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Research article

Antioxidant properties of flavonol glycosides from green beans

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We have examined the antioxidant activity of one class of polyphenolic compounds in green beans: two novel flavonol glycosides (quercetin 3-*O*-[xylosyl(1→2)]-rhamnosyl (1→6)-glucoside and the corresponding kaempferol analogue), quercetin 3-*O*-glucuronide, kaempferol 3-*O*-glucuronide, quercetin 3-*O*-rutinoside and kaempferol 3-*O*-rutinoside. 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 glucuronide and rutinoside of quercetin were good antioxidants, but not as effective as the quercetin aglycone. TEAC values for the glucuronide and other glycosides of kaempferol were much lower than the corresponding quercetin species but similar to that of the kaempferol aglycone. Quercetin 3-*O*-glucuronide and quercetin 3-*O*-rutinoside were both potent inhibitors of lipid peroxidation, in contrast to the those of kaempferol. The compounds described herein demonstrate the antioxidant activity of the flavonols present in green beans and indicate the effect on antioxidant activity of sugar substitutions in the phenolic C ring.

INTRODUCTION

The association between a diet rich in fruit and vegetables and a decreased risk of cardiovascular disease and certain forms of cancer is supported by a considerable amount of epidemiological evidence.[1,2] Recent work has highlighted that, in addition to the antioxidant nutrients, the polyphenolic components of higher plants (such as flavonoids) may also contribute to the beneficial health effects of fruit and vegetables.[3,4] Flavonoids are a class of compounds distributed widely in the plant kingdom,[5] individual compounds of which have demonstrated high antioxidant activity,[6] anti-mutagenic activity[7] and the ability to act as vasodilators.[8] The antioxidant action of individual flavonoid compounds includes both the ability to scavenge free radicals[9,10] and to induce phase II enzymes, such as quinone reductase.[11]

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There are five foods in the UK diet (tea, onion, broccoli, apple and green bean) which supply the majority of flavonols (a sub-class of flavonoids) in our diet,[5] the composition and content of which has received much attention.[12,13] To begin to understand the role of these flavonols as phytoprotectants, their properties in *in vitro* studies are of considerable importance. Recent studies have demonstrated the chemical nature of the flavonols present in the plant tissue (a multiplicity of conjugates) is key to their bioactivity and bioavailability. The degree of hydroxylation is important in determining the antioxidant activity[14] and induction of quinone reductase,[11] whereas the degree of glycosylation may be important in determining their ability to cross the intestinal wall.[15]

Recent studies have identified six flavonol conjugates from commercially available varieties of green bean grown in the UK.[16] Two of these compounds (quercetin 3-*O*-[xylosyl(1→2)]-rhamnosyl(1→6)-glucoside and the corresponding kaempferol analogue), were found to be novel to both the green bean and the human food chain. The other compounds isolated were quercetin 3-*O*-glucuronide, quercetin 3-*O*-rutinoside, kaempferol 3-*O*-glucuronide and kaempferol 3-*O*-rutinoside. This paper reports the antioxidant properties of these compounds.

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Materials and Methods

Green bean (*Phaseolus vulgaris*) varieties Labrador, Matador, Lasso and Montano were grown on a commercial scale in Norfolk and collected at the normal time of commercial harvest (19–28 August 1998). All solvents were of Analar or HPLC grade where appropriate. MN polyamide SC6 was supplied by Macherey-Nagel GmbH & Co.

Extraction of flavonol conjugate mixture

Fresh beans (1 kg) were freeze dried and powdered. Ten portions (100 g) were each homogenised 3 times in 70% methanol (650 ml and cooled in ice) at 1200 rpm for 5 min (Pro400 homogeniser, Radleys, Saffron Walden, UK) and the homogenate filtered under reduced pressure through filter paper (Whatman No. 541). The combined fractions were partially evaporated *in vacuo* at 40°C (1 l) to remove the methanol and the resultant aqueous phase was de-fatted by serial extraction with 3 portions of hexane (750 ml). The aqueous layer was fractionated in 2 portions (500 ml) on a polyamide column (100 g) which had been preconditioned with methanol (1 l) followed by water (2 l). The column was washed with water (1 l) and further eluted, under a pressure of 10 psi nitrogen, with methanol (1.2 l) to elute the neutral flavonols and with methanol: ammonia (99.5:0.5, 1 l) to elute the acidic flavonols. Each extract was evaporated to dryness under reduced pressure at 40°C to yield 2.17 g and 1.59 g, respectively, for the neutral and acid fractions.

Isolation of individual flavonol conjugates

Preparative high performance liquid chromatography (HPLC) was performed using a Prodigy 5 µ ODS3 reversed phase silica (250 mm x 21.2 mm i.d., Phenomenex Ltd, Macclesfield, UK) column with an isocratic solvent [(8:2) acetonitrile/0.1% trifluoroacetic acid (TFA)] at a flow rate of 5 ml/min. The column effluent was monitored at 270 nm and 370 nm, and the fractions were collected using a Gilson fraction collector. Structures of individual compounds were confirmed using a combination of NMR and spectroscopy as described previously.[16]

Lipid phase antioxidant activity

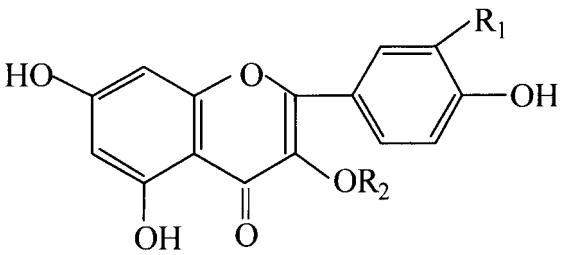
Phospholipid liposomes (final concentration 1 mg/ml) were suspended in 150 mM KCl containing 0.2 mM $FeCl_3$ and test compound at a range of concentrations. Peroxidation was started as described previously[17] with ascorbate (final concentration 0.05 mM), in a final volume of 0.4 ml. Samples were incubated 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 (BHT) in ethanol. The production of thiobarbituric acid reactive substances (TBARS) was measured after incubation at 80°C for 20 min.[17] 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. Calculations of IC_{50} values were 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.*[18] Values are expressed relative to a standard of Trolox, 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′-azinobis(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. Quercetin was used as a positive control, and the TEAC value obtained agreed well with published data.[6]

Results and Discussion

The TEAC values for the green bean flavonols (structures shown in Fig. 1) are listed in Table 1 in addition to the values for their respective aglycones for comparative purposes. Quercetin is a highly effective antioxidant in this assay. Loss of the free 3-OH group in the C ring of quercetin, as in the glucuronide and the rutinoside, drastically decreased antioxidant activity in the aqueous phase, reducing the TEAC value to approximately 50% of that for the aglycone. The presence of a branched trisaccharide at the 3-*O*-position, such as the quercetin 3-*O*-[xylosyl(1→2)]-rhamnosyl (1→6)-glucoside, further reduced the antioxidant activity of the quercetin moiety. These results are consistent with previous studies which reported similar decreases in TEAC value with 3-*O*-glucosylation and 3-*O*-rutinosylation.[9–11] For maximum effectiveness in this assay, there is a requirement for a free –OH group at the 3-*O*-position attached to the 2,3 double bond and adjacent to the 4-carbonyl in the phenolic



R_1	R_2
-OH	Quercetin 3-0-glucuronide
-OH	Quercetin 3-0-rhamnosyl(1->6)-glucoside. (rutinoside)
-OH	Quercetin 3-0-[xylosyl(1->2)]-rhamnosyl(1->6)-glucoside
-H	Kaempferol 3-0-glucuronide
-H	Kaempferol 3-0-rhamnosyl(1->6)-glucoside. (rutinoside)
-H	Kaempferol 3-0-[xylosyl(1->2)]-rhamnosyl(1->6)-glucoside

Fig. 1. Structures of the six flavonol conjugates isolated from green bean.

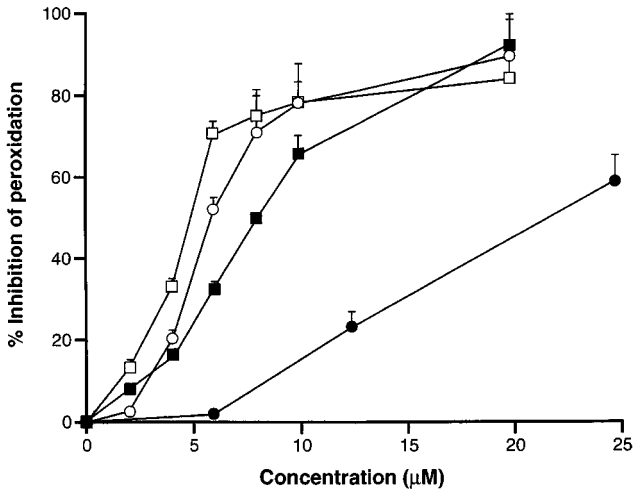


Fig. 2. Effect of quercetin and quercetin conjugates on the inhibition of iron/ascorbate-induced lipid peroxidation of phosphatidyl choline. The peroxidation was performed in the presence of (filled squares) quercetin, (open squares) quercetin 3-*O*-glucuronide, (filled circles) quercetin 3-*O*-[xylosyl(1→2)]-rhamnosyl (1→6)-glucoside, (open circles) quercetin 3-*O*-rutinoside. Values represent the mean and standard deviations of three determinations.

C ring.[6] Removal of any of these features drastically reduces antioxidant activity. Clearly, substitution at the 3-*O*-position with a sugar residue affects the ability of the B ring hydroxyl groups to donate hydrogen, whilst substitution with a larger sugar group further hinders this process. 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 phenolic B ring (as found in quercetin) is an essential motif for efficient antioxidant action. A single hydroxyl group in the B ring (as in kaempferol) has a much smaller contribution to the antioxidant capacity in this assay.

The effect of flavonols on the inhibition of lipid peroxidation of phosphatidyl choline is shown in Figures 2 and 3. The degree of inhibition was measured by estimating the IC_{50} values (concentration of test compound which inhibits peroxidation by 50%) which are listed in Table 1. As a comparison to the flavonols under study, the IC_{50} values for butylated hydroxytoluene and Trolox were 5.0 ± 0.2 μM and 12.6 ± 1.4 μM, respectively.

Figure 2 shows that the quercetin diglycoside and glucuronide from green bean were potent inhibitors of lipid peroxidation, possessing comparable IC_{50} values (5.1–5.7 μM) to the quercetin aglycone. The quercetin triglycoside (quercetin 3-*O*-[xylosyl(1→2)]-rhamnosyl (1→6)-glucoside) was less potent in this assay, possessing a lower IC_{50} value. Figure 3 shows that the kaempferol diglycoside, triglycoside and glucuronide were relatively ineffective inhibitors of lipid peroxidation in this system (IC_{50}s >79.4

Table 1. Levels of individual flavonols, their TEAC values and IC_{50} concentrations from inhibition of lipid peroxidation assay.

Compound	Trolox equivalent antioxidant capacity (TEAC)*	Inhibition of lipid peroxidation IC_{50} (μM)	Level (μg/g fresh weight)
Quercetin	4.43 ± 0.02	7.7 ± 0.6	–
Quercetin-3-O-gluA	2.02 ± 0.02	5.1 ± 0.2	3.5–15.1
Quercetin-3-O-glu-rha	2.09 ± 0.07	5.7 ± 0.2	0.2–4.3
Quercetin-3-O-glu(xyl)rha	1.51 ± 0.01	19.9 ± 1.2	1.0–2.0
Kaempferol	1.35 ± 0.02	12.6 ± 0.6	–
Kaempferol-3-O-gluA	1.23 ± 0.06	>100	0.5–1.3
Kaempferol-3-O-glu-rha	1.05 ± 0.02	79.4 ± 1.3	0.8
Kaempferol-3-O-glu(xyl)rha	1.19 ± 0.03	91.3 ± 3.9	0.3–0.7

*Relative to the antioxidant activity of Trolox.
The values shown for the antioxidant assays are the mean and standard deviation of at least three determinations.
The values obtained for quercetin and kaempferol are consistent with previous studies.[6]

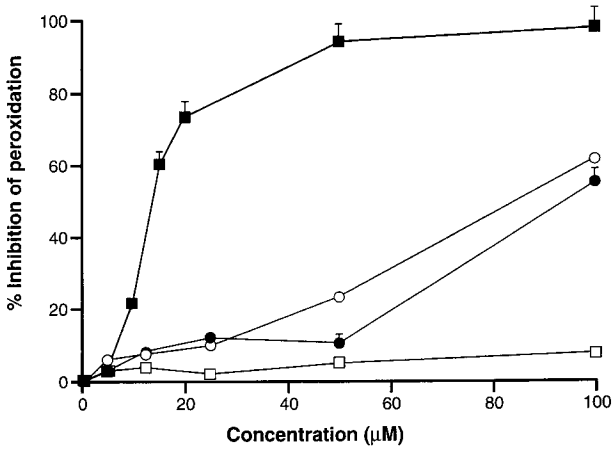


Fig. 3. Effect of kaempferol and kaempferol conjugates on the inhibition of iron/ascorbate-induced lipid peroxidation of phosphatidyl choline. The peroxidation was performed in the presence of (filled squares) kaempferol, (open squares) kaempferol 3-*O*-glucuronide, (filled circles) kaempferol 3-*O*-[xylosyl(1→2)]-rhamnosyl (1→6)-glucoside, (open circles) kaempferol 3-*O*-rutinoside. Values represent the mean and standard deviations of three determinations.

μM), even though kaempferol is a good inhibitor. These data are consistent with previous studies on the antioxidant activities of quercetin glycosides[9–11] and kaempferol glycosides.[9,10] The individual polyphenols in this assay may exert their protective effect in a variety of ways. They may scavenge reactive oxygen species, chelate Fe^{3+} ions, act as a chain breaking antioxidant by scavenging lipid peroxyl radicals or partition into the lipid bilayer to prevent lipid damage. The aglycone kaempferol is more hydrophobic than its glycosylated conjugates and, therefore, much more likely to partition into the phosphatidyl choline vesicles to inhibit peroxidation. This may account for the differential antioxidant potency between the kaempferol aglycone and its conjugates. In contrast, two conjugates of quercetin, quercetin 3-*O*-glucuronide and quercetin 3-*O*-rutinoside, were equally as effective as the aglycone at preventing peroxidation even though they differ in degrees of hydrophobicity. This difference can be explained however by the position and availability of –OH groups on the phenolic rings. When quercetin reacts with a lipid radical to form a phenoxyl radical, the *O*-dihyroxy substitution in the B ring is an important determinant in affording the resulting free radical stability by delocalisation of unpaired electrons around the aromatic ring. Substitution of the hydroxyl group at the 3-*O*-position of the C ring with glucuronic acid or a disaccharide had no effect on the antioxidant potency of quercetin, demonstrating the 3-OH makes only a minor contribution to the stabilisation of the quercetin radical. In addition, studies have demonstrated that the torsion angle of the B phenolic ring with the rest of the flavonoid molecule is correlated with the free radical scavenging ability of the molecule.[19] The hydroxyl group at the 3-*O*-position of quercetin anchors the position of the phenolic B ring in the same plane as the A and C rings via hydrogen bonding. The resulting torsion angle of the phenolic B ring with the rest of the molecule is approximately 0°, yielding increased coplanarity of the phenolic B ring. Substitution of the hydroxyl moiety at the 3-*O*-position induces a torsion angle of approximately 20° of the phenolic B ring with the rest of the molecule, resulting in a loss of coplanarity and decreasing the conjugation, thus diminishing the antioxidant ability. When kaempferol reacts with a lipid radical, stabilisation of the resulting radical may occur through the 3-OH, 5-OH, and 4-oxo groups and conjugation from the A ring to the B ring, but the important *O*-dihydroxy motif in the B ring is absent. Kaempferol is, therefore, a poorer inhibitor of lipid peroxidation. The combination of a missing *O*-dihydroxy motif in the B ring and substitution of the 3-OH with sugar residues (as in the kaempferol conjugates), results in the antioxidant activity being substantially reduced.

The compounds reported herein represent the major flavonols found in green bean. The quercetin conjugates, especially quercetin 3-*O*-glucuronide, dominated in all the varieties assayed (see Table 1), whilst the novel triglycosides of both quercetin and kaempferol were not present in one of the varieties.[16] Since two of these compounds have only been recently isolated and characterised,[16] there is no information on their metabolism or absorption. However, a study on the uptake of other quercetin glycosides suggested that some of these conjugates may be preferentially absorbed through the small intestine as intact glycosides.[20] The remaining unabsorbed conjugates passing into the colon where they are broken down by the gut flora into a range of phenolic acids.

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, does 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.

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