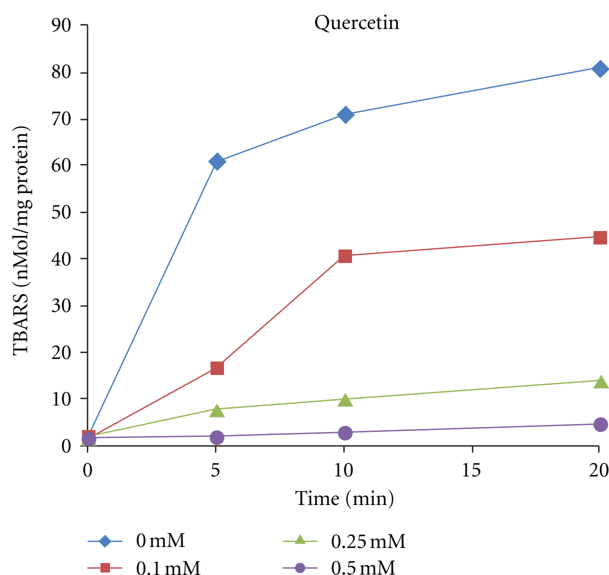

Figure 2: Example of preincubation of hepatic microsomal preparations from vitamin E deficient rats with a polyphenol aglycone (quercetin) on production of thiobarbituric reactive substances (TBARS) following initiation of peroxidation with Fe/ADP.

Table 3: Weights and plasma concentrations of flavonoids and α-tocopherol in vitamin E deficient rats repleted for 2 weeks with 100 mg/kg of either α-tocopherol, quercetin, kaempferol or myricetin.

Parameter	Dietary inclusion				
	−Vitamin E	+dα-tocopherol	+Quercetin	+Kaempferol	+Myricetin
Weight (g)	398 ± 11	394 ± 14	393 ± 11	398 ± 9	397 ± 7
dα-tocopherol (nmol/L)	1.16 ± 0.12	12.91 ± 0.12*	0.91 ± 0.12	1.00 ± 0.07	1.04 ± 0.09
Quercetin (μmol/L)	Trace	Trace	1.48 ± 0.18	Trace	Trace
Kaempferol (μmol/L)	Trace	Trace	Trace	0.47 ± 0.06	Trace
Myricetin (μmol/L)	nd	nd	nd	nd	nd

Results are mean ± SEM of 6 rats/group. *Significantly different from −Vitamin E group ($P < 0.001$). Trace indicates that peak heights were too small to quantify; nd: no detectable compound.

Table 4: Effects of 2 weeks repletion with either 100 mg α-tocopherol, quercetin, kaempferol or myricetin/kg diet on indices of muscle damage, lipid peroxidation and antioxidant capacity of vitamin E deficient rats.

Parameter	Dietary inclusion				
	−Vitamin E	+dα-tocopherol	+Quercetin	+Kaempferol	+Myricetin
Pyruvate kinase (U/L)	807 ± 76	132 ± 13***	632 ± 98	617 ± 108	742 ± 81
TBARS (nmol/mL plasma)	1.66 ± 0.13	1.13 ± 0.09*	2.14 ± 0.51	2.33 ± 0.34	2.08 ± 0.21
TBARS (nmol/mg hepatic protein)	15.3 ± 0.7	2.4 ± 0.6***	13.4 ± 0.5	13.8 ± 0.8	12.5 ± 0.3
Erythrocyte lipid peroxidation (nmol/mgHb)	50 ± 7	3 ± 1***	53 ± 4	63 ± 5	64 ± 4
EPR signal height (RU/mg hepatic protein)	22423 ± 1133	1036 ± 71***	19950 ± 1458	18833 ± 448	17425 ± 1892

Results are mean ± SEM of 6 rats/group. Significantly different from −Vitamin E group (*$P < 0.05$; ***$P < 0.001$). TBARS: thiobarbituric acid reactive substances Hb: haemoglobin, EPR: Electron paramagnetic resonance spectroscopy, RU: relative units.

no analogous effects on these indices were observed in those rats that were fed any of the flavonoids (Table 4).

4. Discussion

The ability of many of the flavonoids to inhibit peroxidation of hepatic microsomal preparations from vitamin E deficient rats might indicate that these dietary compounds could have significant "vitamin E-like" activity in biological systems. As in chemical systems [2, 17], antioxidant potency may depend in part on the number and position of the OH groups on the molecule. For example, using a DPPH radical scavenging system, structure-activity studies indicate that the O-dihydroxy group on ring B of flavonoids plays a crucial role. A double bond at 2-3 position conjugated with a 4-oxo function and hydroxyl groups at positions 3 and

5 also contribute towards antiradical activity of flavonoids [17]. However, within biological samples such as tissue homogenates, antioxidant activity may differ from structure-function relationships apparent in chemical systems [8, 17]. Other factors including molecular charge, solubility, and partitioning coefficients may also be important determinants of antioxidant activity. Within microsomal membranes, in particular, appropriate orientation of the compound within the membranes to allow access of hydroxyl groups to peroxyl radicals within phospholipid moieties may be relevant. This may explain the greater efficacy of dα-tocopherol at the lower concentration as the phytol chain may intercalate within the membrane thus conferring optimum orientation of OH groups on the chromanol ring structure [18].

There appears to be an important distinction between the *in vitro* antioxidant effectiveness of flavonoids and their ability to suppress indices of oxidation *in vivo*. Compared with dα-tocopherol, repletion of vitamin E deficient rats with quercetin, kaempferol or myricetin did not significantly affect indices of lipid peroxidation and tissue damage in plasma and liver. This likely reflects the relatively low bioavailability and/or rapid systemic clearance of the aglycones as their inclusion in the diet did not result in marked increase in concentrations in plasma. Studies aimed at elucidating the degree and mechanisms of the absorption of flavonoid aglycones are contradictory [7, 19, 20], but recent reviews indicate that bioavailability is mostly $<1\%$ of the administered dose [21]. Consequently, the dietary relevant concentrations used in the present study may be insufficient to illicit an antioxidant effect *in vivo*. Bioactivity may only be apparent at greater doses which are not nutritionally achievable. For example, decreased serum TBARS has been observed in rats fed 2–10-fold greater doses of quercetin than used in the present study [22]. However, the results of the present study support previous observations of limited *in vivo* bioactivity of dietary flavonoids. For example, studies in growing rats did not show any vitamin E sparing effects of flavonoids such as quercetin and catechin [23], and supplementation of pigs with green tea polyphenols did not enhance tissue vitamin E levels or plasma antioxidant capacity [24]. Moreover, consumption of green tea extracts for 3 weeks did not influence plasma vitamin E concentrations and cardiovascular risk markers in healthy men [25].

In conclusion, several flavonoid aglycones effectively suppressed lipid peroxidation of microsomal preparations but analogous effects were not observed *in vivo*. Consequently, quercetin, myricetin, and kaempferol are not effective nutritional antioxidants in the vitamin E deficient rat model when compared with the effectiveness of dα-tocopherol. However, in the present study we did not determine potential metabolites derived from the compounds. Consequently indirect redox effects not involving suppression of lipid peroxidation such as stimulation of the antioxidant response element cannot be excluded [26]. Never the less, results of the present study support the conclusion of a recent review [5] that a direct antioxidant effect of flavonoids *in vivo* with dietary achievable intakes is questionable. This does not exclude the possibility that the marked antioxidant efficacy of some flavonoids *in vitro* can be exploited in future drug development aimed at addressing clinical conditions where oxidative stress is an underlying pathology. For example, a systematic review of medicinal plants used in folk medicine [27] indicated that several increased serum antioxidant capacity and/or decreased indices of lipid peroxidation in animals and humans. Whether such effects can be ascribed to flavonoids *per se* is currently unclear. However, studies assessing their use as potential chemopreventative agents appear warranted.

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