Antioxidant Activity of Flavonol Aglycones and Their Glycosides in Methyl Linoleate

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ABSTRACT: The antioxidant activities of the flavonol aglycones, quercetin and myricetin, and their selected glycosides were compared in bulk methyl linoleate oxidized at 40°C. Methyl linoleate hydroperoxide formation, hydroperoxide isomer distribution, and ketodiene formation were followed by using high-performance liquid chromatography (HPLC) analysis. The aglycones, quercetin and myricetin, were consistently more active in bulk methyl linoleate than their glycosides and more active than α-tocopherol at 500 and 1000 μM. At 50 μM, the order of activity was myricetin > α-tocopherol > quercetin, and the order of activity of quercetin and its derivatives was quercetin > quercitrin > isoquercitrin > rutin. Myricitrin was slightly less active than myricetin. The sugar moiety was shown to have a marked effect on the antioxidant activity of flavonols. The rhamnoside derivatives, quercitrin and myricitrin, both possessed activity close to that of their corresponding aglycones. The different activities of glycosides could be partly explained by different solubilities and by differences in oxidizability of glycosides containing a monosaccharide or disaccharide at the C_3 position. The effect on hydroperoxide isomer distribution indicates that α-tocopherol was a more effective hydrogen donor than flavonoids, although flavonoids were more effective in inhibiting oxidation of methyl linoleate.

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KEY WORDS: Antioxidants, flavonols, hydroperoxides, lipid oxidation, methyl linoleate.

Flavonoids are ubiquitous in plants, forming a heterogeneous group of phenolic compounds having benzo-γ-pyrone structure in the molecule. Flavonols are one subgroup of flavonoids that have a double bond between carbons C_2 and C_3, a keto group in position C_4, and a hydroxyl group in position C_3.

Approximately 90% of flavonoids in plants occur as glycosides (1) and are suggested to be absorbed as glycosides from the human intestine (2). Quercetin (3′,4′-dihydroxyflavonol, Fig. 1) glycosides are common flavonols in plant materials (1). Myricetin (3′,4′,5′-trihydroxyflavonol, Fig. 1) and its glycosides, although less frequently present, are very important because of their high antioxidant capacity (3). Quercetin-3-glucoside (isoquercitrin), quercetin-3-rhamnoside (quercitrin), and quercetin-3-rutinoside (rutin) are common glycosides of quercetin (1).

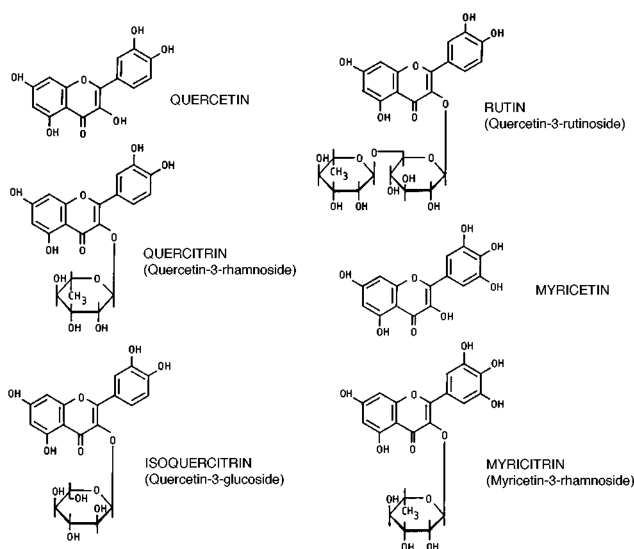

FIG. 1. Structures of the flavonols, quercetin and myricetin, and their glycosides: quercitrin, isoquercitrin, rutin, and myricitrin.

Flavonols are effective radical scavengers and, thus, potential chain-breaking antioxidants in lipid oxidation reactions. Reduction potentials of flavonoid radicals were reported to be comparable to that of trolox (4) which indicates that they are potential chain-breaking antioxidants. Bors *et al.* (3) concluded that for maximal radical scavenging activity a flavonoid molecule needs to meet the following criteria: (i) 3′,4′-dihydroxy structure in the B-ring, (ii) 2,3-double bond in conjunction with a 4-oxo group in the C-ring, and (iii) presence of a 3-hydroxyl group in the C-ring and a 5-hydroxyl group in the A-ring. Flavonols quercetin and myricetin meet these structural requirements. However, studies on relative antioxidant activity of flavonol aglycones and their glycosides in different types of lipid systems are somewhat controversial. The relative activities of flavonoids in quenching a water-soluble radical cation $ABTS^{\cdot+}$ [ABTS = 2,2′-azino-bis(3-ethyl benzothiazoline-6-sulfonate)] decreased in the order quercetin > myricetin > rutin > α-tocopherol (5). In lipid systems flavonol aglycones are generally reported to be more active than their glycosides; quercetin was more active

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than quercitrin and rutin in ferrous-induced oxidation of rat brain mitochondrial suspension (6). In Cu^+-induced oxidation of low density lipoprotein cholesterol, rutin was as active as quercetin (7), but more active than quercetin in oxidation of mouse liver homogenate (8). Contradictory results are apparently due to differences in methodology and experimental conditions. These differences cause varying reactivity of antioxidants toward chain-carrying radicals, different partitioning of compounds in heterophasic systems, and varying pro- and antioxidants in test systems. It is obvious that although optimal chemical structure for maximal radical scavenging potentials can be determined (4), experimental conditions such as lipid structure, solvent, initiation of oxidation, and the analytical measurement chosen have a significant effect on the relative antioxidant activity of compounds.

Studies on antioxidant activity of flavonoids have mostly been performed in heterogeneous hydrophilic systems where oxidation is often initiated by metal ions or radicals. The number of studies on lipid models which contain no added initiator and are devoid of other antioxidants often present in lipid models is limited. However, to study the mechanisms of antioxidants on the oxidation reactions of lipids, a model free from other antioxidants as well as high levels of initiating radicals is needed.

Methyl linoleate is a nonpolar lipid model free from other biological antioxidants and was widely used in antioxidant activity testing (9,10). When used without added initiator, reactions between antioxidants and metal ions or artificial radicals are limited and the main inhibiting reaction takes place between antioxidants and lipid peroxyl radicals. Thus, the main oxidation phase in this lipid model is the propagation phase rather than initiation. Further, the oxidation pathway of methyl linoleate easily can be followed by simple high-performance liquid chromatography (HPLC) analysis (11) and information about lipid oxidation products, such as hydroperoxide isomer distribution and formation of selected decomposition products of hydroperoxides, can be obtained.

This study compared antioxidant activity of two important flavonol aglycones, myricetin and quercetin, and their glycoside forms quercitrin, isoquercitrin, rutin, and myricitrin in oxidizing methyl linoleate at 40°C. Methyl linoleate hydroperoxide formation was monitored by HPLC analysis with ultraviolet (UV) detection. Both hydroperoxide formation and isomer distribution, as well as selected decomposition products, were monitored by HPLC analysis to further characterize the antioxidant mechanism of flavonols in a hydrophobic lipid model.

EXPERIMENTAL PROCEDURES

Materials. Methyl linoleate (MeLo) (Nu-Chek-Prep. Inc, Elysian, MN) was used as the oxidizing substrate without further purification. Initial hydroperoxide content was <5 mmol/kg. Myricetin (purity 85%), quercetin (98.9%), and rutin (95%) were supplied by Sigma Chemical Co. (St. Louis, MO). HPLC-purified quercitrin, isoquercitrin, and myricitrin were supplied by Extrasynthese (Genau Cedex, France). α-Tocopherol (98.8%) was supplied by Merck (Darmstadt, Germany). The solvents were from Rathburn Chemicals (Walkerburn, United Kingdom) and of HPLC-grade. Diethyl ether was purified by silica solid-phase extraction cartridge prior to HPLC analysis to remove peroxides and the stabilizing agent.

Methods. MeLo samples (0.5 g) were oxidized in the presence of antioxidants at levels of 50, 500, and 1000 μM (flavonoids) or 50 and 1000 μM (α-tocopherol) at 40°C in the dark in 15-mL open vials. MeLo without antioxidant addition served as the control. The relatively large air/lipid surface of this system promoted rapid oxidation of MeLo. Antioxidants were added into oil as freshly prepared ethanol solutions (50–200 μL solution). Ethanol was evaporated by purging the oil with nitrogen. Pure ethanol (200 μL) was added to the control sample and purged by nitrogen flow.

Conjugated diene (CD) analysis was used to estimate the initial hydroperoxide content having CD structure in the molecule. Initial hydroperoxide content of MeLo was measured by dissolving approximately 10 mg of sample into isooctane and measuring the absorbance of CD structure of hydroperoxides by ultraviolet (UV) absorption at 234 nm (Perkin Elmer, Lambda 11/Bio UV/Vis spectrophotometer, Überlingen, Germany). Hydroperoxide formation, isomer distribution, and formation of ketodiene compounds were monitored by HPLC. The analysis and peak identification were performed as described previously (11). The HPLC apparatus was composed of an autosampler (Waters 700 Satellite WISP; Millipore Corporation, Milford, MA), one pump (Waters 501), a UV–VIS photodiode array detector (Waters 996 PDA), and a computer work station. Data handling was performed by Millennium 2010 software (Waters). A column of 5 μm particle size, Supelcosil LC-SI 57930 (250 × 2.1 mm) with a Supelcosil pre column of 20 × 2.1 mm (Supelco, Bellefonte, PA), was used.

Each oxidation experiment was performed in duplicate to confirm the order of activity of antioxidants studied. Samples were analyzed as duplicates and a reference sample of MeLo hydroperoxide mixture was used to follow the detector response of the HPLC analysis. One-way analysis of variance (Minitab Statistical Software; Addison-Wesley, Reading, MA) was used to test statistically the differences in antioxidant activities. The significance level used was 5% ($P < 0.05$).

RESULTS

Effect in inhibiting MeLo oxidation. Both aglycones, quercetin (Fig. 2) and myricetin (Fig. 3), were more active in MeLo than were their corresponding glycoside derivatives. The sugar moiety in the flavonol glycosides had a marked effect on the antioxidant activity of flavonols. At each level tested (50 μM in Fig. 2A and 500 or 1000 μM in Fig. 2B), the order of activity of quercetin and its glycosides was: quercetin

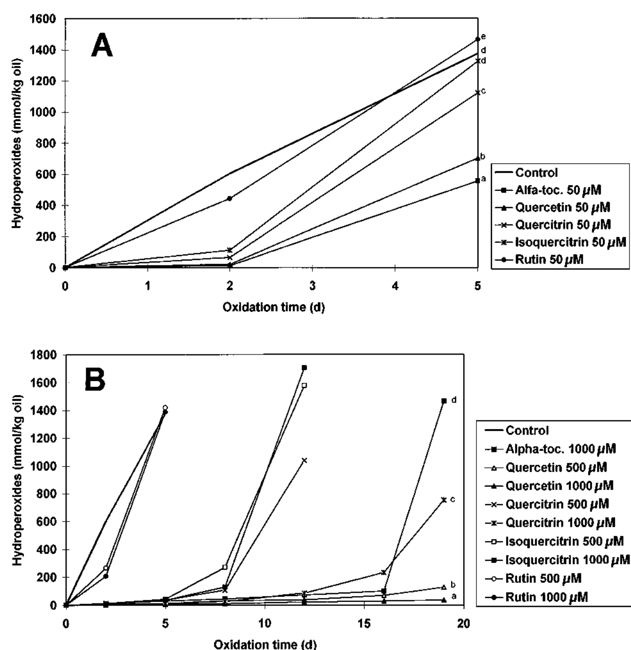


FIG. 2. Effect of α-tocopherol, quercetin, quercitrin, isoquercitrin, and rutin in inhibiting hydroperoxide formation as measured by high-performance liquid chromatography (HPLC) in methyl linoleate at 40°C. (A) Concentration 50 μM, (B) concentrations 500 and 1000 μM. Each point represents a mean value of duplicate analyses. Samples followed by different letters are significantly different ($P < 0.05$).

> quercitrin > isoquercitrin > rutin. The significance of the differences was calculated by one-way analysis of variance after 5 d of oxidation (Fig. 2A) and after 19 d of oxidation (Fig. 2B). Rutin possessed low antioxidant activity. At a low concentration (50 μM), α-tocopherol was more active than quercetin (Fig. 2A), but at a higher concentration (1000 μM), the order of activity was reversed (Fig. 2B).

The difference between the antioxidant activity of myricetin and its rhamnoside derivative, myricitrin, was small but statistically significant at both the 50 μM (Fig. 3A) and the 500 μM levels (Fig. 3B). Both myricetin and myricitrin were more active than equal molar concentrations of α-tocopherol.

Effect on the distribution of MeLo hydroperoxide isomers. Formation of the four isomers of MeLo hydroperoxides, MeLoOOH, (13-*cis,trans* MeLoOOH, 13-*trans,trans* MeLoOOH, 9-*cis,trans* MeLoOOH, and 9-*trans,trans* MeLoOOH) was followed by HPLC analysis. In addition to total hydroperoxide formation, the relative amount of *cis*,*trans* isomers formed was followed. The percentage of *cis*,*trans* isomers at three stages of oxidation is presented in Table 1. The levels of *cis*,*trans* isomers initially, during the lag phase, and during the propagation phase of oxidation are reported. The oxidation time required for each sample to reach these oxidation stages varied. Thus, for control and rutin samples the lag phase was evaluated after 2 d of oxidation, for α-tocopherol, quercetin, quercitrin, and isoquercitrin

TABLE 1
Effect of Antioxidants on Geometrical Isomer Distribution of Methyl Linoleate Hydroperoxides During Oxidation at 40°C[a]

Antioxidant	Oxidation time (d) *cis,trans* isomers of total hydroperoxides (%)		
Control	0	2	5
	69	42	40
α-Tocopherol	0	5	16
1000 μM	71	72	63
Quercetin	0	5	16
500 μM	72	51	44
1000 μM	72	57	48
Quercitrin	0	5	12
500 μM	71	45	41
1000 μM	71	50	42 (16 d)
Isoquercitrin	0	5	12
500 μM	71	44	40
1000 μM	71	45	40
Rutin	0	2	5
500 μM	70	41	41
1000 μM	71	42	41
Myricetin	0	7	17
500 μM	74	55	48
1000 μM	75	58	53
Myricitrin	0	7	17
500 μM	70	50	45
1000 μM	70	52	46

[a]The results are average of duplicate analysis (SD% <5%). SD, standard deviation.

after 5 d, and for myricetin and myricitrin after 7 d of oxidation. Similarly, control and rutin samples reached the propagation phase after 5 d of oxidation, α-tocopherol, quercetin, quercitrin, and isoquercitrin after 12 or 15 d of oxidation, and myricetin and myricitrin after 17 d of oxidation.

The formation of *cis*,*trans* isomers at each stage of oxidation was significantly ($P < 0.05$) higher in the presence of α-tocopherol than in the presence of either quercetin and its derivatives or myricetin and its rhamnoside. In the presence of α-tocopherol, the relative amount of *cis*,*trans* isomers was 63–72% of the total hydroperoxides during the first 15 d, whereas, in the presence of quercetin and its derivatives, the relative amount of *cis*,*trans* hydroperoxides was 51% after 5 d and 44% after 16 d of oxidation. In the presence of myricetin and myricitrin the relative amount of *cis*,*trans* hydroperoxide isomers after 17 d of oxidation was 48 and 45%, respectively.

Effect of antioxidants on the formation of hydroperoxide decomposition products. Ketodienes are one important group of hydroperoxide decomposition products (12). In this study, formation of ketodiene compounds was used as an indicator of hydroperoxide decomposition. Inhibition of antioxidants in ketodiene formation followed their activities in inhibiting hydroperoxide formation. For quercetin and its derivatives,

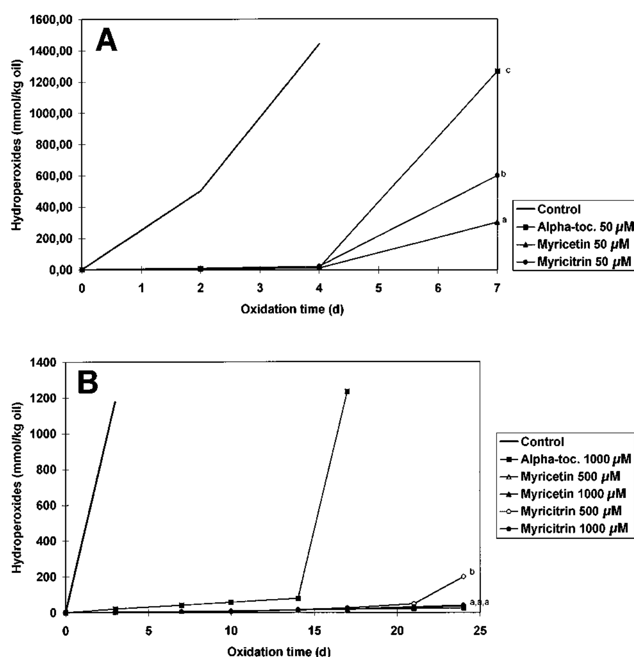


FIG. 3. Effect of α-tocopherol, myricetin, and myricitrin on hydroperoxide formation as measured by HPLC in methyl linoleate at 40°C. (A) Concentration 50 μM, (B) concentrations 500 and 1000 μM. Each point represents a mean value of duplicate analyses. Samples followed by different letters are significantly different ($P < 0.05$). See Figure 2 for abbreviation.

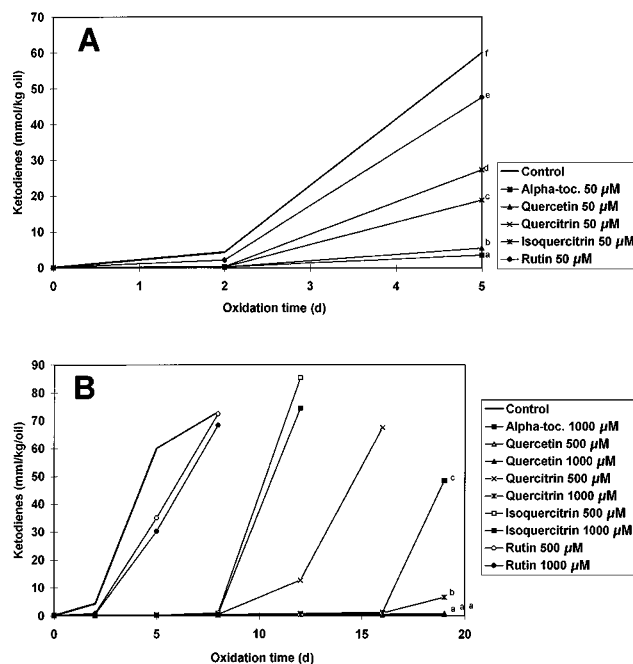
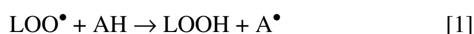


FIG. 4. Effect of α-tocopherol, quercetin, quercitrin, isoquercitrin, and rutin in inhibiting formation of ketodiene compounds as measured by HPLC in methyl linoleate at 40°C. (A) Concentration 50 μM, (B) concentrations 500 and 1000 μM. Each point represents a mean value of duplicate analyses. Samples followed by different letters are significantly different ($P < 0.05$). See Figure 2 for abbreviation.

the order of activity was: quercetin > quercitrin > isoquercetrin > rutin at all levels tested (Figs. 4A and 4B). At low concentrations α-tocopherol was slightly more active and at higher concentrations slightly less active than quercetin and its derivatives. Also, with myricetin and myricitrin the inhibiting effect of ketodiene formation followed their order of activity in inhibiting hydroperoxide formation and was: myricetin > myricitrin > α-tocopherol at all levels tested (Figs. 5A and 5B). High levels of myricetin and myricitrin totally inhibited the ketodiene formation (Fig. 5B).

DISCUSSION

Flavonols are known to act as antioxidants, both as radical scavengers (8,13) and as metal chelators (14). In this study, no radical or metal ion initiator was added to samples and oxidation was assumed to be initiated by the traces (4–5 mmol/kg) of hydroperoxides initially present in MeLo. Therefore, in this study the flavonols were assumed to act as chain-breaking antioxidants during the propagation phase of MeLo oxidation, and the observed main inhibitory reactions would occur between the lipid peroxyl radical (LOO$^\bullet$) and the antioxidants (A$^\bullet$) (Eq. 1):



$$LOO^\bullet + AH \rightarrow LOOH + A^\bullet \quad [1]$$

Activity of flavonoid aglycones vs. *glycosides.* Myricetin and quercetin were the two most active compounds in retarding hydroperoxide formation, having activity equal to that of α-tocopherol. At 50 μM, α-tocopherol was slightly more active than the flavonols studied, whereas at 500 and 1000 μM both myricetin and quercetin were slightly more active than α-tocopherol. Glycosides were consistently less active than their corresponding aglycones.

According to Bors *et al.* (3) the presence of a free 3-hydroxyl group in the C-ring is a requirement for the maximal radical scavenging activity of flavonoids. Hedrickson *et al.* (15) measured redox potential by glassy carbon electrode, and reported that the presence of a hydroxyl group in the C_3 position is less oxidizable than the hydroxyl groups in ring B but more oxidizable than phenolic hydroxyl groups in ring A. Therefore, the improved antioxidant activity of flavonols and the higher activity of aglycones in MeLo may be partly explained by the free hydroxyl group at C_3. However, in this study quercitrin and myricitrin, which both possess rhamnose at C_3, showed antioxidant activity close to that of their aglycones.

Effect of the sugar moiety on the antioxidant activity of flavonoids. There was a marked difference between activities of the three glycosides of quercetin, indicating that the chemical character of the sugar was important in the antioxidant activity of flavonols. The effect of the sugar moiety is reported to have an effect on the oxidizability of flavonoid glycosides. When comparing the kinetics of oxidation of quercetin and its two glycosides, Hedrickson *et al.* (15) reported that quercetin oxidized fastest, followed by quercitrin and rutin, as measured by rotating ring-disk voltametry. The

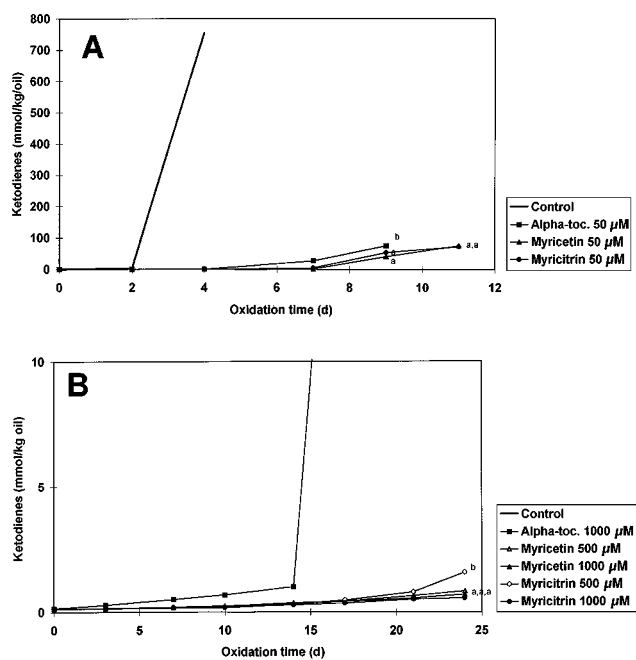


FIG. 5. Effect of α-tocopherol, myricetin, and myricitrin on formation of ketodiene compounds as measured by HPLC in methyl linoleate at 40°C. (A) Concentration 50 μM, (B) concentrations 500 and 1000 μM. Each point represents a mean value of duplicate analyses. Samples followed by different letters were significantly different ($P < 0.05$). See Figure 2 for abbreviation.

oxidation rate of compounds decreased as the substituent at C_3 became a poorer leaving group; disaccharide is a poorer leaving group than monosaccharide, thus rutin is less oxidizable than monosaccharide glycosides. This observation may explain the lower antioxidant activity of rutin than quercitrin and isoquercitrin. Hedrickson *et al.* (15) suggested that, during electrochemically generated oxidation, the C_3 hydroxyl group of oxidized quercetin interacts with C_6' carbon and forms a 3′,4′,6′-trihydroxylated derivative which can donate two new hydrogens, thus forming a quinone as a reaction product. This interaction increases the oxidation rate of quercetin and its monosaccharide glycosides. The higher antioxidant activity of flavonol aglycones and monosaccharide glycosides observed in this study may partly be explained by the higher oxidizability of these compounds than flavonol disaccharide glycosides such as rutin.

The activities of quercitrin and isoquercitrin, which are both monosaccharide glycosides, differed markedly. Quercitrin contains the sugar rhamnose and possessed markedly higher activity than isoquercitrin, a glucose-containing glycoside. Further, the rhamnoside derivative of myricetin (myricitrin) possessed very high activity in bulk MeLo indicating that the rhamnoside-containing glycosides possess higher activities than other glycosides. Rhamnose contains a methyl group instead of CH_2OH in position C_5, as seen in molecular structures of quercitrin and myricitrin (Fig. 1), and is less polar than other monosaccharides. This polarity may induce better solubility of quercitrin and myricitrin into the lipid medium and, thus, partly explain the higher activity of rhamnoside derivatives in a bulk lipid system.

The finding that rutin had very low activity is controversial in the literature. Most studies report lower activity for rutin than for other glycosides. Findings from this study support the previous observations that rutin is less active than α-tocopherol or quercetin; quercetin, myricetin, and α-tocopherol had the greatest activity, whereas the activity of rutin was very limited. It is well-documented that relative activity of antioxidants differs markedly in different types of lipid systems (16). It is obvious that in bulk lipid systems the mechanism of antioxidant action is markedly different from that in water-soluble systems.

Effect of antioxidants on lipid oxidation product distribution. When comparing the distribution of *cis,trans* and *trans,trans* isomers at equal levels of oxidation, it was shown that the relative amount of hydroperoxide isomers containing *cis,trans* configuration in the molecule was higher in the presence of α-tocopherol than with either of the flavonols (Table 1). The initial relative amount of *cis,trans* hydroperoxides in each of the samples was 69–75% of hydroperoxides. In the presence of α-tocopherol, as much as 70% of hydroperoxides were in the *cis,trans* form even at relatively high oxidation levels, whereas in the control sample only 40% and in flavonol samples 40–45% had *cis,trans* conformation.

The effect of α-tocopherol on isomer distribution was reported to be due to its high hydrogen donor activity (17). These results indicate that although α-tocopherol is slightly less active than quercetin and myricetin at inhibiting oxidation of MeLo, it possesses higher hydrogen donor activity than the flavonols tested.

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