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Research article

Antioxidant properties of flavonol glycosides from tea

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We have determined the antioxidant activity of the major flavonols found in tea: a monoglycoside, a diglycoside and two triglycosides of kaempferol and three monoglycosides, a diglycoside and two triglycosides of quercetin. The Trolox equivalent antioxidant capacity (TEAC) and inhibition of iron/ascorbate-induced lipid peroxidation of phosphatidyl choline vesicles were measured. In the aqueous phase TEAC assay, the quercetin monoglycosides and diglycoside were approximately half as effective as quercetin aglycone. The quercetin triglycosides were much less effective than the monoglycosides and the diglycoside. The kaempferol glycosides were 32–39% less effective in the aqueous phase antioxidant assay compared to the kaempferol aglycone. Quercetin monoglycosides and diglycoside were potent inhibitors of lipid peroxidation, in contrast to the triglycoside which was much less effective. All the kaempferol glycosides were significantly less potent inhibitors of lipid peroxidation compared to the kaempferol aglycone. The compounds described herein demonstrate the antioxidant activity of the major flavonols in tea and indicate the effect of substituting a range of sugar moieties in the phenolic C ring.

Introduction

It has become established that diets rich in fruit and vegetables are related to a reduced incidence of cardiovascular disease and certain forms of cancer.[1,2] Such diets contain a rich supply of flavonoids which may play a significant role in this protective effect. Epidemiological studies analysing the dietary effect of one class of flavonoids, the flavonols, suggested that a high dietary intake of flavonols correlated with a reduced risk of coronary heart disease.[3,4] Other studies indicated that the type of flavonol, rather than the flavonols as a class, may be important in understanding this effect.[5] Flavonols exist in plants as conjugates of the aglycones, such as quercetin and kaempferol, and have been shown to exhibit high antioxidant activity,[6] inhibit oxidation of low density lipoprotein *in vitro* and reduce both platelet aggregation and plasma cholesterol,[7,8] which are consistent with a protective role against coronary heart disease.

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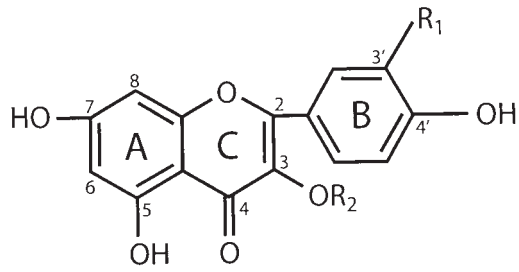
The chemical form of the flavonols before and after digestion is an important factor in determining both the bioavailability and bioactivity, since studies have shown that some quercetin glycosides are preferentially absorbed compared to the aglycone.[9] As a first step to understanding the role of the flavonols in the diet, we have analysed the antioxidant properties of the major flavonols in tea, one of the major sources of flavonols in many diets. Recent studies have identified these flavonol species found in tea (Fig. 1) as five quercetin glycosides and four kaempferol glycosides.[10] This paper reports the antioxidant properties of these compounds.

Materials and Methods

Materials

Teas were purchased from a local supermarket and a specialist tea store in both loose form and in tea bags. Six varieties of tea (Lapsang souchong, Assam, Darjeeling, Keemun, Ceylon and Nunjo) were studied. Quercetin, kaempferol and daidzein were purchased from Apin Chemicals Ltd (Abingdon, UK). Phospholipid liposomes were obtained from Sigma Chemical Co. (Poole, UK). All solvents were of AnalaR grade or HPLC grade where appropriate.



Compound	R_1	R_2
Quercetin (Q)	-OH	-H
Q 3-0-gal (Hyperin)	-OH	-gal
Q 3-0-glu (Isoquercitrin)	-OH	-glu
Q 3-0-glu-rha (Rutin)	-OH	-glu-rha
Q 3-0-gal-rha-glu	-OH	-gal-rha-glu
Q 3-0-glu-rha-glu	-OH	-glu-rha-glu
Kaempferol (K)	-H	-H
K 3-0-glu	-H	-glu
K 3-0-glu-rha	-H	-glu-rha
K 3-0-gal-rha-glu	-H	-gal-rha-glu
K 3-0-glu-rha-glu	-H	-glu-rha-glu

Fig. 1. Structures of the flavonol glycosides isolated from tea. Key: gal, galactose; glu, glucose; rha, rhamnose.

Extraction, quantification and isolation of flavonol conjugates

The flavonols were extracted from the tea leaf by hot water infusion as described previously.[10] Isolation and analysis of individual conjugates were carried out using preparative high performance liquid chromatography (HPLC) and analytical HPLC, respectively, as described previously.[10] Structures of individual compounds were confirmed using a combination of NMR and spectroscopy.[10]

Lipid phase antioxidant activity

Phospholipid liposomes (final concentration 1 mg/ml) were suspended in 150 mM KCl containing 0.2 mM $FeCl_3$ and test compound at a range of concentrations. Peroxidation was started as described previously[11] with ascorbate (final concentration 0.05 mM), in a final volume of 0.4 ml. Samples were incubated at 37°C for 40 min and the reactions terminated by the addition of 0.8 ml of 20% (w/v) trichloroacetic acid (TCA)/0.4% (w/v) thiobarbituric acid (TBA)/0.25 N HCl and 0.01 ml of butylated hydroxytoluene in ethanol. The production of thiobarbituric acid reactive substances (TBARS) was measured after incubation at 80°C for 20 min.[11] Results are expressed as percentage inhibition of peroxidation, where 100% inhibition is defined as baseline peroxidation of liposomes without added iron/ascorbate, and 0% inhibition is defined as peroxidation of liposomes with added iron/ascorbate. Calculation of IC_{50} values was performed by fitting a third order polynomial curve to fit the data.

Aqueous phase antioxidant activity

The Trolox equivalent antioxidant capacity (TEAC) was measured by the method of Salah *et al.*[12] Values are expressed relative to a standard of Trolox C, the water soluble analogue of vitamin E. The assay is based on the relative ability of antioxidants to scavenge the radical cation of 2,2′-azino*bis*(3-ethyl-benzothiazoline-6-sulphonate (ABTS). Since the radical is generated by interaction with activated metmyoglobin and H_2O_2, then the assay is also influenced by how well the test compound inhibits formation of the radical. The extent of quenching of the ABTS radical is measured spectrophotometrically at 734 nm and compared to standard amounts of Trolox C. Kaempferol and quercetin were used as positive controls as described previously,[13] and the TEAC values obtained agreed well with published data.[14]

Table 1. Levels of individual flavonols and their TEAC values.

Flavonol	Level (mg/l fresh weight as aglycone)	TEAC
Q	0	4.43 ± 0.02
Q-3-O-gal (Hyperin)	2–13	2.22 ± 0.07
Q-3-O-glu (Isoquercitrin)	2–12	2.15 ± 0.09
Q-3-O-glu-rha (Rutin)	0–15	2.09 ± 0.07
Q-3-O-gal-rha-glu	1–6	1.25 ± 0.02
Q-3-O-glu-rha-glu	2–17	1.24 ± 0.05
K	0	1.35 ± 0.02
K-3-O-glu	1–9	1.06 ± 0.01
K-3-O-glu-rha	0–7	1.05 ± 0.02
K-3-O-gal-rha-glu	0–6	0.96 ± 0.01
K-3-O-glu-rha-glu	0–6	0.96 ± 0.02

Results and Discussion

The chemical structures of the individual quercetin and kaempferol conjugates found in the six teas tested are shown in Figure 1. The total flavonol content of the six teas ranged only by a factor of two, from 36.5 mg/l for Keemun to 75.8 mg/l for Darjeeling. However, the variation in the individual flavonols (shown in Table 1) was much greater, which could be attributed to the degree of fermentation during preparation of the tea. The TEAC values for each of the quercetin and kaempferol glycosides are listed in Table 1. Quercetin is a highly effective antioxidant in this assay, having a TEAC value of 4.43 ± 0.02. In the case of the quercetin glycosides, the values are substantially lower (50–72% less) than quercetin aglycone, showing a decrease in antioxidant potency in the aqueous phase. This is consistent with previous studies which reported similar decreases in TEAC value with 3-O-glucosylation and 3-O-rutinosylation.[13–15] There is a trend for a decrease in TEAC value with the number of sugar moieties attached to the 3-O-position in the phenolic C ring, since the two triglycosides of quercetin possess a much lower TEAC value than the monoglycosides and the diglycoside. Kaempferol is a relatively poor antioxidant in this assay, with a TEAC value of 1.35 ± 0.02. The glycosides of kaempferol showed a small but significant ($P < 0.05$) decrease in TEAC value, and the triglycosides, diglycosides and monoglycosides possess similar TEAC values. For maximum effectiveness as an antioxidant in this assay, there is a requirement for a free –OH group at the 3-position attached to the 2,3 double bond and adjacent to the carbonyl in the C ring.[6] Removal of any of these features drastically reduces antioxidant activity. In the case of quercetin, substitution at the 3-position with a sugar residue affects the ability of the B ring hydroxyl groups to donate hydrogen, reducing the TEAC value, and substitution with two or more sugar residues further diminishes this ability. In the case of kaempferol, which has only one free hydroxyl group in the B ring, the effect of adding a sugar moiety to the C ring on antioxidant activity is less pronounced since the 3′,4′ dihydroxy structure in the B ring (as found in quercetin) is an essential motif for higher antioxidant action. A single hydroxyl group in the B ring (as found in kaempferol) has only a small contribution to the action of the molecule in this assay.[14]

The effect of flavonols on lipid peroxidation of phosphatidyl choline is shown in Figure 2. The degree of inhibition was measured by estimating the IC_{50} value (concentration of test compound which inhibits peroxidation by 50%). As a comparison to the flavonols under study, the IC_{50} values for butylated hydroxytoluene (BHT) and Trolox C were 5.0 ± 0.2 μM and 12.6 ± 1.4 μM, respectively. Figure 2A shows the two quercetin monoglycosides, hyperin (IC_{50} = 4.48 ± 0.12 μM) and isoquercitrin

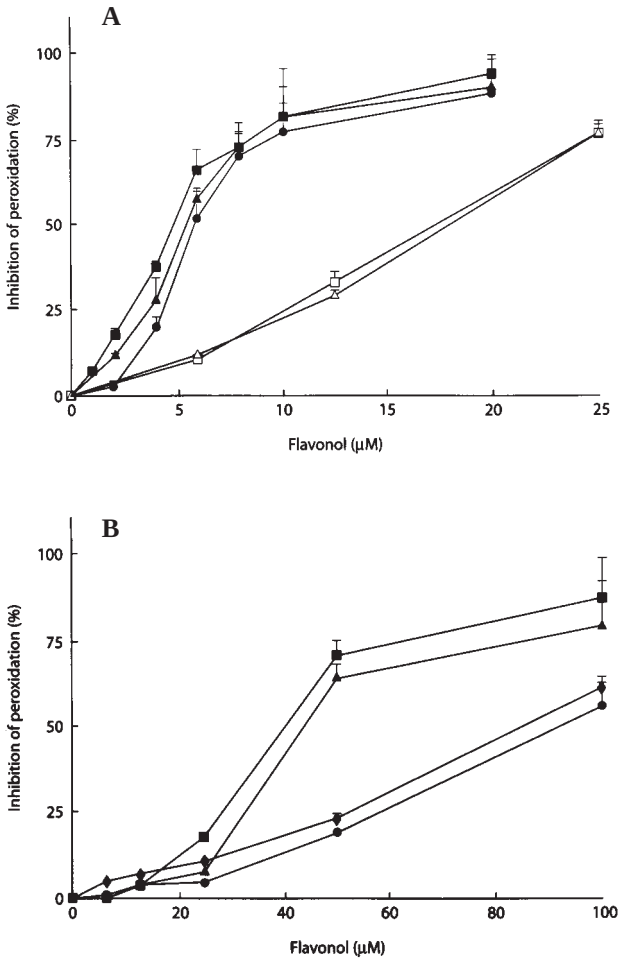


Fig. 2. (**A**) Effect of quercetin glycosides on the inhibition of iron/ascorbate-induced lipid peroxidation of phosphatidyl choline. The peroxidation was performed in the presence of (filled squares) Q 3-O-gal, (filled triangles) Q 3-O-glu, (filled circles) Q 3-O-glu-rha, (open squares) Q 3-O-gal-rha-glu, and (open triangles) Q 3-O-glu-rha-glu. Values represent the mean and standard deviations of three determinations. (**B**) Effect of kaempferol glycosides on the inhibition of iron/ascorbate-induced lipid peroxidation of phosphatidyl choline. The peroxidation was performed in the presence of (filled circles) K 3-O-glu, (filled diamonds) K 3-O-glu-rha, (filled triangles) K 3-O-gal-rha-glu, (filled squares) K 3-O-glu-rha-glu. Values represent the mean and standard deviations of three determinations.

(IC_{50} = 5.06 ± 0.41 μM) and the diglycoside rutin (IC_{50} = 5.66 ± 0.21 μM) were all potent inhibitors, similar to the quercetin aglycone (IC_{50} = 7.7 ± 0.3 μM) and comparable to BHT and Trolox C. This is consistent with previous studies which demonstrated that 3-O-glycosylation of quercetin has only a small effect on the ability to inhibit lipid peroxidation.[13,15] The two quercetin triglycosides (Q 3-gal-rha-glu and Q 3-O-glu-rha-glu) were less effective at inhibiting lipid peroxidation, with IC_{50} values of 13.7 ± 0.5 μM and 19.0 ± 1.1 μM, respectively. Glycosylation with one or two sugar moieties at the 3-position did not adversely affect the ability of quercetin to inhibit lipid

peroxidation, but 'adding' a third sugar group drastically reduces this effect. Figure 2B shows the glycosides of kaempferol were relatively ineffective at preventing peroxidation in this system (IC_{50}s 32.7–100 μM), even though kaempferol is a relatively good inhibitor (12.6 ± 0.6 μM). These data are consistent with the activity of kaempferol sophoroside.[15]

Tea is a major source of flavonols in many countries and although the total amount of these compounds does not vary greatly in different teas (when expressed in terms of their total aglycone content), there are large differences in both the individual conjugates present and the proportion of conjugates for each flavonol. For example, the variety Keemun, possesses predominantly kaempferol conjugates whilst the other five teas have the quercetin conjugates as the major components. In addition, the number of sugar groups conjugated to the flavonols varies according to the processing of the tea. The triglycosides predominate in the teas which are least fermented, e.g. Darjeeling and the di- and mono-glycosides predominate in the most highly fermented teas, e.g. Nunjo. A knowledge of these differences is, therefore, important when assessing the antioxidant activity of tea because of the reduced potency of the triglycosides compared to the di- and mono-glycosides of quercetin, and the greater activity of quercetin conjugates compared to those of kaempferol.

The compounds described herein represent the major flavonols found in tea and, although much is known about their activity *in vitro*, there is no information on their uptake, absorption or metabolism. However, studies employing a range of quercetin glycosides suggested that some of these glycosides are absorbed through the small intestine,[9,16] the remainder passing into the colon where they are metabolised by the gut microflora. This is consistent with the observation that unidentified flavonol glycosides have been observed in human plasma.[17] These studies also suggest that the terminal sugar of the glycoside is an important determinant for absorption via the small intestine. Therefore terminal glucose, which is a feature of the triglycosides from tea, may enhance absorption, whereas a terminal rhamnose (as in rutin) inhibits absorption.

It is important to determine the nature of conjugation of the flavonols using a combination of chromatography and spectroscopy, since a simple analysis after acid hydrolysis, to determine total phenolics as aglycones, would not provide an appropriate indication of antioxidant activity since almost all the flavonols present in the plant tissue are in conjugated forms. Therefore, the biological activity of these compounds actually present in the tissue is important when considering antioxidant action in the gut, transport into the blood and metabolic action in the liver. Further, we predict that metabolism by conjugation would also dramatically affect the antioxidant activity of flavonols *in vivo*.

This study supplies information that is needed to increase our understanding of the biological significance of dietary flavonols and the relationship between their consumption and disease prevention.

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