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Antioxidant Properties of the Major Polyphenolic Compounds in Broccoli

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We have examined the antioxidant activity of the major phenolic compounds in Broccoli: two flavonol glycosides (quercetin 3-O-sophoroside and kaempferol 3-O-sophoroside) and four hydroxycinnamic acid esters (1,2′-disinapoyl-2-feruloyl gentiobiose, 1-sinapoyl-2-feruloyl gentiobiose, 1,2,2′-trisinapoyl gentiobiose and 1,2-disinapoyl gentiobiose). The Trolox C 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 two flavonol glycosides were less active than their respective aglycones. TEAC values for the hydroxycinnamic acid esters were less than the sum of their constituent hydroxycinnamic acids on a molar basis. Quercetin 3-O-sophoroside was a potent inhibitor of lipid peroxidation, in contrast to kaempferol 3-O-sophoroside. The hydroxycinnamic acid esters were highly effective at preventing lipid damage with the exception of 1,2,2′-trisinapoyl gentiobiose. The six compounds analysed herein demonstrate the antioxidant activity of the major phenolics in broccoli and indicate the effect on antioxidant activity of sugar substitutions in the phenolic B ring.

Keywords: broccoli, hydroxycinnamic acid esters, flavonol glycosides, quercetin, ferulic acid, sinapic acid, antioxidant, lipid peroxidation, TEAC

INTRODUCTION

The implication of flavonoids and hydroxycinnamic acids in the diet is receiving much interest and the biological activity of these polyphenolic components is of great importance in understanding their mode of action *in vivo*. Interest has been focused on the health benefits derived from a diet rich in flavonoids. These compounds can be found in many fruits and vegetables, and may play a role in reducing the risk of heart disease.[1,2] In addition, individual compounds have been shown to exhibit high antioxidant activity,[3] anti-mutagenic activity[4] and the ability to act as vasodilators.[5]

Broccoli is becoming increasingly popular as a fresh vegetable and is a significant source of biologically active dietary components such as the flavonol glycosides,[6] hydroxycinnamic acids[7] and sulphur-containing compounds such as the glucosinolates.[8] Recent studies have identified six novel compounds from broccoli florets

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(Figure 1) which represent up to 75% of the phenolic fraction; two flavonol glycosides (quercetin 3-O-sophoroside and kaempferol 3-O-sophoroside)[9] and four hydroxycinnamic acid esters (1,2'-disinapoyl-2-feruloyl gentiobiose, 1-sinapoyl-2-feruloyl gentiobiose, 1,2,2'-trisinapoyl gentiobiose and 1,2-disinapoyl gentiobiose.[10] This paper reports the antioxidant properties of these compounds.

MATERIALS AND METHODS

All chemicals were of the highest grade available supplied by Sigma Chemical Company (Poole, UK). Florets of Broccoli were taken from freshly harvested plants of *Brassica oleraceae* (cv. Marathon) grown under commercial conditions in the UK and immediately immersed in liquid nitrogen for subsequent freeze-drying.

Preparation of Samples

Four hydroxycinnamic acid esters were isolated from freeze dried broccoli florets by maceration with 70% aqueous methanol at room temperature. The methanolic extract was subjected to preparative reverse phase high performance liquid chromatography (HPLC) using a C18 silica column and a water:acetonitrile eluate as described by Price *et al.*[10] Two novel flavonol glycosides were similarly isolated according to the method of Price *et al.*[9] Chemical identification of all compounds was achieved using a combination of NMR spectroscopy, U.V. spectroscopy and mass spectroscopy.[9,10] Chromatographic techniques such as HPLC in reversed mode (C18 silica), monitored by UV absorption using a diode array detector, and thin layer chromatography in normal phase mode (silica gel) visualised with sprays specific for plant glycosides, were used to assess the purity of these compounds. For quantification purposes, the HPLC detector was operated at a wavelength of 270 nm and the purity of each compound was >95% with the exception of 1-sinapoyl-2-feruloyl gentiobiose (91%) and 1,2-disinapoyl gentiobiose (88%) by peak area measurements. The impurities in each case were identified as either one or more of the other gentiobiose compounds or the other flavonol sophoroside respectively.

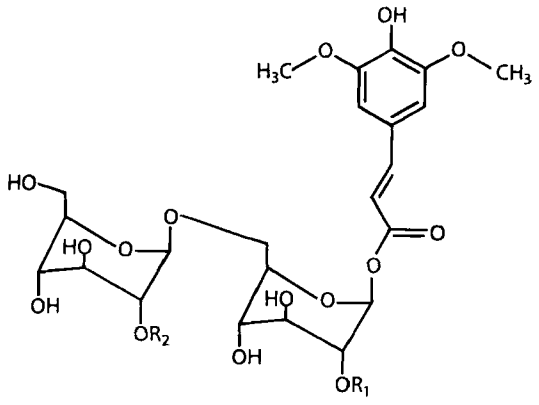
Lipid Phase Antioxidant Activity

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 % 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 the data.

Aqueous Phase Antioxidant Activity

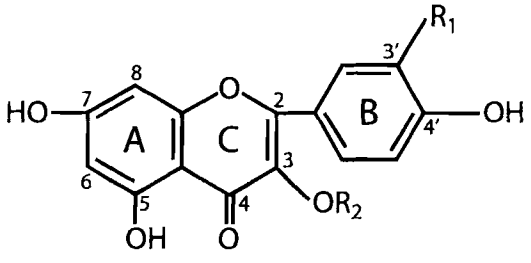
The Trolox equivalent antioxidant capacity (TEAC) was measured by the method of Salah *et al.*[12] Values are expressed relative to a standard of Trolox C, the water soluble analogue of vitamin E. The assay is based on the relative ability of antioxidants to scavenge the radical cation of 2,2'-azinobis(3-ethyl-benzothiazoline-6-sulphonate (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 spec-

A



Compound	R_1	R_2	Content in broccoli florets µg/g fresh weight
1,2'-disinapoyl-2-feruloyl gentiobiose	feruloyl	sinapoyl	62
1-sinapoyl-2-feruloyl gentiobiose	feruloyl	H	148
1,2,2'-trisinapoyl gentiobiose	sinapoyl	sinapoyl	62
1,2-disinapoyl gentiobiose	sinapoyl	H	64

B



Compound	R_1	R_2	Content in broccoli florets µg/g fresh weight
Quercetin 3 - O - sophoroside	- OH	- sophorose	65
Kaempferol 3 - O - sophoroside	- H	-sophorose	166
Quercetin	- OH	- H	-
Kaempferol	- H	- H	-

FIGURE 1 (**A**) Structures of the four hydroxycinnamic acid esters isolated from broccoli. (**B**) Structures of the two flavonol glycosides isolated from broccoli and their respective aglycones.

trophotometrically at 734 nm and compared to standard amounts of Trolox C. Positive controls were performed as described previously.[13]

RESULTS AND DISCUSSION

The TEAC values for the flavonol glycosides and hydroxycinnamic acid esters (structures shown in Figure 1) are shown in Table I. In the case of the flavonol glycosides, the values for both are strikingly lower than their corresponding aglycones, particularly for quercetin 3-O-sophoroside. This is consistent with previous studies which reported that 3-O-glucosylation or 3-O-rutinosylation of quercetin decreased the TEAC value by two fold.[3,13] 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 4-carbonyl in the C-ring.[3] Removal of any of these features drastically reduces antioxidant activity. Clearly substitution at the 3-position with a glucose residue affects the ability of the B ring hydroxyl groups to donate hydrogen. Substitution with two sugar residues further decreases TEAC values.

The TEAC values for the hydroxycinnamic acid esters are compared to their constituent monomeric, free hydroxycinnamic acids in Table I. Caffeic acid (3,4-dihydroxy-cinnamic acid) has a TEAC value of 1.33 ± 0.021. Methylation of the 3-hydroxyl group (ferulic acid) considerably enhances the TEAC value. Adding a further methoxy group (sinapic acid) does not increase the TEAC value appreciably. Of the hydroxycinnamic acid esters, disinapoyl feruloyl and trisinapoyl esters possessed significantly higher TEAC values than the disinapoyl and sinapoyl feruloyl esters. For all the esters, the TEAC value is not simply a sum of component phenolics, i.e. the TEAC value for the disinapoyl ester is much less than twice the sinapic acid value.

Esterification of hydroxycinnamates and glycosylation of flavonols modifies partition coefficients/solubility and radical scavenging.[3] 3-O-rutinosylation of quercetin either increases or decreases the antioxidant action depending on the assay used,[14,15] and so predicting the effect of glycosylation is difficult. However, the antioxidant activity of phenolics is very approximately due to the total number of free phenolic hydroxyl moieties in the molecule[3] which affects both metal binding (depending on location) and

TABLE I Trolox C equivalent antioxidant values

Compound	Trolox Equivalent Antioxidant Capacity (TEAC)*	Number of free phenolic -OH groups
Kaempferol	1.35 ± 0.024	4
Kaempferol 3-O-sophoroside	1.03 ± 0.025	3
Quercetin	4.43 ± 0.027	5
Quercetin 3-O-sophoroside	1.45 ± 0.020	4
1,2-disinapoyl gentiobiose	1.67 ± 0.016	2
1-sinapoyl-2-feruloyl gentiobiose	1.79 ± 0.025	2
1,2,2′-trisinapoyl gentiobiose	2.15 ± 0.016	3
1,2′-disinapoyl-2-feruloyl gentiobiose	2.26 ± 0.013	3
Caffeic Acid	1.33 ± 0.021	2
Ferulic Acid	1.96 ± 0.009	1
Sinapic Acid	1.84 ± 0.009	1

* Relative to the antioxidant activity of Trolox C (mM).[3] The values shown are the mean and standard deviation of at least three determinations. The values obtained for quercetin, kaempferol, ferulic and caffeic acid are consistent with previous studies.[3]

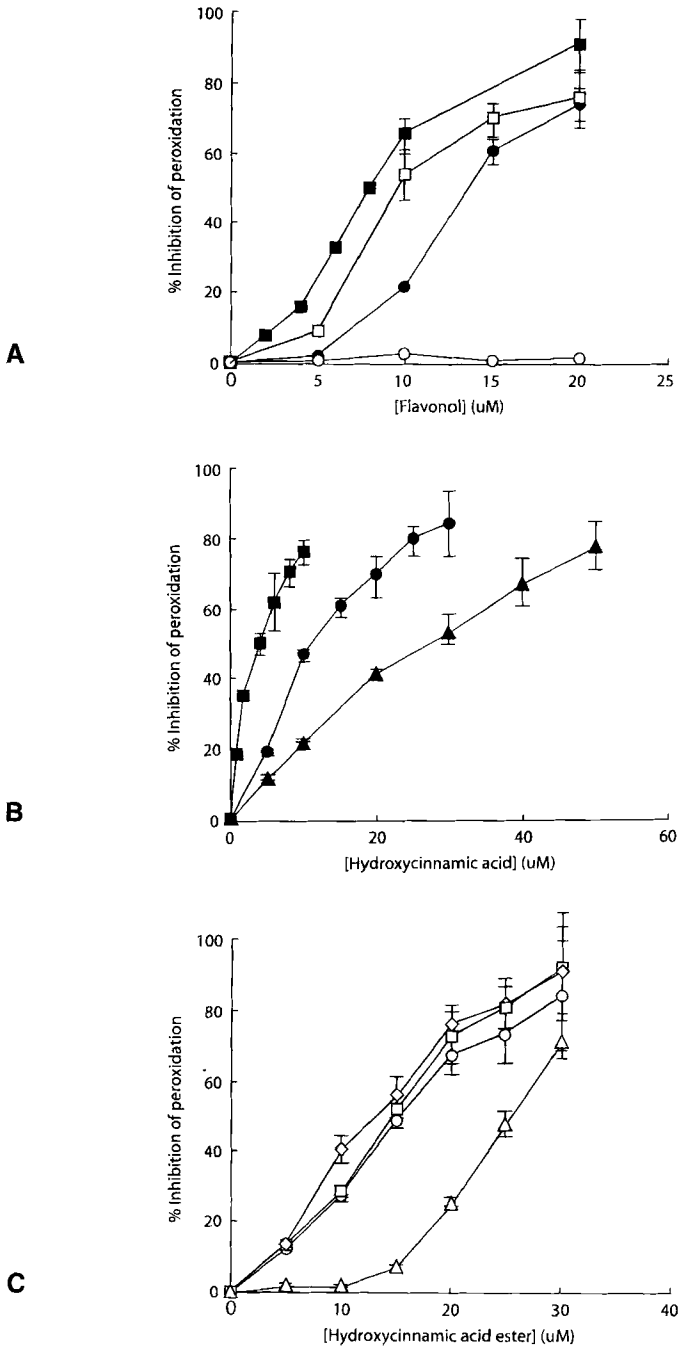


FIGURE 2 (**A**)Effect of flavonols and flavonol glycosides on the inhibition of iron/ascorbate-induced lipid peroxidation of phosphatidyl choline. The peroxidation reaction was performed in the presence of (■) quercetin, (□) quercetin 3-O-sophoroside, (●) kaempferol and (○) kaempferol 3-O-sophoroside. (**B**) Effect of hydroxycinnamic acids on the inhibition of iron/ascorbate-induced lipid peroxidation of phosphatidyl choline. The peroxidation reaction was performed in the presence of (■) caffeic acid, (●) ferulic acid, and (▲) sinapic acid. (**C**) Effect of hydroxycinnamic acid esters on the inhibition of iron/ascorbate-induced lipid peroxidation of phosphatidyl choline. The peroxidation reaction was performed in the presence of (○) 1,2′-disinapoyl-2-feruloyl gentiobiose, (△) 1-sinapoyl-2-feruloyl gentiobiose, (□) 1,2,2′-trisinapoyl gentiobiose and (◊) 1,2-disinapoyl gentiobiose. Values represent the mean and standard deviations of three determinations.

hydrogen donation (depending on pK_a). The number of hydroxyls in each of the compounds tested here is indicated in Table I and there is a trend to increasing TEAC value with the number of hydroxyls within the flavonol group.

The effect of the flavonols on lipid peroxidation of phosphatidyl choline is shown in Figure 2a. The degree of inhibition was measured by obtaining the IC_{50} (concentration of test compound which inhibits peroxidation 50%). As a comparison to the flavonols under study, the IC_{50} values for the antioxidants butylated hydroxytoluene and Trolox C were 5.0 ± 0.2 and 12.6 ± 1.4 μM respectively. Quercetin 3-O-sophoroside was a potent inhibitor with IC_{50} = 9.2 ± 0.3 μM, similar to the quercetin aglycone (7.7 ± 0.3 μM).[12] This is consistent with a previous study which demonstrated that 3-O-glycosylation of quercetin has only a small effect on the ability to inhibit lipid peroxidation. In contrast, kaempferol 3-O-sophoroside was ineffective at preventing peroxidation in this system, even though kaempferol is a relatively good inhibitor (IC_{50} = 12.6 ± 0.6 μM). Figure 2b shows the effect of hydroxycinnamic acids on lipid peroxidation. Caffeic acid was the most potent inhibitor of lipid peroxidation (IC_{50} = 3.9 ± 0.1 μM) followed by ferulic acid (IC_{50} = 11.6 ± 0.5 μM) and sinapic acid (IC_{50} = 26.6 ± 0.4 μM). In Figure 2c, three of the hydroxycinnamic acid esters were also good inhibitors of lipid peroxidation with IC_{50} values in the range 12–15 μM. The sinapoyl feruloyl ester was considerably less effective (IC_{50} = 23.9 ± 2.5 μM).

The compounds described herein comprise approximately 75% of the total phenolics in broccoli as analysed by HPLC after methanol extraction and partial purification using polyamide chromatography. It is possible to analyse for flavonols and hyroxycinnamates after acid/alkaline hydrolysis and to determine total phenolics as aglycones. However, this does not provide an appropriate indication of the antioxidant activity of a plant extract or food, since almost all of these phenolics are present in the plant tissue in conjugated forms. Based on the composition of the polyphenolic compounds in broccoli (Figure 1), a theoretical TEAC value of approximately 1.2 would be obtained for the unmodified glycosides in 100 g fresh weight of broccoli florets (assuming that the water content is 90%). If the same calculation is made after the complete hydrolysis of these compounds (addition of the TEAC values of the resulting aglycones and free hydroxycinnamic acids), then the TEAC value increases to approximately 2.6. These data indicate the importance of measuring the biological activity of compounds which are actually present in the tissue rather than the equivalent aglycones resulting from chemical hydrolysis.

These novel compounds have only recently been isolated and characterised, and so there is no information on the uptake and absorption in animals or human, and no information on the metabolism. However, comparison with other flavonol glycosides such as quercetin-4'-glucoside, and with other hydroxycinnamate esters such as chlorogenic acid, suggest that the compounds are absorbed through the small intestine as glycosides or esters.[16,17] Some portion of the compounds are not absorbed in the small intestine, and these pass into the colon where they are broken down by gut microbes. This is supported by the observation that unidentified flavonoid glycosides can be observed in human plasma.[18]

Acknowledgements

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