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Comparison of Quercetin and a Non-Orthohydroxy Flavonol As Antioxidants by Competing In Vitro Oxidation Reactions

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Two structurally related flavonols, quercetin and morin, along with protocatechuic acid (PA), β-resorcylic acid (DHBA), and phloroglucinol carboxylic acid (PCA), which represent quercetin and morin degradation products, were assessed with respect to their antioxidant potency by chemical comparisons in competing oxidation reactions. The measurement of the antioxidant capacity was performed with the β-carotene bleaching method, and the compounds were also tested with respect to their abilities to prevent lipid, protein, and DNA oxidation. The effect of concentration was also considered. The results obtained strongly suggested that quercetin is a powerful antioxidant in every system used, whereas morin is a much weaker antioxidant and in some cases may also have pro-oxidant action. PA and PCA were always inferior antioxidants compared to the parent molecule quercetin; DHBA and PCA exhibited activities comparable to that of morin in reaction comparisons.

Keywords: *Antioxidant activity; flavonols; morin; phenolic acids; phloroglucinol carboxylic acid; protocatechuic acid; quercetin; β-resorcylic acid*

INTRODUCTION

Currently available information indicates that flavonoids are relatively stable compounds with respect to various modes of processing (e.g., boiling, frying, etc.), but it has also been noted that processing may result in an appreciable loss of these components. Hence, it might be supported that flavonoid degradation during processing of a given plant food may be influenced by several factors, but the fate of flavonoids in the processed foods, in terms of degradation mechanism and product formation, is rather obscure. The complexity of plant tissues with regard to chemical composition does not facilitate the detection of products that may arise from flavonoid decomposition, and it is also probable that such products undergo further changes and/or react with other food constituents. Thus the impact of processing on plant foods can only be estimated indirectly, i.e., by comparing the antioxidant activity of extracts of a plant tissue before and after treatment. Such an approach, however, may be of limited value because the factors that may alter the chemical and biological activities of the plant extract would remain unknown.

The hydroxyl free-radical-mediated oxidative degradation of quercetin and morin has been shown to yield a wide range of products (*1*). As regards quercetin, the cleavage of its skeleton gives characteristic fragments, such as PA and PCA (Figure 1). Although these two phenolic acids do not always represent major degradation products, in terms of quantity, they are formed even under very different conditions. For example, PA was one of the major products arising from quercetin decomposition in alkaline and buffer solutions (*2*, *3*). This is evidence of their importance in the degradation mechanism of quercetin, and it suggests that both components could represent, under certain circumstances, intermediary as well as end products in quercetin oxidation. In a similar way, oxidation of morin gives PCA and DHBA, which represents the ex-B ring of morin skeleton and corresponds to PA.

On the basis of those considerations, PA, DHBA, and PCA were subjected to some representative in vitro biochemical tests (*4*), to obtain clues about their potencies as biological antioxidants, and to compare their properties with those of the parent molecules quercetin and morin. These tests were carried out to investigate whether cleavage of flavonols could result in other antioxidant phenolics, and how this cleavage could affect the antioxidant activities of flavonols. BHT was used as a reference compound because its antioxidant properties are well studied. The tests employed were concerned with the oxidation of molecules of biological importance such as lipids, proteins, and DNA. Oxidations were induced through a citrate−Fe^{2+} system which is capable of triggering Fenton-type reactions (*3*) which are known for their deleterious effects in living tissues. In some cases, H_2O_2 was also included in reaction mixtures to assist oxidations.

MATERIALS AND METHODS

Chemicals. Acetic acid, chloroform, ethyl acetate (EtOAc), ethylene glycol monomethyl ether (EGME), and methanol (MeOH) were from BDH Chemicals Ltd. (Poole, England). All solvents were of analytical grade. 2,4-Dihydroxybenzoic acid (β-resorcylic acid, DHBA) and 2,4,6-trihydroxybenzoic acid (phloroglucinol carboxylic acid, PCA) were from Aldrich Chemical Co Ltd. (Gillingham-Dorset, U.K.). Bovine serum albumin (BSA, fraction V, 96%), butylated hydroxytoluene (BHT), *trans*-β-carotene, 3,4-dihydroxybenzoic acid (protocatechuic acid, PA), DNA (from calf thymus, ctDNA), guanidine, α-L-lecithin (α-L-phosphatidylcholine, 60%, type X-T, from egg), linoleic acid, morin, quercetin, 2-thiobarbituric acid (TBA), and Tween

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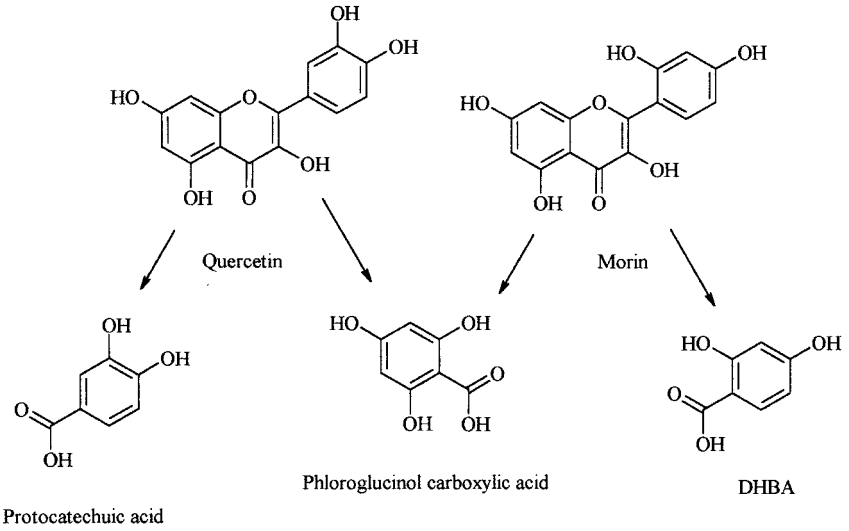

Figure 1. Phenolic acids arising from quercetin and morin HFR-mediated decomposition.

20 were from Sigma Chemical Co. (St. Louis, MO). Disodium-EDTA, citric acid, 2,4-dinitrophenylhydrazine (2,4-DNPH), ferrous sulfate ($FeSO_4$), hydrogen peroxide (H_2O_2), orthophosphoric acid, potassium dihydrogen orthophosphate, and trichloroacetic acid (TCA) were from BDH Chemicals Ltd. (Poole, England).

Evaluation of Antioxidant Capacity. For the evaluation of antioxidant capacity, the well-established method of thermally induced β-carotene bleaching was used, as described previously (*5*, *6*). In a 100-mL, round-bottom flask, 0.02 mL of linoleic acid, 0.2 mL of Tween 20, 1.0 mL of *trans*-β-carotene (0.2 mg mL^{-1} in chloroform), and 0.2 mL of the compound under examination (final concentration 1.1 μM) were added, and the mixture was taken to dryness with nitrogen. To the resulting residue 50 mL of oxygenated distilled water was added and mixed, and the absorbance was read at 470 nm. The solution was then incubated in a water bath at 50 °C to induce autoxidation. Samples were taken at 10-min intervals to monitor the evolution of A_{470}. Flasks were covered with aluminum foil throughout experiments to avoid light-induced oxidation of β-carotene. Each experiment was repeated twice.

Antioxidant potency was based upon three different calculations. Antioxidant activity (A_A) was determined as percent inhibition relative to control sample using the following equation (*7*):

$$A_A = [(R_{control} - R_{sample})/(R_{control})] \times 100 \qquad (a)$$

where $R_{control}$ and R_{sample} represent the bleaching rates of β-carotene without and with the addition of antioxidant, respectively. Degradation rates (R_D) were calculated according to first-order kinetics:

$$R_D = ln(A_I/A_X) \times 1/t \qquad (a\text{-}1)$$

where *ln* is natural log, A_I is the initial A_{470} ($t = 0$), and A_X is the A_{470} at $t = 10$, 20, 30, etc. min.

The oxidation rate ratio (R_{OR}) was calculated using the equation (*8*)

$$R_{OR} = R_{sample}/R_{control} \qquad (b)$$

where R_{sample} and $R_{control}$ are as described previously.

The antioxidant activity coefficient (C_{AA}) was calculated using the equation (*6*)

$$C_{AA} = [(A_{s(120)} - A_{c(120)})/(A_{c(0)} - A_{c(120)})] \times 1000 \qquad (c)$$

where $A_{s(120)}$ is the absorbance of the sample containing antioxidant at $t = 120$ min, $A_{c(120)}$ is the absorbance of the control at $t = 120$ min, and $A_{c(0)}$ the absorbance of the control at $t = 0$ min.

Prevention of Oxidative Damage to Lipids. The ability of the compounds to prevent metal-catalyzed lipid peroxidation was tested by using an assay based on lecithin liposomes as peroxidation substrate (*9*).

Preparation of Lecithin Liposomes. Lecithin (47.3 mg) was dissolved in 1 mL of chloroform. Chloroform was evaporated with a stream of nitrogen, and the residue was suspended in 10 mL of phosphate buffer, pH 7.4. The suspension was sonicated for 1 min, and the resulting liposome was used as stock solution. The liposome was stored at 4 °C and used within 5 days.

Analytical Procedure. All solutions were prepared in 0.05 M phosphate buffer, pH 7.4. The sample (0.2 mL; final concentration 12.5, 25.0, and 50.0 μM), 0.2 mL citric acid/$FeSO_4$ (1.5/1.65 mM), and 0.2 mL of lecithin (0.09 mg mL^{-1}), were mixed in a 1.5-mL Eppendorf tube (total volume 0.6 mL), and incubated at 37 °C in a water bath for 60 min. To the solution was added 0.1 mL of TCA (30%), 0.04 mL of Na-EDTA (0.11 M), and 0.2 mL of TBA (0.8% w/v in 0.05 M NaOH), and heated at 90 °C in a water bath for 30 min. Samples were spun down for 5 min, and the absorbance was read at 532 nm. The % inhibition in TBARS formation relative to control was calculated on the basis of A_{532} measurements.

Prevention of Oxidative Damage to Proteins. A system based on Fenton-reaction-mediated BSA modification was used to evaluate the efficiency of the compounds to inhibit protein oxidation. The system was established on the basis of previously published data (*10*−*12*).

Analytical Procedure. Oxidation of BSA was performed in phosphate buffer, pH 7.4. BSA (0.05 mL; 20.3 mg mL^{-1}), 0.05 mL of $FeSO_4$/citric acid (4.4/4.0 mM), 0.05 mL of H_2O_2 (4.4 mM), and 0.05 mL of a compound solution (final concentration 12.5, 25.0, and 50.0 μM), were placed in a 1.5-mL Eppendorf tube (final volume 0.2 mL) and vortexed. The mixture was incubated in a water bath at 37 °C for 60 min.

Determination of Carbonyl Content. Carbonyl content of oxidized BSA was determined according to a previously published method (*13*). To the samples was added 0.5 mL of 2,4-DNPH (10 mM in 2 N HCl), and the resulting mixture was allowed to react for 60 min at room temperature, with vortexing every 15 min. After the completion of the reaction, 0.5 mL of TCA (20%) was added to precipitate protein, samples were centrifuged for 5 min, and the supernatant was discarded. The precipitate was washed three times with 1 mL of EtOH/EtOAc (1:1), each time centrifuging and discarding the supernatant. The washed precipitate was dissolved in 0.6 mL of guanidine (6 M in phosphate buffer, adjusted to pH 2.3 with TFA), incubated at 37 °C for 15−20 min, and centrifuged.

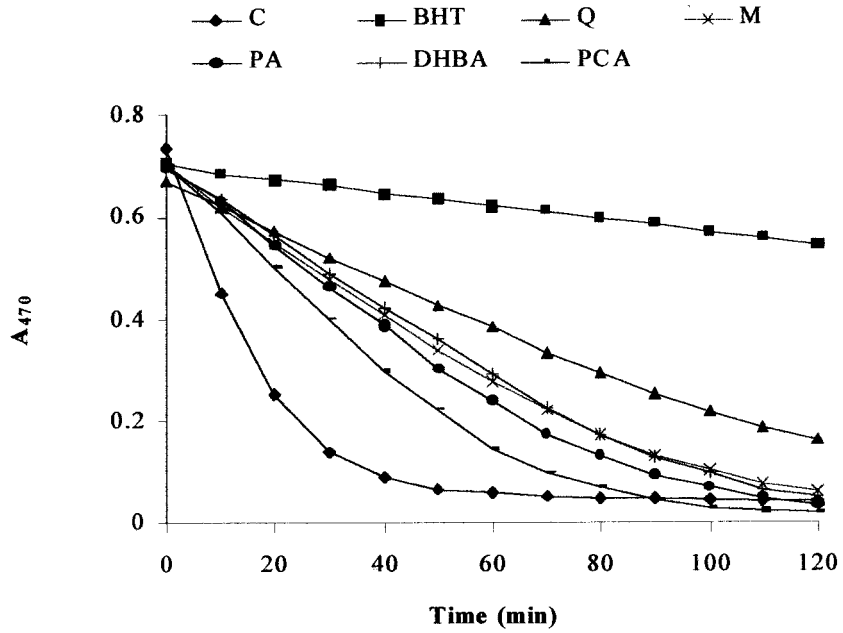

Figure 2. β-Carotene bleaching as affected by the addition of quercetin, morin, and the degradation products thereof. All compounds were tested at a final concentration of 1.1 μM. C, control; BHT, butylated hydroxytoluene; Q, quercetin; M, morin; PA, protocatechuic acid; PCA, phloroglucinol carboxylic acid; DHBA, 2,4-dihydroxybenzoic acid.

Results are expressed as % inhibition in carbonyl formation, relative to control, from the absorbance at 390 nm.

Prevention of Oxidative Damage to DNA. The evaluation of the preventive action of compounds against DNA damage was accomplished using a Fenton-reaction-based assay. Fenton-reaction-induced oxidation of DNA was performed as described previously (*14*), in phosphate buffer, pH 7.4. In a 1.5-mL Eppendorf tube, 0.15 mL of ctDNA (0.5 mg mL^{-1}), 0.15 mL of $FeSO_4$/citric acid (1.1/1.0 mM), 0.15 mL of H_2O_2 (0.44 mM), and 0.15 mL of a compound solution (final concentration 0.25, 1.0, and 4.0 μM), were added and mixed (final volume 0.6 mL). Samples were incubated in a water bath at 37 °C for 30 min, and DNA damage was estimated from thiobarbituric acid-reactive substances (TBARS) (*15*−*17*), determined as described for lipid peroxidation.

Statistics. Values represent means of triplicate analysis, except in the case of antioxidant capacity determinations, for which analyses were run in duplicate. Differences in the antioxidant parameters and in the % inhibition of oxidation of lipids, protein, and DNA among the compounds tested were calculated using Student's *t* test at 5% significance level.

RESULTS

Antioxidant Capacity. The test used to assess potency of the compounds as antioxidants is a well-established model system; based on β-carotene coupled reaction with autoxidized linoleic acid, there is a gradual decrease in A_{470} with carotene bleaching. Based on testing with the reference compound BHT, the concentration of 1.1 μM which produced measurable inhibition in carotene bleaching was set as the amount to be used for testing compounds in this assay system.

On the basis of the bleaching rates (Figure 2), three parameters relative to the control were calculated in order to compare the antioxidant abilities of the compounds. The calculation of all three parameters was judged necessary for comparison because similar data are available in the literature (*6*). Table 1 illustrates analytically the values obtained. BHT was the most effective antioxidant of the set and the most efficient compound with respect to all parameters ($P < 0.05$), as it gave the highest A_A and C_{AA}, and the lower R_{OR}. Quercetin gave significantly different values of A_A and R_{OR}. On the other hand, PA was found to be the less effective compound with regard to A_A and R_{OR} ($P < 0.05$). Similar results were found for PCA, but only for A_A.

Table 1. Parameters Used to Assess the Antioxidant Capacity of the Compounds Tested[a]

	parameters		
compound	A_A[b]	R_{OR}[c]	C_{AA}[d]
control	0.00	1.0000	0.00
BHT	94.55*	0.0545*	722.54*
quercetin	75.85*	0.2415*	173.41*
morin	59.50	0.4050	30.35
PA	26.75*	0.7325*	−11.56
DHBA	61.50	0.4600	15.90
PCA	37.75*	0.3850	−28.90

[a] Values are means of duplicate analysis. [b] Antioxidant activity. [c] Oxidation rate ratio. [d] Antioxidant activity coefficient. *Statistically different values.

It was observed that in all cases PA appeared to be a less powerful antioxidant as compared to the other two phenolic acids. Further, taking into consideration the antioxidant activity coefficient (C_{AA}), PA and PCA showed pro-oxidant activity, as both compounds gave negative values. Whereas quercetin was by far the most potent antioxidant in comparison with its degradation products PA and PCA by measurements of A_A and R_{OR}, DHBA and PCA were more efficient than the parent molecule morin.

Following is the overall ranking of the compounds with respect to A_A:

BHT > quercetin > DHBA > morin > PCA > PA

On the basis of R_{OR}, PCA and DHBA flip-flopped their rankings, with the order being:

BHT > quercetin > PCA > morin > DHBA > PA

Finally, C_{AA} values shifted the order of all compounds except BHT and quercetin:

BHT > quercetin > morin > DHBA > PA > PCA

Table 2. Efficiency of Quercetin, Morin, and Their Oxidation Products in Inhibiting Fe^{2+}-Catalyzed TBARS Formation, in a Lecithin Liposome, in Phosphate Buffer, pH 7.4[a]

	% inhibition[b]		
compound	12.5 μM	25.0 μM	50.0 μM
BHT	86.3*	87.3*	87.7*
quercetin	67.2*	83.8*	84.5*
morin	58.4	69.4	79.0
PA	8.6	11.9	10.9
DHBA	6.1	5.5	4.4
PCA	10.5	14.0	0.5*

[a] Values represent means of triplicate determinations. [b] Values are expressed as % inhibition in A_{532} relative to control. *Statistically different values ($P < 0.05$).

Prevention of Oxidative Damage to Lipids. The ability of the compounds to prevent lipid peroxidation was tested using liposomes of egg lecithin. Lipid peroxidation is known to result in the formation of malonaldehyde and other structurally similar compounds, which react with thiobarbituric acid (thiobarbituric acid-reactive substances, TBARS) to produce a chromophore that absorbs at 532 nm. The inhibition of TBARS formation provides sound evidence concerning the potency of a compound to protect against lipid peroxidation.

To obtain a more reliable picture, all the compounds were tested at concentrations of 12.5, 25.0, and 50.0 μM. From the data presented in Table 2 it can be seen that BHT and quercetin were proven once again very efficient antioxidants, their inhibition being significantly different from that of any other compound at 12.5 μM ($P < 0.05$). The impact of all the other compounds on lipid peroxidation was very weak, as PCA, PA, and DHBA gave 10.5, 8.6, and 6.1% inhibition, respectively.

A 2-fold increase in concentration of each compound (25.0 μM) yielded higher inhibition for all the compounds, with that of BHT and quercetin being significant. Notable differences were found when the concentration of the compounds was increased by another 2-fold (50.0 μM). In particular, a reduction in % inhibition was found for PA, DHBA, and PCA, with the latter acid giving significantly low levels ($P < 0.05$) of inhibition. In contrast, morin gave even higher inhibition, reaching 79.0%. As for 25.0 μM, BHT and quercetin exhibited significant inhibitions. The majority of the compounds yielded higher values of inhibition at 50.0 μM, and so these values were used to rank the potency of the compounds against lipid peroxidation:

BHT > quercetin > morin > PA > DHBA > PCA

Prevention of Oxidative Damage to Proteins. The efficacy of the compounds to prevent protein oxidation was estimated by the inhibition in carbonyl group formation, which occurs when proteins are attacked by oxygen free radicals. In this case, H_2O_2 was also included in the reaction mixtures to enable free radical generation. As for the lipid peroxidation test, compounds were tested at 12.5, 25.0, and 50.0 μM.

The data presented in Table 3 are illustrative of the kind of interactions among BSA, citrate−Fe^{2+}, and individual compounds. At 12.5 μM concentration only morin and quercetin acted as antioxidants and inhibited carbonyl formation, whereas all the other compounds had pro-oxidant effects, the most important being those

Table 3. Efficiency of Quercetin, Morin, and Their Oxidation Products in Inhibiting BSA Oxidation by a Citrate−Fe^{2+}/H_2O_2 System, in Phosphate Buffer, pH 7.4[a]

	% inhibition[b]		
compound	12.5 μM	25.0 μM	50.0 μM
BHT	−55.5*	−45.3*	−43.2*
quercetin	13.3	44.1*	57.8*
morin	34.1	14.7	−52.9*
PA	−7.0	−1.9	21.7
DHBA	−28.0*	−27.3	−11.2
PCA	−33.0*	−14.3	−22.6

[a] Values represent means of triplicate determinations. [b] Values are expressed as % inhibition relative to control. *Statistically different values ($P < 0.05$).

Table 4. Efficiency of Quercetin, Morin, and Their Oxidation Products in Inhibiting Citrate/Fe^{2+}-Catalyzed ctDNA Damage, as Estimated from TBARS Formation, in Phosphate Buffer, pH 7.4[a]

	% inhibition[b]		
compound	0.25 μM	1.0 μM	4.0 μM
BHT	29.3	43.7*	44.8
quercetin	29.0	44.1*	47.5*
morin	30.7*	41.7	42.7
PA	28.0	42.1	43.0
DHBA	26.9	42.2	44.7
PCA	23.1*	41.8	41.9*

[a] Values represent means of triplicate determinations. [b] Values are expressed as % inhibition in A_{532} relative to control. *Statistically different values ($P < 0.05$).

of DHBA, PCA, and BHT ($P < 0.05$), which promoted BSA oxidation by 28.0, 33.0, and 55.5%, respectively. The increase in concentration by 2- and 4-fold revealed different behavior for each compound: quercetin exhibited by far the highest antioxidant activity at both 25.0 and 50.0 μM, whereas an increasing effect was seen for PA which had an inhibitory action at 50.0 μM. BHT and DHBA gave negative values of inhibition, but their pro-oxidant action appeared to decrease as their concentration increased. Contrary to this, morin showed an enhanced pro-oxidant activity, which at 50.0 μM resulted in a very important promotion of BSA oxidation ($P < 0.05$). As regards PCA, the results drawn were rather confusing. The pro-oxidant effect that PCA exhibited at 12.5 μM (−33.0%) was reduced to −14.3% when 25.0 μM was used, but it recovered to −22.6% at 50.0 μM.

Prevention of Oxidative Damage to DNA. Hydroxyl free radical may attack DNA on both base and sugar moiety. In the latter instance, TBARS are formed, and thus DNA damage can be estimated from the absorbance at 532 nm (*15*−*17*). As it can be seen in Table 4, the addition of every compound tested at a final concentration 0.25 μM caused inhibition in TBARS formation, the highest being that of morin, which reached 30.7% ($P < 0.05$). The lowest inhibition was found for PCA (23.1%), and it was significantly different from the other values. A 4-fold increase in concentration (1.0 μM) resulted in increased inhibitions for all compounds. BHT and quercetin were the most efficient ($P < 0.05$), as they inhibited TBARS formation by 43.7 and 44.1%, respectively. The increase in concentration by another 4-fold for each compound (4.0 μM) also resulted in higher inhibitions for all compounds. Quercetin was the only compound which gave a significant decrease in A_{532}, whereas PCA was of lower efficacy ($P < 0.05$).

It is noteworthy, however, that in no case did inhibition exceed 48%.

DISCUSSION

Reactive oxygen species (ROS) are responsible for the oxidative damage of biological macromolecules, such as carbohydrates, lipids, proteins, and DNA (*18*). Some of the most relevant ROS include peroxyl radicals ($ROO^{\bullet}$), the nitric oxide radical ($NO^{\bullet}$), the superoxide anion radical ($O_2^{\bullet -}$), peroxynitrile ($ONOO^-$), hydrogen peroxide (H_2O_2), and hydroxyl free radical, HFR ($^{\bullet}OH$). The latter species appears to be of particular importance because it can react with extremely high reaction rates with all the macromolecules mentioned above (*19*). Thus, HFRs react with whatever biological molecule is in their vicinity as soon as they are formed, producing secondary radicals of variable reactivity. Transition metal ions that occur widely in living tissues, such as Fe^{2+} and Cu^{2+}, play an important role in this respect because they are able of catalyzing reactions leading to HFR generation (*19*−*21*).

Flavonols are a class of flavonoids that exhibit powerful antioxidant effects in biological systems, including free radicals scavenging and metal ion sequestering, but their effectiveness greatly depends on particular chemical features (*22*−*24*). However, it is widely accepted that phenols have complex pro- and antioxidant effects in vitro, depending on their structure and the assay system used, and it is often hard to predict their actual action. Therefore, four different systems were employed to compare the relative antioxidant activities of quercetin, morin, and the degradation products thereof.

Antioxidant Capacity. The results show that both BHT and quercetin were capable of acting as antioxidants because they inhibited β-carotene bleaching to a significant extent. On the other hand, morin did not exhibit profound antioxidant effects, indicating that the substitution pattern in the B-ring is a very important determinant in this respect, in accordance with previous data (*22*, *25*). This finding, however, contrasts previous results (*26*) where morin was found to be more efficient than quercetin in inhibiting linoleic acid and methyl linolenate autoxidation.

DHBA and PCA were shown to exert more powerful antioxidant effects than PA. Comparing DHBA and PA, these results appear somewhat surprising, because the latter possesses an *o*-diphenol structure, and it would be expected to be a superior antioxidant, as previously proposed for PA and pyrocatechol (*27*), but also for caffeic acid, which is the hydroxycinnamic analogue of PA (*27*, *28*). Gallic acid (3,4,5-trihydroxybenzoic acid) was demonstrated to be a better antioxidant than PA, possibly because it bears one more hydroxyl group (*27*). On such a basis, the superior efficiency of PCA compared to PA could be explained. However, DHBA exhibited higher A_A and C_{AA} than PCA, although the former has two hydroxyl groups. Therefore, the positions of hydroxyl groups relative to the carboxyl group appear to be critical factors in estimating the antioxidant effects of phenolics of similar structure.

Effect on Lipid Peroxidation. Lipid peroxidation is initiated by the attack on a fatty acid or fatty acyl side chain of any chemical species that has sufficient reactivity to abstract a hydrogen atom from a methylene carbon in the side chain (*29*, *30*). Thus, HFRs can attack lipids (*19*):

$$\text{Lipid-H} + {}^{\bullet}\text{OH} \rightarrow \text{Lipid}^{\bullet} + H_2O \quad \text{(a)}$$

$$\text{Lipid}^{\bullet} + O_2 \rightarrow \text{Lipid-}O_2^{\bullet} \quad \text{(b)}$$

$$\text{Lipid-}O_2^{\bullet} + \text{Lipid-H} \rightarrow \text{Lipid-}O_2\text{H} + \text{Lipid}^{\bullet} \quad \text{(c)}$$

Therefore, in such a system rapid donation of a hydrogen atom or scavenging of HFRs would be crucial to avoiding chain reactions which result in lipid peroxidation and TBARS formation.

Quercetin is able to form inactive complexes with Fe^{2+}, and these complexes are not capable of generating HFRs in Fenton-type reactions (*9*). In addition, quercetin may promote oxidation of Fe^{2+} to Fe^{3+}, which is less effective in generating HFRs (*31*). Thus, the inhibitory action of quercetin and morin, in addition to their HFR-scavenging ability, may be ascribed to their chelating properties. This consideration explains the stronger effect of quercetin, which has an *o*-diphenol B-ring structure, and may exhibit higher chelating potency than morin (*24*, *32*). Moreover, quercetin is known to be a more potent HFR scavenger than morin (*33*). BHT is not a metal chelator, and therefore it acts rather as a scavenger or a hydrogen donor. The high efficiency of BHT, however, could be mainly attributed to its lipophilicity which would facilitate incorporation into the liposomes, thereby conferring a greater protective activity.

This may explain why the very polar (hydrophilic) phenolic acids PA, DHBA, and PCA had very little impact and caused insignificant inhibition at any concentration applied. Further, their efficacy decreased with increasing concentration, and it could be supported that these phenolic acids may exhibit concentration-dependent pro-oxidant action in the lipid system employed. PA has been shown to exhibit potent iron-reducing ability, protecting Fe^{2+} from oxidation (*31*). This fact could account for increased HFR generation. However, PA could effectively inhibit TBARS formation in rat liver microsomes (*31*). Similar results for PA, but also for *p*-hydroxybenzoic, vanillic, and sinapic acids were found when LDL was used as substrate for Cu^{2+}-mediated peroxidation (*34*).

It has been stated that the antioxidant effect of monophenols, such as *p*-hydroxybenzoic acid, against lipid peroxidation is strongly enhanced by the introduction of a second hydroxyl group, and is increased by one or two methoxy substitutions in *ortho* position to the hydroxyl group. In support of this, data on metal-ion-induced methyl linoleate breakdown (*35*) suggested gallic acid (trihydroxy substituted) to be a more efficient alkoxy radical scavenger than vanillic and salicylic acids. This may explain the slightly higher inhibition of PCA, which was achieved at concentrations of 12.5 and 25.0 μM, although at 50.0 μM PCA was the less effective phenolic acid. It appears therefore, that the expression of the antioxidant ability is substantially dependent on the relative concentrations of Fe^{2+} and the phenolic compound.

Effect on Protein Oxidation. Protein oxidation may be achieved through a Fenton system, such as Fe^{2+}/H_2O_2 (*36*−*38*), or Cu^{2+}/H_2O_2 (*11*, *39*, *40*). It is believed that the reduced metal ion binds to a metal binding site on the protein, after which the protein−metal ion complex reacts with H_2O_2 to generate in situ an activated oxygen species, i.e., HFR. This species then reacts with the side chains of amino acid residues at

the metal binding site (*10, 41*). Among other modifications, some amino acids residues are converted to carbonyl derivatives.

On the basis of these considerations, it is evident that hindrance of HFR generation or their efficient scavenging would reduce formation of carbonyl derivatives. Quercetin inhibited carbonyl formation at any concentration tested, and PA at 50.0 μM also exhibited an inhibition. Both compounds possess an *o*-diphenol structure, which is required for efficient chelation, and thus it is assumed that they hindered HFR formation by chelating with Fe^{2+}.

All the other compounds tested were found to promote BSA oxidation, a finding which evidences enhanced HFR generation. Once again, however, the concentration appeared to play a very important role, because morin at 12.5 μM exhibited the highest antioxidant effect, which was turned into pro-oxidant when higher concentrations were used. Morin has been shown to stimulate HFR generation under certain conditions, but this has been demonstrated for quercetin as well (*42*).

The interactions of the compounds tested with Fe^{2+} and BSA are more complicated than those observed in lecithin liposomes, evidencing the profound influence of various amino acid residues on BSA, which could participate in redox reactions, such as those bearing double bonds, amino, hydroxyl, and sulfhydryl groups. In fact, some of the most vulnerable amino acids to metal-catalyzed oxidation were shown to be histidine (*10, 12*), serine, glycine, and arginine (*37*), which bear heterocyclic nitrogen and conjugated double bonds, hydroxyl, amino, and imino groups, respectively. Further, the hydrophilicity of the protein system used in combination with citric acid may enable enhanced solubilization of Fe^{2+} and therefore increased HFR formation.

The failure of most of the compounds tested to inhibit BSA oxidation is not surprising, as it is known that HFR scavengers have little or no ability to inhibit protein MCO (*41*). This is corroborated by the finding that quercetin inhibition did not exceed 58%. It is worth mentioning, however, that phenolics such as those used in this study may appreciably stimulate MCO.

Effect on DNA Damage. Methodologies for estimating oxidative DNA modification are mainly based on HPLC coupled with electrochemical detector, which relies on measuring 8-oxoG, GC−MS techniques, and recently LC−MS (*43*). However, TBARS measurement constitutes a rapid test, which provides information concerning the extent of HFR generation and HFR-induced DNA damage (*15*−*17, 44*). On the basis of this test, the effect of the compounds on ctDNA damage induced by citrate−Fe^{2+}/H_2O_2 was estimated.

The protective action of compounds such as quercetin, morin, and PA may be attributed to chelation of Fe^{2+}, which results in limiting HFR production. This mechanism was implicated in DNA protection by PA and catechin from HFR attacks, induced by a Cu^{2+}/H_2O_2 system (*45*). However, it has been proposed that cyanidin, an anthocyanidin structurally related to quercetin, interacts with DNA forming a co-pigmentation complex, which can effectively block the DNA sites susceptible to HFR attack (*46*). Such a mechanism has also been proposed for the isoflavonoid genistein (*14*).

On the basis of structural criteria, quercetin could act as antioxidant by both mechanisms. In fact, at 4.0 μM quercetin was the most efficient antioxidant, indicating its high potential in preventing DNA oxidative damage. Nevertheless, all the compounds tested were able to limit TBARS formation to various extents, evidencing their inhibitory effect against HFR-mediated oxidations. This effect appeared to be concentration-dependent, because increased amounts yielded higher inhibitions. It could be supported, therefore, that all the compounds tested may indeed interact with DNA, protecting sites prone to oxidative modifications. It should also be highlighted that inhibition in TBARS formation as high as 30.7% could be achieved with particularly low amounts (0.25 μM morin). In no case, however, did inhibition exceed 48%.

Despite those findings, flavonoids under certain conditions have been reported to stimulate DNA damage. Quercetin and myricetin accelerated bleomycin-dependent DNA damage in the presence of Fe^{3+} (*44*), flavan-3-ols and procyanidins were found to cleave DNA in the presence of Cu^{2+} (*47*), and myricetin induced significant DNA degradation, which was enhanced by Cu^{2+} or Fe^{3+} (*48*). In all cases the effects were shown to be concentration-dependent.

CONCLUSIONS

The examination of the antioxidant properties of the compounds tested revealed that quercetin is a powerful antioxidant in every system used. In most cases morin possesses antioxidant properties inferior to those of quercetin. Quercetin degradation products are much less effective antioxidants compared to quercetin, but this does not hold true for morin. It appears that the anti/pro-oxidant effect of some phenolics depends to a great extent on the system used and the relative amounts of the phenolic.

ABBREVIATIONS USED

BSA, Bovine serum albumin; ctDNA,: calf thymus DNA; DHBA, 2, 4-dihydroxybenzoic acid; HFRs, hydroxyl free radicals; MCO, metal-catalyzed oxidation; PA, protocatechuic acid; PCA, phloroglucinol carboxylic acid; TBARS, thiobarbituric acid-reactive substances.

LITERATURE CITED

(1) Makris, D. P.; Rossiter, J. T. High-performance liquid chromatography studies on free-radical oxidation of flavonols. *Spec. Publ. - Royal Soc. Chem*. **2000**, *255*, 249−251.

(2) Makris, D. P.; Rossiter, J. T. Quercetin and rutin (quercetin 3-*O*-rhamnosylglucoside) thermal degradation in aqueous media under alkaline conditions. In *Functional Foods II - Claims and Evidence*; Buttriss J., Saltmarsh, M., Eds.; Royal Society of Chemistry Press: London, 2000; pp 216−238.

(3) Makris, D. P.; Rossiter, J. T. Heat-induced, metal-catalyzed oxidative degradation of quercetin and rutin (quercetin 3-*O*-rhamnosylglucoside) in aqueous model systems. *J. Agric. Food Chem*. **2000**, *48* (9), 3830−3838.

(4) Fahey, J. W.; Clevidence, B. A.; Russell, R. M. Methods for assessing the biological effects of specific plant components. *Nutr. Rev*. **1999**, *57* (9), S34−S40.

(5) Igile, G. O.; Oleszek, W.; Jurzysta, M.; Burda, S.; Fafunso, M.; Fasanmade, A. A. Flavonoids from *Vernonia amygdalina* and their antioxidant activities. *J. Agric. Food Chem*. **1994**, *42*, 2445−2448.

(6) Velioglou, Y. S.; Mazza, G.; Gao, L.; Oomah, B. D. Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. *J. Agric. Food Chem*. **1998**, *46*, 4113−4117.

(7) Al-Saikhan, M. S.; Howard, L. R.; Miller, J. C., Jr. Antioxidant activity and total phenolics in different genotypes of potato (*Solanum tuberosum*, L.). *J. Food Sci*. **1995**, *60* (2), 341−343.
(8) Marinova, E. M.; Yanishlieva, N. V.; Kostova, I. N. Antioxidative action of the ethanolic extract and some hydroxycoumarins of *Fraxinus ornus* bark. *Food Chem*. **1994**, *51*, 125−132.
(9) Afanas'ev, I. B.; Dorozhko, A. I.; Brodskii, A. V.; Kostyuk, V. A.; Potapovitch, A. I. Chelating and free radical scavenging mechanisms of inhibitory action of rutin and quercetin in lipid peroxidation. *Biochem. Pharmacol*. **1989**, *38* (11), 1763−1769.
(10) Fucci, L.; Oliver, C. N.; Coon, M. J.; Stadtman, E. R. Inactivation of key metabolic enzymes by mixed-function oxidation reactions: Possible implication in protein turnover and aging. *Proc. Natl. Acad. Sci. U.S.A*. **1983**, *80*, 1521−1525.
(11) Cooper, B.; Creeth, J. M.; Donald, A. S., R. Studies of the limited degradation of mucus glycoproteins. The mechanism of the peroxide reaction. *Biochem. J*. **1985**, *228*, 615−626.
(12) Amici, A.; Levine, R. L.; Tsai, L.; Stadtman, E. R. Conversion of amino acid residues in proteins and amino acid homopolymers to carbonyl derivatives by metal-catalyzed oxidation reactions. *J. Biol. Chem*. **1989**, *264* (6), 3341−3346.
(13) Levine, R. L.; Garland, D.; Oliver, C. N.; Amici, A.; Climent, I.; Lenz, A.-G.; Ahn, B.-W.; Shaltiel, S.; Stadtman, E. R. Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol*. **1990**, *186*, 464−480.
(14) Harper, A.; Kerr, D. J.; Gescher, A.; Chipman, K. Antioxidant effects of isoflavonoids and lignans, and protection against DNA oxidation. *Free Radical Res*. **1999**, *31*, 149−160.
(15) Floyd, R. A. DNA-ferrous iron catalyzed hydroxyl free radical formation from hydrogen peroxide. *Biochem. Biophys. Res. Commun*. **1981**, *99* (4), 1209−1215.
(16) Halliwell, B.; Gutteridge, J. M. C. Formation of a thiobarbituric-acid-reactive substance from deoxyribose in the presence of iron salts. *FEBS Lett*. **1981**, *128* (2), 347−352.
(17) Aruoma, O. I.; Murcia, A.; Butler, J.; Halliwell, B. Evaluation of the antioxidant and prooxidant actions of gallic acid and its derivatives. *J. Agric. Food Chem*. **1993**, *41*, 1880−1885.
(18) Diplock, A. T.; Charleux, J.-L.; Crozier-Willi, G.; Kok, F. J.; Rice-Evans, C.; Robefroid, M.; Stahl, W.; Viña-Ribes, J. Functional food science and defence against reactive oxidative species. *Br. J. Nutr*. **1998**, *80*, suppl. 1, S77−S112.
(19) Halliwell, B.; Gutteridge, M. C. Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem. J*. **1984**, *219*, 1−14.
(20) Halliwell, B.; Gutteridge, M. C. Role of iron in oxygen radical reactions. *Methods Enzymol*. **1984**, *105*, 47−56.
(21) Halliwell, B.; Gutteridge, M. C. Oxygen free radicals and iron in relation to biology and medicine: Some problems and concepts. *Arch. Biochem. Biophys*. **1986**, *246* (2), 501−514.
(22) Rice-Evans, C.; Miller, N. J.; Bolwell, P. G.; Bramley, P. M.; Pridham, J. B. The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radical Res*. **1995**, *22* (4), 375−383.
(23) Rice-Evans, C.; Miller, N. J. Antioxidant activities of flavonoids as bioactive components of food. *Biochem. Soc. Trans*. **1996**, *24*, 790−794.
(24) van Acker, S. A. B. E.; van de Berg, D.-J.; Tromp, M. N. J. L.; Griffioen, D. H.; van Bennekom, W.; van der Vijgh, W. J. F.; Bast, A. Structural aspects of antioxidant activity of flavonoids. *Free Radical Biol. Med*. **1996**, *20* (3), 331−342.
(25) Bors, W.; Heller, W.; Michel, C.; Saran, M. Flavonoids as antioxidants: Determination of radical-scavenging efficiencies. *Methods Enzymol*. **1990**, *186*, 343−355.
(26) Torel, J.; Cillard, J.; Cillard, P. Antioxidant activity of flavonoids and reactivity with peroxy radical. *Phytochemistry* **1986**, *25* (2), 383−385.
(27) Cuvelier, M.-E.; Richard, H.; Berset, C. Comparison of the antioxidative activity of some acid-phenols: Structure−activity relationship. *Biosci. Biotechnol. Biochem*. **1992**, *56* (2), 324−325.
(28) Foti, M.; Piattelli, M.; Baratta, M.-T.; Ruberto, G. Flavonoids, coumarins, and cinnamic acids as antioxidants in a micellar system. Structure−activity relationship. *J. Agric. Food Chem*. **1996**, *44*, 497−501.
(29) Halliwell, B.; Chirico, S. Lipid peroxidation: its mechanism, measurement, and significance. *Am. J. Clin. Nutr*. **1993**, *57* (suppl.), 715S−725S.
(30) Porter, N. A.; Caldwell, S. E.; Mills, K. A. Mechanisms of free radical oxidation of unsaturated lipids. *Lipids* **1995**, *30* (4), 277−290.
(31) Yoshino, M.; Murakami, K. Interaction of iron with polyphenolic compounds: Application to antioxidant characterization. *Anal. Biochem*. **1998**, *257*, 40−44.
(32) Brown, J. E.; Khodr, H.; Hider, R. C.; Rice-Evans, C. A. Structural dependence of flavonoid interactions with Cu^{2+} ions: implications for their antioxidant properties. *Biochem. J*. **1998**, *330*, 1173−1178.
(33) Husain, S. R.; Cillard, J.; Cillard, P. Hydroxyl radical scavenging activity of flavonoids. *Phytochemistry* **1987**, *26* (9), 2489−2491.
(34) Natella, F.; Nardini, M.; Di Felice, M.; Scaccini, C. Benzoic and cinnamic acid derivatives as antioxidants: Structure−activity relation. *J. Agric. Food Chem*. **1999**, *47*, 1453−1459.
(35) Milic, B. Lj.; Djilas, S. M.; Canadanovic-Brunet, J. M. Antioxidative activity of phenolic compounds on the metal-ion breakdown of lipid peroxidation system. *Food Chem*. **1998**, *61* (4), 443−447.
(36) Creeth, J. M.; Cooper, B.; Donald, A. S. R.; Clamp, J. R. Studies of the limited degradation of mucus glycoproteins. The effect of dilute hydrogen peroxide. *Biochem. J*. **1983**, *211*, 323−332.
(37) Stadtman, E. R.; Berlett, B. S. Fenton chemistry. Amino acid oxidation. *J. Biol. Chem*. **1993**, *266* (26), 17201−17211.
(38) Gebicki, S.; Gebicki, J. M. Formation of peroxides in amino acids and proteins exposed to oxygen free radicals. *Biochem. J*. **1993**, *289*, 743−749.
(39) Hunt, J. V.; Simpson, J. A.; Dean, R. T. Hydroperoxide-mediated fragmentation of proteins. *Biochem. J*. **1988**, *250*, 87−93.
(40) Huggins, T. G.; Wells-Knecht, M. C.; Detorie, N. A.; Baynes, J. W.; Thorpe, S. R. Formation of *o*-tyrosine and dityrosine in proteins during radiolytic and metal-catalyzed oxidation. *J. Biol. Chem*. **1993**, *268* (17), 12341−12347.
(41) Stadtman, E. R.; Olivers, C. N. Metal-catalyzed oxidation of proteins. *J. Biol. Chem*. **1991**, *266* (4), 2005−2008.
(42) Puppo, A. Effect of flavonoids on hydroxyl radical formation by Fenton-type reactions; Influence of the iron chelator. *Phytochemistry* **1992**, *31* (1), 85−88.
(43) Poulsen, H. E.; Weinmann, A.; Loft, S. Methods to detect DNA damage by free radicals: relation to exercise. *Proc. Nutr. Soc*. **1999**, *58*, 1007−1014.
(44) Laughton, M. J.; Halliwell, B.; Evans, P. J.; Hoult, J. R. S. Antioxidant and pro-oxidant actions of the plant phenolics quercetin, gossypol and myricetin. *Biochem. Pharmacol*. **1989**, *38* (17), 2859−2865.
(45) Ueda, J.-I.; Saito, N.; Shimazu, Y.; Ozawa, T. A comparison of scavenging abilities of antioxidants against hydroxyl radicals. *Arch. Biochem. Biophys*. **1996**, *333* (2), 377−384.

(46) Sarma, A. D.; Sharma, R. Anthocyanin-DNA copigmentation complex: mutual protection against oxidative damage. *Phytochemistry* **1999**, *52*, 1313−1318.
(47) Shirahata, S.; Murakami, H.; Nishiyama, K.; Yamada, K.; Nonaka, G.; Nishioka, I.; Omura, H. DNA breakage by flavan-3-ols and procyanidins in the presence of cupric ions. *J. Agric. Food Chem*. **1989**, *37*, 299−303.
(48) Sahu, S. C.; Gray, G. C. Interactions of flavonoids, trace metals, and oxygen: nuclear DNA damage and lipid peroxidation induced by myricetin. *Cancer Lett*. **1993**, *70*, 73−79.

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