J. Pharm. Pharmacol. 1991, 43: 486–491
Received January 9, 1991

Effect of Hydroxyethyl Rutosides and Related Compounds on Lipid Peroxidation and Free Radical Scavenging Activity. Some Structural Aspects

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Abstract—Four hydroxyethyl rutosides, 7,3′,4′-trihydroxyethyl quercetin, quercetin and a commercial standardized mixture of hydroxyethyl rutosides were investigated on non-enzymatic lipid peroxidation, for hydroxyl radical scavenging activity and interaction with 1,1-diphenyl-2-picrylhydrazyl stable free radical (DPPH). It was found that the tested compounds exhibited a considerable inhibition of microsomal lipid peroxidation. They were less active than the reference compound quercetin, and this was attributed to their structural characteristics. They were also found to be potent hydroxyl radical scavengers and to interact with DPPH. As hydroxyl radical scavengers, they were more potent than the known hydroxyl radical scavengers mannitol and dimethyl sulphoxide. These properties could be considered as a useful and exploitable combination.

Flavonoids constitute a group of naturally occurring benzo-γ-pyrone derivatives which have been found to possess several biological properties, e.g. hepatoprotective (Perrissoud & Testa 1982), antithrombotic (Gryglewski et al 1987), anti-inflammatory (Corvazier & Maclouf 1985) and antiviral (Middleton 1984) activities. Many of these actions have been correlated with their ability to scavenge oxygen-generated free radicals and to inhibit lipid peroxidation in-vitro (e.g. Younes & Siegers 1980; Robak & Gryglewski 1988).

The peroxidation of cellular membrane lipids can lead to cell necrosis and is considered to be implicated in a number of pathophysiological conditions as well as in the toxicity of many xenobiotics (Kappus 1985, 1987). The hydroxyl radical, included among the most reactive oxygen species, is thought to be a major factor responsible for oxidative injury of enzymes, lipid membranes and DNA (Fridovich 1988).

Hydroxyethylated derivatives of the natural flavonoid rutin are employed mainly in the treatment of vascular disorders, since they have been found to affect capillary permeability (Roztocil et al 1977). Mixtures of hydroxyethyl rutosides are licensed preparations in many countries. Their flavonoid skeleton, as well as existing evidence concerning their protective effect against adriamycin-induced toxicity (Gulati et al 1985) and their dismutase-like activity (McGinness et al 1982) could justify an investigation of the effect of various hydroxyethyl rutosides on lipid peroxidation and on their ability to scavenge hydroxyl radicals. Thus, the contribution of the structural features of these compounds to antioxidant activity could be evaluated and may indicate a structure-antioxidant activity relationship. Furthermore, a potential interrelationship between lipid peroxidation and hydroxyl radical scavenging activity could be studied.

Materials and Methods

Hepatic microsomal fractions were prepared from untreated female Wistar rats, 200–250 g. Fractions were heat-inactivated (90°C for 90 s) and suspended in Tris-HCl/KCl buffer (50 mM/150 mM, pH 7·4). The flavonoids tested were kindly donated by Zyma SA, Switzerland. All chemicals used were of analytical grade.

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In-vitro lipid peroxidation

For the in-vitro lipid peroxidation experiments, the incubation mixtures contained the microsomal fraction, corresponding to 0·125 g liver mL^{-1}, ascorbic acid (0·2 mM) in Tris buffer, and various concentrations (10–1000 μM) of the tested compounds dissolved in dimethyl sulphoxide (DMSO). An equal volume of this solvent (100 μL) was added to the control incubate. The reaction was started by the addition of a freshly prepared $FeSO_4$ solution (10 μM). The mixture was incubated at 37°C for 45 min. Aliquots (0·3 mL) of the incubation mixture (final volume 4 mL) were taken at various time intervals. Lipid peroxidation was assayed spectrophotometrically (535 nm against 600 nm) by determination of the 2-thiobarbituric acid reactive material (Rekka et al 1989). Each experiment was performed at least in duplicate.

Competition of the tested flavonoids with DMSO for hydroxyl radicals

The formaldehyde formed during the oxidation of dimethyl sulphoxide by the Fe^{3+}/ascorbic acid system was used to detect hydroxyl radicals (Klein et al 1981). The reaction mixture contained EDTA, 0·1 mM Fe^{3+} (as a 1:2 mixture with EDTA) 167 μM, DMSO (at concentrations 3·3–66 mM) in phosphate buffer (50 mM, pH 7·4). The tested compounds were added at various concentrations (1–5 mM) and the reaction was started by the addition of 2 mM ascorbic acid. The mixture (final volume 375 μL) was incubated at 37°C for 30 min, then the reaction was stopped by the addition of 125 μL trichloroacetic acid (17·5% w/v) and the formaldehyde formed was assayed spectrophotometrically by the method of Nash (1953). Each experiment was performed at least in duplicate and the deviation of absorbance values was less than 10%.

Interaction of the tested flavonoids with 1,1-diphenyl-2-picrylhydrazyl stable free radical (DPPH)

To an ethanolic solution of DPPH (final concentration 200 μM) an equal volume of the compounds dissolved in ethanol was added at various concentrations (10–200 μM). Ethanol (analytical grade, iron content less than 10^{-5}%) was added to the control solution. After 20 min at room temperature, absorbance was recorded at 517 nm (Kato et al 1988). Each experiment was performed at least in duplicate and the deviation of absorbance values was less than 10%.

Results

In this study we examined the involvement of five hydroxyethylated flavonoids in lipid peroxidation and free radical scavenging activity. We also included quercetin (compound 6, Table 1) as a reference compound, as well as a commercial standardized mixture of hydroxyethyl rutosides (Venoruton, compound 7) used therapeutically. For this mixture we used in our calculations an estimated molecular weight of 742·68, corresponding to its major constituent, troxerutin (compound 3).

Almost all compounds tested inhibited non-enzymatic lipid peroxidation. All the studied compounds, as well as DMSO were tested and found not to interfere with the assay.

Compound 4 had only a slight effect (ca. 7% inhibition) on lipid peroxidation at 1 mM after 45 min of incubation. This effect disappeared at 0·5 mM. Compound 3 inhibited lipid peroxidation by 33·3% at 1 mM under the same experimental conditions. All other compounds tested caused more than 50% inhibition of lipid peroxidation at 1 mM. Time and concentration dependence of lipid peroxidation is shown in Fig. 1. From these results the IC50 values after 45 min of incubation could be calculated, as shown in Table 1.

The effect of the tested compounds on lipid peroxidation could be summarized in the following order, according to decreasing antioxidant activity: 6 > 5 > 1 > 7 > 2 > 3 > 4.

In order to examine the hydroxyl radical scavenging activity of these compounds, the competition between them and DMSO for HO· generated from the ascorbic acid/Fe^{3+} system, expressed as percent inhibition of formaldehyde production, was estimated. Because of their poor aqueous solubility, compounds 5 and 6 were not included in this series of experiments. All the other compounds were studied at three concentrations (5, 2·5 and 1 mM) and their competition with a standard DMSO concentration was tested. Results are shown in Table 2.

It can be seen that, with the exception of compound 4, all derivatives inhibited more than 50% the oxidation of DMSO at 5 mM. This effect was concentration dependent. Mannitol, a known hydroxyl radical scavenger, was found to inhibit the hydroxyl radical-induced oxidation of DMSO (33 mM) by 13·5% at 25 mM.

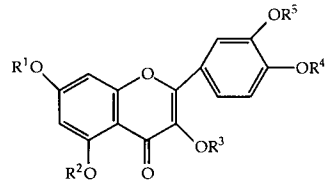
The order of decreasing protection against hydroxyl radicals, at 2·5 mM concentration, was 1 > 7 > 2 > 3 > 4.

In order to investigate whether the observed effect was a result of the HO· scavenging activity of the tested compounds, or whether an interference with radical generation was involved, the competition was measured at a fixed concentration of the flavonoids (2·5 mM) and the DMSO concentration varied (3·3, 6·6, 33 and 66 mM). A decreasing inhibition of the formaldehyde production was observed with increasing DMSO concentrations.

The second-order rate constant for the reaction of a given scavenger with HO· can be determined (Halliwell et al 1987). Formaldehyde generated from DMSO was determined spectroscopically, and the absorbance (A) recorded after the incubation with a given concentration of the scavenger, S, was related to the absorbance without the scavenger (A_o). The equation which can give the rate constant, k_s for the reaction of a scavenger with HO· is:

$$\frac{A_o}{A} = 1 + \frac{k_s[S]}{k_{DMSO}[DMSO]}$$

Table 1. Structures of the flavonoid derivatives tested and their IC50 values for lipid peroxidation (after 45 min of incubation).



No.*	R^1	R^2	R^3	R^4	R^5	IC50 (mM)
1	$HOCH_2CH_2$	H	Rutinose	H	H	0·032
2	$HOCH_2CH_2$	H	Rutinose	$HOCH_2CH_2$	H	0·70
3	$HOCH_2CH_2$	H	Rutinose	$HOCH_2CH_2$	$HOCH_2CH_2$	> 1
4	$HOCH_2CH_2$	$HOCH_2CH_2$	Rutinose	$HOCH_2CH_2$	$HOCH_2CH_2$	> 1
5	$HOCH_2CH_2$	H	H	$HOCH_2CH_2$	$HOCH_2CH_2$	0·026
6	H	H	H	H	H	0·017
7	Mixture of compounds 1, 2, 3 and 4					0·50

*1. 7-Mono-hydroxyethyl rutoside.
2. 7,4′-Di-hydroxyethyl rutoside.
3. 7,3′,4′-Tri-hydroxyethyl rutoside.
4. 5,7,3′,4′-Tetra-hydroxyethyl rutoside.
5. 7,3′,4′-Tri-hydroxyethyl quercetin.
6. Quercetin.

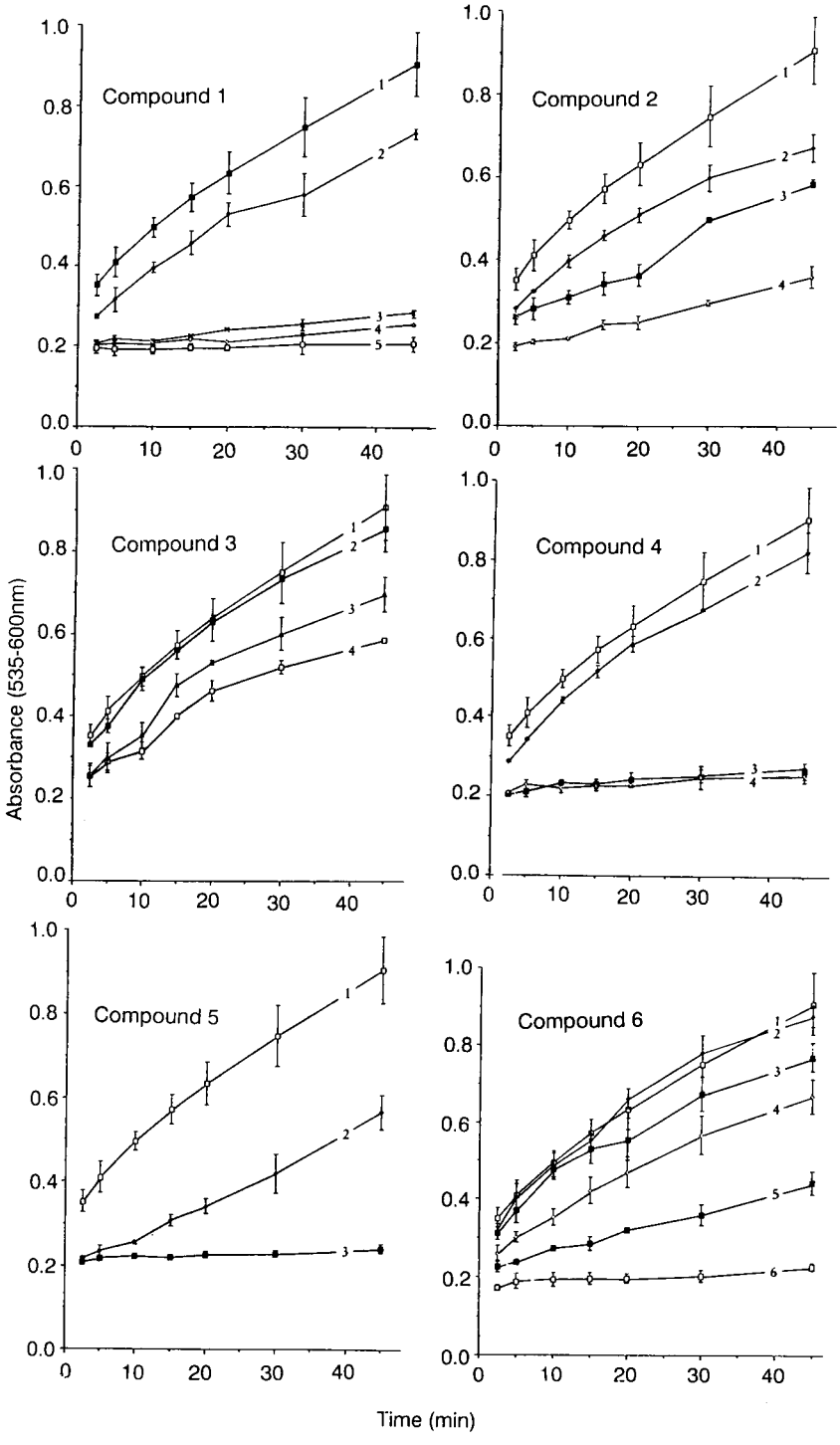


FIG. 1. Time course of lipid peroxidation, as affected by various concentrations of the tested compounds. Compound 1:1: Controls; 2: 0·01 mM; 3: 0·05 mM; 4: 0·1 mM; 5: 0·25 mM. Compound 2:1: Controls; 2: 0·25 mM; 3: 0·5 mM; 4: 1 mM. Compound 3:1: Controls; 2: 0·25 mM; 3: 0·5 mM; 4: 1 mM. Compound 5:1: Controls; 2: 0·01 mM; 3: 0·05 mM; 4: 0·1 mM. Compound 6:1: Controls; 2: 0·01 mM; 3: 0·05 mM. Compound 7:1: Controls; 2: 0·05 mM; 3: 0·01 mM; 4: 0·25 mM; 5: 0.5 mM; 6: 1 mM.

For the calculations of the rate constants for the reaction of the tested compounds with HO· we used the reported rate constant for this reaction of DMSO, $0{\cdot}7 \times 10^{10}\ M^{-1}s^{-1}$, which has been determined colorimetrically in a similar way from the degradation of deoxyribose by HO· (Halliwell et al 1987).

A plot of A_o/A against 1/[DMSO] was constructed using the various DMSO concentrations. According to the above equation, a straight line with a slope of k_s/k_{DMSO} and an intercept on the y-axis of 1 should be obtained. For the flavonoids tested this was not the case, and a deviation from the straight line was observed, resulting in higher apparent

Table 2. Effect of the tested compounds on the hydroxyl radical-mediated oxidation of dimethyl sulphoxide (33 mM) and their k_s values.

	Inhibition (%)			
Compound*	5 mM	2·5 mM	1 mM	k_s ($\times 10^{10}$ $M^{-1}s^{-1}$)
1	95	80	65	12·8–14·4
2	88	59	28	1·3–2·7
3	96	51	26	1·3–2·9
4	36	27	20	1·3–2·3
7	82	64	29	–

*For structures see Table 1.

Table 3. Interaction of the tested compounds with DPPH stable free radical (200 μM).

	Interaction (%)			
Compound*	200 μM	100 μM	50 μM	10 μM
1	92·8	92·1	88·8	87·7
5	85·7	83·0	66·2	31·1
6	92·3	91·1	89·6	72·8
7	46·5	22·8	11·4	4·0

*For structures see Table 1.

$k_s$values at high DMSO concentrations (66 mM). Thus, for the calculation of k_s values we used the lowest two DMSO concentrations (3·3 and 6·6 mM) and the results are shown in Table 2.

For compounds 5 and 6, which were insoluble in the buffer even at very low concentrations (< 1 mM), an alternative method permitting use of ethanolic solutions was applied for estimation of their reducing activity. Their interaction with the stable free radical DPPH (200 μM), using four different concentrations of the compounds (10, 20, 100 and 200 μM) was studied. In this series of experiments, substances 1 and 7 were also included, as a link with the DMSO assays. Results are shown in Table 3.

Concerning the lowest concentration tested (10 μM), the order of decreasing reducing activity was: 1 > 6 > 3 > 7.

Discussion

Oxygen-generated free radicals have been shown to be implicated in many pathophysiological conditions (Marx 1987) and in the toxicity of xenobiotics e.g. adriamycin (Kappus et al 1984) and halothane (Younes et al 1988). The autoxidation of membrane lipids, initiated by oxygen radicals can lead to cell injury. An important intermediate in biological oxidations is the superoxide anion radical, which is formed in-vivo from the reduction of molecular oxygen. This species can produce H_2O_2, a highly toxic product, which in turn gives rise to HO· by reaction with transition metal ions in the body. The HO· radical is very reactive and one of the strongest oxidizing agents. Many physiological processes, such as phagocytosis, are known to involve the formation of HO· radicals and their toxicity is apparently due to the subsequent reactions of this species. HO· radicals have been shown to cause DNA strand breaks (Imlay & Linn 1988) and to induce K^+ loss from cell membranes (Maridonneau-Parini et al 1986).

Antioxidant treatment seems to offer protection against free radical-induced injury. However, many synthetic antioxidants, although more efficient in-vitro than biological antioxidants of the body defense system, produce unwanted side reactions unrelated to their biological functions (Scott 1985). Flavonoids are naturally occurring, non-toxic substances shown to possess many pharmacological actions and therapeutic applications (Havsteen 1983). However, the efficacy of the oral route for the administration of bioflavonoids has been questioned for many years (Clark & Mackay 1950), mainly because of their inadequate absorption from the gastrointestinal tract. Semisynthetic hydroxyethylated flavonols can be more easily absorbed, and, at the same time, hydroxyethylation increases their hydrophilicity, thus preventing precipitation of the substance in the bile duct and renal channels, reported to occur after rutin administration (Balant et al 1984).

In this study we investigated the effect of 7-mono-, 7,4′-di-, 7,3′,4′-tri- and 5,7,3′,4′-tetra-hydroxyethyl rutosides and 7,3′,4′-tri-hydroxyethyl quercetin on non-enzymatic lipid peroxidation in-vitro. We compared their antioxidant activity with that of quercetin, as well as of standardized mixture of *O*-β-(-hydroxyethyl)-rutosides. Quercetin was found to be the most potent antioxidant. Its calculated IC50 value (17 μM) is in agreement with that reported in the literature (15 μM), obtained from similar experiments (Robak et al 1986). Concerning the structures of the tested compounds in relation to their antioxidant activity, our results indicate that quercetin and its trihydroxyethyl derivative are more potent antioxidants than the other compounds, which are glycosides. This is evident when we compare compounds 3 and 5 (Table 1). Their only structural difference is that the former is a glycoside. This difference, however, can produce a considerable decrease of activity. These results confirm previous observations (Ratty & Das 1988) that the sugar moiety masked the antioxidant activity of flavonoids. A possible explanation of this could be that the lower lipophilicity of the glycoside prevents its access to the lipid membranes, the site of lipid peroxidation.

A more important structural feature that seems closely related to antioxidant activity is the presence of phenolic groups. A number of phenolic compounds possess antioxidant activity (Burton & Ingold 1981). Concerning flavonoid antioxidants, it has been reported that the presence of a hydroxyl group on both aromatic rings is necessary (Affany et al 1987). It seems that this is not always the case, since compound 5, one of the most potent antioxidants in our series, has no phenolic group on ring C. From our results it can be concluded that the number rather than the position of phenolic groups is more important for the antioxidant activity of the tested compounds. Comparison of compounds 1, 2, 3, 4 shows that the progressive substitution on phenolic groups proceeded in parallel with the decrease in the produced inhibition of lipid peroxidation. The activity of the tested mixture was comparable with that of compound 2 and higher than that of troxerutin, its major constituent.

The HO· radical scavenging activity of some of these compounds was also investigated. As a HO· radical-generating system the Fe^{3+}/EDTA-catalysed autoxidation of ascorbic acid was used. HO· radicals thus formed, mediate the oxidation of DMSO to yield formaldehyde. The

formaldehyde production from DMSO represents a convenient method to detect HO· (Klein et al 1981). From the DMSO results it can be seen that, with the exception of compound 4, all compounds tested inhibited very significantly the oxidation of DMSO. This inhibition was principally due to HO· scavenging ability, since it was dependent upon the concentration of DMSO used.

We calculated the second-order rate constant for the reaction of the tested flavonoids with HO·. Use of mannitol as a scavenger gave a k_s value of $1{\cdot}7 \times 10^9$ M^{-1}s^{-1}, which was in accordance with the values given in the literature, $(1{\cdot}0$–$2{\cdot}0) \times 10^9$ M^{-1}s^{-1} (Goldstein & Czapski 1984; Halliwell et al 1987). For the compounds tested, higher than expected apparent k_s values were obtained at the highest DMSO concentrations (33 and 66 mM) and a fixed concentration of the scavenger (2·5 mM). This may be attributed to a simultaneous inhibition of HO· generation, and is further confirmed by the deviation from the straight line at high DMSO concentrations, when we plotted A_0/A vs 1/[DMSO], as described. We therefore used the low DMSO concentrations (3·3 and 6·6 mM) for a better approximation of more reliable k_s values.

For some of the flavonoids, their interaction with the stable free radical DPPH was studied. This interaction expresses the reducing activity of the compounds tested and indicates their ability to scavenge free radicals (Ratty et al 1988). In these experiments, compound 1 and quercetin (compound 4) presented the highest reducing activity and were found to be equipotent free radical scavengers.

From all the results obtained it can be concluded that the tested compounds exhibit a considerable inhibition of microsomal membrane lipid peroxidation. In addition, they are potent HO· radical scavengers and they interact with the stable free radical DPPH. In the lipid peroxidation experiments, these compounds were found to be less active than the reference compound quercetin, and this could be attributed to their structural characteristics. As HO· scavengers, the tested compounds were more potent than mannitol and DMSO, both known HO· scavengers.

In the series tested, the presence of phenolic groups, as well as of the proper substitution at positions 3′ and 4′ would justify the assumption that the antioxidant action of these compounds could be correlated with their ability to chelate iron. Since, in the experiments on lipid peroxidation and HO· radical scavenging activity, iron ions catalyse the free radical generation, it could be possible that the antioxidant effect of these compounds is mediated via their chelating properties. Our observation that, in the kinetic experiment, the deviation from the straight line may be attributed to interference with HO· generation could further support the possibility that this effect is partly due to iron complexation. However, we have reported (Rekka et al 1990) that *o*-methoxy-phenol (25 μM) had no effect on lipid peroxidation under the same experimental conditions. In addition, the fact that compound 4, which cannot chelate iron, although not very potent, can still inhibit the oxidation of DMSO (33 mM) by 36% at 5 mM indicates that binding to iron is not the decisive factor in HO· scavenging activity. Furthermore, the interaction of a potential scavenger with DPPH is a process which does not depend on iron. Our results show that all compounds tested interact with DPPH and the order of activity correlates with those of the other two series of experiments. The above indicate that iron chelation is not the primary cause of the antioxidant properties of these compounds. In any case, antioxidant action is considered to be a complex process which can include prevention of formation or scavenging of free radicals (Rekka et al 1990).

A direct relationship between HO· radicals and initiation of lipid peroxidation has been questioned (Robak & Gryglewski 1988). In addition, DMSO and mannitol were found to have no effect on the peroxidation of microsomal membrane lipids (Demopoulos et al 1990). The simultaneous presence of these properties in the series of compounds tested, and with about the same order of activity, constitutes an exploitable and apparently useful combination.

Acknowledgements

We acknowledge Zyma SA, Switzerland, for kindly providing the substrates and some of the reagents used.

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