JPP Journal of Pharmacy and Pharmacology

JPP 2003, 55: 131–142

Received May 13, 2002
Accepted October 1, 2002
DOI 10.1211/002235702559
ISSN 0022-3573

Anti- and pro-oxidant activity of rutin and quercetin derivatives

Marc Kessler, Geneviève Ubeaud and Louis Jung

Abstract

Some semi-synthetic flavonoids, particularly derivatives of rutin, are used as therapeutic agents in the treatment of diseases involving free radicals. Here, for the first time, a complete study has been made of the relationship between the structure of such molecules and their superoxide, hydroxyl and peroxyl radical scavenging activity. The molecules chosen for this study were rutin, its aglycone (quercetin), and their methyl ethyl and hydroxyl-ethyl derivatives. Our results are consistent with the general agreement on the structural requirements for free radical scavenging activity. Moreover, we have shown that alkylation of the hydroxyl in position 7 enhanced the scavenging, and also that in a Fenton reaction system, some quercetin derivatives with free catechol moiety or free hydroxyl in position 3 (or both) were pro-oxidant, through superoxide radical and hydrogen peroxide production. Although the structural features needed for pro-oxidant activity are not entirely clear, it appears that to avoid pro-oxidant behaviour, the hydroxyl group in position 3 should be blocked to prevent its auto-oxidation. Thus, flavonoids cannot only be considered purely as antioxidants, since under certain reaction conditions they can also display pro-oxidant activity. This unexpected behaviour could explain, in part, the observed toxicity of some flavonoids in-vivo.

Introduction

For many years, interest in the study of antioxidants has been increasing. Flavonoids, polyphenolic compounds found in plants, are of particular interest because they are components of many fruits and vegetables present in the diet of man.

Flavonoids have shown a wide range of biological activity, including vasoprotective, anti-inflammatory, anti-hepatotoxic and anti-carcinogenic action. A large part of their pharmacological activity may be attributed to their ability to inhibit certain enzymes and to their antioxidant properties (iron chelating and oxygen free radical scavenging properties).

Actually, oxygen free radicals are natural physiological products, but are also extremely reactive species. They play an important role in biological processes (Halliwell & Gutteridge 1999a), but they can also have deleterious effects when produced in large quantity, or if the natural defenses are overloaded. It is now generally accepted that radical oxygen species are implicated in a wide variety of pathological states (Halliwell & Gutteridge 1999b).

In recent years, several studies have been conducted on the superoxide, hydroxyl and peroxyl radical scavenging activity of natural flavonoids, to identify the structural features necessary for antioxidant activity, and a consensus has been reached (Bors et al 1990; Rice-Evans et al 1996). However, the situation may not be as simple as it seemed, and some results, particularly for hydroxyl radical scavenging, are quite contradictory. Indeed, some flavonoids have sometimes been reported to be pro-oxidant (Laughton et al 1989; Cao et al 1997; Miura et al 1998).

In this study, our interest was focused on specific flavonoids recommended in the treatment of vascular disorders, such as venous ulcer, chronic venous insufficiency and oedema, which involve oxygen free radicals produced by ischaemic conditions (Michiels et al 1994) or by inflammatory reaction during venous hypertension (Heitzer et al 2001).

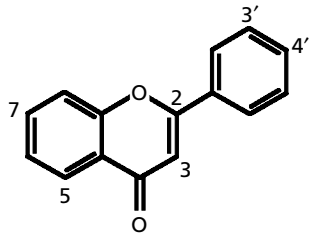
Laboratoire de Chimie Thérapeutique, Faculté de Pharmacie de Strasbourg, 74 route du Rhin, BP 24, F-67401 Illkirch, France

Marc Kessler, Geneviève Ubeaud, Louis Jung

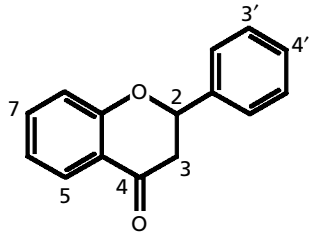
Correspondence: G. Ubeaud, Laboratoire de Chimie Thérapeutique, Faculté de Pharmacie de Strasbourg, 74 route du Rhin, BP 24, F-67401 Illkirch, France. E-mail: ubeaud@pharma.u-strasbg.fr

Acknowledgement: The authors would like to thanks Novartis (Nyon, Switzerland) for the kind provision of some compounds.

Table 1 Substitution patterns of the flavonoids studied.



	3	5	7	3′	4′
Group I					
Rutin	Rutinose	OH	OH	OH	OH
MonoHER	Rutinose	OH	OC_2H_5OH	OH	OH
DiHER	Rutinose	OH	OC_2H_5OH	OH	OC_2H_5OH
TriHER	Rutinose	OH	OC_2H_5OH	OC_2H_5OH	OC_2H_5OH
TetraHER	Rutinose	OC_2H_5OH	OC_2H_5OH	OC_2H_5OH	OC_2H_5OH
Group II					
Quercetin	OH	OH	OH	OH	OH
MonoHEQ	OH	OH	OC_2H_5OH	OH	OH
DiHEQ	OH	OH	OC_2H_5OH	OH	OC_2H_5OH
TriHEQ	OH	OH	OC_2H_5OH	OC_2H_5OH	OC_2H_5OH
TetraHEQ	OC_2H_5OH	OH	OC_2H_5OH	OC_2H_5OH	OC_2H_5OH
PentaHEQ	OC_2H_5OH	OC_2H_5OH	OC_2H_5OH	OC_2H_5OH	OC_2H_5OH
GroupIII					
MonoMeR	Rutinose	OH	CH_3	OH	OH
MonoEthR	Rutinose	OH	CH_2CH_3	OH	OH
MonoMeQ	OH	OH	CH_3	OH	OH
Group IV					
PentaMeQ	CH_3	CH_3	CH_3	CH_3	CH_3
PentaEthQ	CH_2CH_3	CH_2CH_3	CH_2CH_3	CH_2CH_3	CH_2CH_3
Group V					
Luteolin		OH	OH	OH	OH



	3	4	5	7	3′	4′
Group VI						
Taxifolin	OH	C = O	OH	OH	OH	OH
Eriodictyol		C = O	OH	OH	OH	OH
Leucocyanidol	OH	OH	OH	OH	OH	OH

The molecules chosen were hydroxyethyl derivatives of rutin (hydroxy-ethyl rutins or HERs) and their aglycones (Table 1).

HERs act directly on venous tone by increasing capillary resistance, reducing hyperpermeability and oedema (Michel & Kendall 1993). They also improve microvascular endothelial function by their antioxidative effects, preventing free-radical-mediated damage caused by vascular oxidative stress (Janssens et al 1996). Therefore, it is of interest to specify the free radical scavenging activity of HERs and related compounds, to determine the structural elements essential to antioxidant activity, and to determine which modifications lead to improved activity and to the suppression of pro-oxidant behaviour.

These compounds were also chosen for chemical and analytical reasons. Firstly, they form a consistent and homogenous series of compounds that presents enough structural variability to carry out a structure–activity relationship. Secondly, they are obtained by chemical synthesis, thus making it possible to control their purity.

Much evidence of HERs' free radical scavenging activity has already been obtained for peroxyl radicals (Mora et al 1990; Van Acker et al 1996) but practically no research has been carried out on superoxide radicals (Robak & Gryglewsky 1988). Results on hydroxyl radical scavenging have been quite contradictory (Rekka & Kourounakis 1991; Haenen et al 1993) and yet little work has been done on hydroxyethyl derivatives of quercetin (hydroxy-ethyl quercetins or HEQs), which are considered to be HER metabolites.

To extend the field of this study, we have evaluated the activity of the mono- and poly-methyl (Me) and ethyl (Eth) derivatives of rutin and quercetin, and of a flavone (luteolin), a flavanone (eriodictyol), a dihydroflavonol (taxifolin) and a flavan-3,4-diol (leucocyanidol) (Table 1).

Materials and Methods

Reagents

Superoxide dismutase (EC 1.15.1.1) (Mn SOD from *Escherichia coli*) from bovine erythocytes, xanthine oxidase (EC 1.1.3.22) (grade III), hypoxanthine, α-naphthylamine were purchased from Sigma (St Quentin Fallavier, France). Ascorbic acid, 2-deoxy-D-ribose, thiobarbituric and trichloroacetic acid were obtained from Aldrich (St Quentin Fallavier, France), hydrogen peroxide, 2,2′-azobis-(2-amidinopropane) dihydrochloride (ABAP), mannitol and 2,6-di-tertbutyl-*p*-kresol from Fluka (St Quentin Fallavier, France) and Tris (hydromethyl) amino-methane from Merck, France and mannitol from Laboratoire Delagrange, France.

All other chemicals used were of the highest grade available.

Chemistry

All flavonoids were obtained by chemical synthesis in our laboratory apart from (2*R*,3*R*/2*S*,3*S*) taxifolin (Aldrich, Saint Quentin Fallavier), eriodictyol and luteolin (Extrasynthèse, Genay), and diHER and tetraHER, which were generous gifts from Novartis (Nyon, Switzerland).

MonoHER, monoEthR and monoMeR were obtained from rutin (Scheline 1966; Courbat & Albert 1975) by reaction with ethylene oxide, diethyl sulfate or dimethyl sulfate in a sodium tetraborate solution containing a catalytic amount of sodium hydroxide. After deprotection in 6M HCl, the compounds were recrystallized from water (for monoHER) or purified by chromatography on an RP18 column using methanol–water as the mobile phase (for monoMeR and monoEthR).

Troxerutin was obtained by reaction of rutin (Tang & Yuan 1996) with ethylene oxide in alcoholic medium, and was recrystallized from ethanol.

PentaMeQ and pentaEthQ were obtained from rutin or quercetin (Picq et al 1982) by reaction with dimethyl sulfate or diethyl sulfate in acetone, in the presence of an excess of potassium carbonate. The compounds were purified by recrystallization from methanol, ethanol or ethanol–water.

TetraHEQ and pentaHEQ were obtained from the reaction of quercetin (Courbat & Valenza 1973) in water with an excess of ethylene oxide in the presence of a catalytic amount of sodium hydroxide. Depending on reaction time, we could obtain either tetraHEQ or pentaHEQ, compounds which were recrystallized from methanol.

DiHEQ was obtained by the dealkylation of tetraHEQ (Courbat & Valenza 1973) by reaction with aluminum chloride in nitrobenzene and was recrystallized from water.

Reduction of (2*R*,3*R*/2*S*,3*S*) taxifolin in methanol with excess of $NaBH_4$ gave leucocyanidol.

The purity of all synthetic flavonoids was assessed by HPLC on reverse-phase C18 column, at a flow rate of 1 mL min^{-1} using a gradient of water, adjusted at pH 2 with orthophosphoric acid, and acetonitrile. All compounds were obtained at 97–98% purity.

Apparatus

The HPLC system consisted of a Beckman System Gold, autosampler module 507, pump 126 solvent module and a programmable detector module 166. The analytical column was a reverse-phase LiChrospher C18 column 120×4 mm, 5 μm particle size (Merck, France) and a guard column C18 10×4 mm (Merck, France).

The absorbance measurements for the free radical assays were performed on a Beckman UV-Visible detector (model 65).

Free radical scavenging activity

Superoxide radical assay

The enzymatic system used to produce superoxide radicals was essentially that described by Hu et al (1995).

To a solution of hypoxanthine (0.2 mM) in phosphate buffer (0.1 M) pH 7.4, hydroxylamine (0.2 mM), ethylenediamine tetra-acetic acid (EDTA) (0.1 mM) and finally xanthine oxidase (2.5 mU mL^{-1}) were added. After incubation at 37°C for 30 min, the dye reagent (300 μg mL^{-1} of sulfanilic acid, 0.75 mg mL^{-1} of α-naphthylamine and 16.7% acetic acid (v/v)) was added. The mixture was allowed to stand for 30 min at room temperature, and the absorbance was measured at 550 nm.

As the absorbance is proportional to the quantity of superoxide produced, the decrease of radical production was determined by the decrease of absorbance observed with (A) or without (A_0) different concentrations of flavonoid and then expressed as a percentage as follows:

$$I = [(A_0 - A)/A_0] \times 100 \quad (1)$$

where I is inhibition of superoxide radical production, A is the absorbance recorded with a given concentration of flavonoid and A_0 is the absorbance without flavonoid.

Xanthine oxidase activity

The interaction between flavonoid derivatives and the xanthine oxidase in our assay was determined by monitoring, at 295 nm, the formation of urate from hypoxanthine at 37°C. The production of uric acid was followed for 2 min in the presence of different concentrations of flavonoids (0, 10, 50 and 100 μM).

The ratio, R, between the initial rate of production of uric acid with the enzyme alone (Po) and with the enzyme and the flavonoid (Px) was determined (R = Px/Po).

R = 1 implies that the flavonoid has no influence on the enzyme activity under our assay conditions.

R > 1 implies enhanced activity and R < 1 implies inhibition of the enzyme.

Hydroxyl radical assay

The assay procedure was that described by Zhao & Jung (1995). The samples were incubated at 37°C for 15 min in phosphate buffer, pH 7.4 (24 mM NaH_2PO_4–Na_2HPO_4 in 15 mM NaCl) with deoxyribose (0.6 mM), ascorbic acid (0.6 mM), hydrogen peroxide (0.855 mM), the substances to be tested (0–1000 μM) and EDTA (0.02 mM). $(NH4)_2Fe(SO_4)_2$ (0.02 mM) was used to initiate the reaction.

EDTA and $(NH_4)_2Fe(SO_4)_2$ were pre-mixed just before addition to the reaction mixture.

Cold trichloroacetic acid (1.5 mL of a 2.8% (w/v) solution) was then added. A 1-mL sample of the incubation mixture was mixed with 1 mL of thiobarbituric acid (TBA) reagent (1% w/v, in 0.05 M NaOH), followed by heating at 100°C for 15 min and subsequent cooling to room temperature. The absorbance at 532 nm was measured against appropriate blanks.

For the substances with low water solubility, we prepared a dispersion of the substance in gum arabic.

The method is based on the specific reaction of deoxyribose with hydroxyl radicals generated from ascorbic acid/Fe^{2+}/EDTA. This produces malondialdehyde (MDA) which can be evaluated by reaction with TBA.

The absorbance at 532 nm is proportional to the quantity of MDA produced. This is proportional to the degradation of deoxyribose, which is in turn proportional to the production of hydroxyl radicals, as follows:

$$dA/dt \propto -d[D]/dt \propto d[\cdot OH]/dt \quad (2)$$

If the production of hydroxyl radicals is independent of the presence or absence of the scavenger (S) (i.e. if $V^S_{\cdot OH} = V_{\cdot OH}$) the rate constant for the reaction of a given scavenger with hydroxyl radicals can be determined using the following equation:

$$1/A = 1/A_0(1+\{k_S[S]/(k_D\{D\}+k_X)\}) \quad (3)$$

where A is the absorbance recorded with a given concentration of the scavenger (S), A_0 is the absorbance without the scavenger, [D] is the concentration of deoxyribose (0.6 mM in our experiment), k_D and k_S are the rate constants of deoxyribose (Buxton et al 1988) and of studied compound, respectively and k_X is a constant depending on the experimental conditions and representing the part of hydroxyl radical that reacts with reagents in the assay mixture other than deoxyribose, such as Fe^{2+}-EDTA, H_2O_2.

So, the rate constant can be expressed as:

$$A_0/A = 1(1+\{k_S[S]/(k_D\{D\}+k_X)\}) \quad (4)$$

and determined by plotting of A_0/A against 1/[S] for various concentrations of test compounds (between 100 and 600 μM).

Hydroxyl radical production

The degradation of deoxyribose by flavonoids was followed by the procedure described above, either in pH 7.4 phosphate buffer either without any radical generating system, or with iron-EDTA–ascorbic acid. Each experiment was then repeated with 300 U of SOD or 1000 U of catalase.

Inhibition of lipid peroxidation

Rat liver microsomal preparation

Liver microsomes were prepared according to Ubeaud et al (1995) from male Wistar rats, 240–260 g. Pooled liver samples (n = 5) were thawed and homogenized at 4°C at 10 000 *g* in a Tris-HCl buffer (50 mM, pH 7) containing 250 mM sucrose (40 mL buffer for 10 g liver). The homogenate was centrifuged at 9000 *g* for 15 min at 4°C. The supernatant was then centrifuged at 27 000 *g* for 15 min at 4°C. The new supernatant was centrifuged at 105 000 *g* for 60 min at 4°C, then washed in a pH 7.25 buffer at containing 0.1 M sodium pyrophosphate and 0.001 M EDTA. After a second centrifugation at 105 000 *g* for 60 min at 4°C, the pellet was resuspended in an appropriate volume of pH 7.4 buffer containing 0.1 M sodium pyrophosphate. Microsomal suspensions were then stored shock-frozen in liquid nitrogen.

The microsomal protein content was determined using Bradford's method (Bradford 1976). The incubations for the ABAP model were performed by using 1 mg mL^{-1} of microsomal protein in 0.1 M phosphate pH 7.4 buffer.

Non-enzymatic lipid peroxidation

Before use, the microsomal fractions were heated at 100°C for 90 s to deactivate the enzymatic factors. All solutions were freshly prepared in dimethyl sulfoxide (DMSO) or distilled water. The maximum DMSO concentration in the final incubation mixture was 2%, which was found to have no influence on the assay. The reaction was initiated by the addition of ABAP, (10 mM final concentration) prepared in Tris-HCl buffer just before use. The mixture was then incubated at 37°C for 90 min and lipid peroxidation was evaluated by detection of the MDA formed by the TBA assay.

As the absorbance is proportional to the quantity of peroxyl radicals produced, the decrease of radical production was determined by the decrease of absorbance observed with (A) or without (A_0) different concentrations of flavonoid and then expressed as a percentage as follows:

$$I = [(A_0 - A)/A_0] \times 100 \quad (5)$$

where I is inhibition of peroxyl radical production, A is the absorbance recorded with a given concentration of flavonoid and A_0 is the absorbance without flavonoid.

Statistical analysis

For each concentration of flavonoid tested for anion superoxide and peroxyl radical production (10–100 μM for superoxide anion, 100–1000 μM for peroxyl radical) the inhibition percentage is the average of three different experiments.

The IC50 values (concentration giving 50% inhibition of radical production) were determined by regression analysis. All values were expressed as the mean $\pm$ s.e.m. of three different experiments.

The ratio, R, used to determine the interaction between flavonoid derivatives and the xanthine oxidase activity, was analysed by a variance analysis followed by Student's *t*-test with a level of significance of $P < 0.05$.

The hydroxyl radical scavenging rate constant was determined by linear regression analysis of degradation of deoxyribose versus flavonoid concentration.

The k_S values were expressed as the mean $\pm$ s.e.m. of three different experiments repeated three times.

Results and Discussion

Superoxide radical scavenging activity

According to the IC50 values reported in Table 2, the most potent compounds are those having a free catechol group on the B-ring (i.e. quercetin, rutin and their monoalkylated derivatives). The activity dramatically decreases for compounds where one or both of the two hydroxyls on the C3′ and C4′ positions are substituted (i.e. groups I–IV), confirming the importance of the free catechol moiety.

Blockage of the hydroxyl at C7 has a slightly negative effect on rutin (monoHER, monoMeR and monoEthR vs rutin), but it enhances quercetin activity (monoMeQ and monoHEQ vs quercetin).

However, considering compounds of group III, we observe that the nature of the alkyl group on the C7 hydroxyl does not significantly influence the scavenging activity. This is also true when all hydroxyls are substituted (pentaHEQ vs group IV compounds). Furthermore, the aglycones (quercetin and quercetin derivatives) show a higher activity than the corresponding glycosides (rutin and rutin derivatives), indicating that the 3-OH group is important. However, the effect of this glycoside substitution is of little importance compared with that of the catechol moiety. Rutin retains a good scavenging activity.

The C2–C3 bond can be saturated or unsaturated (quercetin vs taxifolin or luteolin vs eriodictyol). This has no influence on superoxide radical scavenging, while the reduction of the C4 carbonyl function (taxifolin vs leucocyanidol) causes a slight decrease in activity.

Xanthine oxidase activity

To be sure that the observed effect of the flavonoids on our hypoxanthine–xanthine oxidase assay was caused by superoxide scavenging and not by the inhibition of radical production via inhibition of the enzyme, we investigated the possible interactions between flavonoids and xanthine oxidase.

The enzyme activity in our assay was evaluated by spectrophotometric measurement of the uric acid formed from hypoxanthine, in the presence of different concentrations of flavonoids (0, 10, 50 and 100 μM).

It is well known that some flavonoids (Costantino et al 1992; Chang et al 1993; Cotelle et al 1996; Cos et al 1998), especially quercetin, inhibit xanthine oxidase, but in our assay most of the compounds had no influence on enzyme activity. Only triHER and pentaHEQ (at concentrations of 50 and 100 μM) enhanced the enzyme activity, suggesting that their superoxide radical scavenging activity was underestimated in our model. However, these compounds had an IC50 value superior to 100 μM. Although their activity may be better than it appears here, it is still, in any case, low.

Taxifolin, eriodictyol (at 10–100 μM) and monoMeQ (at 100 μM) inhibit the enzyme. For these compounds the scavenging activity was overestimated in the model. However, the IC50 of monoHEQ (< 10 μM) actually reflects scavenging rather than the inhibition of production, since no inhibition of xanthine oxidase was observed at 10 μM.

For taxifolin and eriodictyol, the IC50 values were close to 10 μM (Table 2) and at this concentration an inhibition of xanthine oxidase has been observed. These compounds appear more active than they might be in reality. So, the C2–C3 double bond seems to be an important structural element for free radical scavenging.

In these conditions, the most active compounds appear to be the mono-alkylated derivatives of quercetin, compounds having a catechol moiety, a C4 carbonyl function and the C3 hydroxyl, free or substituted. Literature results are quite contradictory on the subject of the C3 hydroxyl, some (Sichel et al 1991; Hu et al 1995; Cotelle et al 1996) stating that this hydroxyl must be free, while others (Robak & Gryglewsky 1988; Yuting et al 1990) state that it can be substituted.

The role of the C2–C3 double bound is uncertain. It is probably important for the dihydro compounds such as taxifolin and eriodictyol, which inhibit the production of superoxide radicals at a concentration similar to their IC50 value, making them appear more active than they are.

However, we have shown that the C7 hydroxyl can be substituted with different alkyl groups. This enhances the superoxide scavenging activity of the compounds that have free hydroxyl at C3.

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging capacities were evaluated using the Fenton reaction, a simple test tube reaction allowing the rate constant to be determined. However, it was noted that all organic compounds react with hydroxyl radicals with a rate constant (k_S) between 10^7 and 10^{10} M^{-1} s^{-1}. Thus, these values have no significance in isolation but are useful for the comparison of the in-vitro scavenging activity of compounds with similar structures.

The k_S values of hydroxyl radical scavenging are given in Table 3. The k_S value obtained for mannitol, used as a reference, is similar to the values presented in the relevant

Table 2 IC50 values of tested compounds for superoxide radical generation in the hypoxanthine/xanthine oxidase system.

Compounds	IC50 (μM)		Xanthine oxidase activity
	Our results	Robak & Gryglewsky (1988) (μM)	
Group I			
Rutin	17±6	37.8±0.9	NM
MonoHER	31±4		Activated at 100 μM
DiHER	>100		NM
TriHER	>100	39.5±1	Activated at 50 and 100 μM
TetraHER	>100		NM
Group II			
Quercetin	19±8	10.0±1.1	NM
MonoHEQ	6±1		NM
DiHEQ	>100		NM
TriHEQ	>100		NM
TetraHEQ	>100		NM
PentaHEQ	>100		Activated at 50 and 100 μM
Group III			
MonoMeR	34±6		NM
MonoEthR	39±9		NM
MonoMeQ	7±2		Inhibited at 100 μM
Group IV			
PentaMeQ	>100		NM
PentaEthQ	>100		NM
Group V			
Luteolin	17±4		NM
Group VI			
Taxifolin	9.3±0.4		Inhibited at 10 and100 μM
Leucocyanidol	20±1		NM
Eriodictyol	7.4±0.7		Inhibited at 10 and100 μM

IC50 values are expressed as the mean values±s.e.m. of three separate experiments (each experiment was carried out in triplicate). NM, no modification of the enzyme activity by the flavonoid.

literature (Goldstein & Czapski 1984). For rutin and its derivatives, the rate constant of hydroxyl radical scavenging seems to increase with the number of hydroxyethyl groups present, in accordance with Haenen et al (1993).

Surprisingly, in general the hydroxyethyl groups were more effective than free hydroxyl groups in the scavenging activity determined by our Fenton assay. However, the substitution of the hydroxyl on position 5 (tetraHER vs triHER) did not increase the scavenging ability.

For the quercetin derivatives, only the k_S for tetra- and penta-substituted compounds has been determined. For the other compounds, the A_0/A plot was non-linear, making it impossible to determine a constant rate and suggesting that the compounds may interfere with the hydroxyl radical production system. The plot of A_0/A is linear only if the rate of hydroxyl radical production is independent of the presence or absence of the scavenger (S) (i.e., if $V^S_{\cdot OH} = V_{\cdot OH}$). A difference between $V^S_{\cdot OH}$ and $V_{\cdot OH}$ may be caused by the flavonoids interfering with the radical production system, probably either by iron ion chelation or by stimulating radical production. The degradation of deoxyribose for different concentrations of flavonoids was indeed higher than without flavonoids, suggesting that these compounds may interfere with hydroxyl radical generation by being pro-oxidant.

In fact, several flavonoids with a B-ring catechol moiety, such as quercetin, myricetin and baicalein, have been reported to oxidize at physiological pH and then to promote hydroxyl radical generation in the Fenton reaction (Hodnick et al 1988; Canada et al 1990; Hanasaki et al 1994; Miura et al 1998). So, the k_S values determined in Table 3 are apparent rates resulting from two opposite phenomena: hydroxyl radical scavenging and radical production.

Increasing the number of hydroxyethyl groups seems to favour scavenging activity and to decrease the possibility of pro-oxidant behaviour. So the most potent compounds are the poly-alkylated ones, precisely because in these cases, k_S values reflect just the scavenging phenomenon.

The scavenging ability of rutin, triHER and tetraHEQ, compared with the radical generation activity of quercetin and triHEQ, indicate that the free hydroxyl at position 3 is implicated in the pro-oxidant mechanism whether the catechol group is free or substituted.

Haenen et al (1993) have shown that, in the Fenton assay, the order of hydroxyl radical scavenging activity of HER was reversed by omitting EDTA. These authors

Table 3 Rate constants for the reaction of hydroxyl radicals with tested compounds.

Compounds	Rate constant k_s (M^{-1} s^{-1})		
	Our results	Haenen et al (1993)	Rekka & Kourounakis (1991)
Mannitol	$1.88 \pm 0.18 (\times 10^9)$	$2.8 \pm 1.7 \times 10^9$	
Group I			
Rutin	$4.1 \pm 0.4 (\times 10^9)$		
MonoHER	$4.5 \pm 0.2 (\times 10^9)$	$3.9 \pm 2.2 \times 10^9$	$13.6 \pm 1.1 \times 10^{10}$
DiHER	UR*	$8.2 \pm 3.5 \times 10^9$	$2 \pm 1 \times 10^{10}$
TriHER	$8.0 \pm 0.1 (\times 10^9)$	$10.2 \pm 2.9 \times 10^9$	$2.1 \pm 1.1 \times 10^{10}$
TetraHER	$8.0 \pm 0.2 (\times 10^9)$	$11.1 \pm 2.2 \times 10^9$	$1.8 \pm 0.7 \times 10^{10}$
Group II			
Quercetin	UR		
MonoHEQ	UR		
DiHEQ	UR		
TriHEQ	UR		
TetraHEQ	$0.9 \pm 0.1 (\times 10^9)$		
PentaHEQ	$1.3 \pm 0.3 (\times 10^9)$		
Group III			
MonoMeR	$1.1 \pm 0.5 (\times 10^9)$		
MonoMeQ	UR		
Group IV			
PentaMEQ	$1.2 \pm 0.2 (\times 10^9)$		
PentaEthQ	UR*		
Group V			
Luteolin	$1.54 \pm 0.14 (\times 10^9)$		
Group VI			
Taxifolin	$2.3 \pm 0.2 (\times 10^9)$		
Leucocyanidol	$3.87 \pm 1.10 (\times 10^9)$		
Eriodictyol	$3.06 \pm 0.14 (\times 10^9)$		

k_s values are expressed as the mean values±s.e.m. of three separate experiments (each experiment was carried out in triplicate). UR, unexploitable results, UR*, unexploitable results due to water solubility problems.

explained that this change could be due to a difference in iron chelating activity. However, another explanation could be proposed. Earlier reports (Laughton et al 1989; Puppo 1992) indicate that the stimulatory effect of flavonoids on free radical production occurred in the presence of Fe–EDTA complex, but not with iron alone, or with ADP or citrate complexes. So, the generation of hydroxyl radicals depends on the type of chelator and the concentration of the complex (Kachur et al 1998).

It has been suggested that iron complexation with EDTA may alter the redox potential of iron ions and so facilitate the oxidation of flavonoids. The hydroxyl scavenging rate constant would then appear lower than it should be, whereas without EDTA, no pro-oxidant activity would occur and free radical scavenging activity would be favoured.

Hydroxyl radical production

To elucidate the mechanism of quercetin pro-oxidant activity that gives the alkyl derivatives of quercetin their pro-oxidant activity, the hydroxyl radical production in our deoxyribose assay (pH 7.4 phosphate buffer solution) was measured without any radical generating system, but with 300 U of SOD or 1000 U of catalase.

The hydroxyl radical generating activity of monoHEQ and triHEQ was shown to be strongly concentration dependent (Figure 1). The addition of 300 U of SOD or 1000 U of catalase (data not shown) reduced radical formation by monoHEQ and diHEQ, but had no influence on triHEQ.

These results suggest that superoxide radicals and hydrogen peroxide are involved in the pro-oxidant activity of monoHEQ, but not in that of triHEQ.

Several studies (Hodnick et al 1988; Canada et al 1990; Miura et al 1998) have shown that some flavonoids, possessing a pyrogallol or catechol moiety, were able to enhance hydroxyl radical generation (inhibited by catalase and SOD), by producing superoxide radicals and hydrogen peroxide during their auto-oxidation. Based on the auto-oxidation mechanism proposed for quercetin by different authors (Miura et al 1998; Jovanovic et al 1998), we can propose a similar mechanism for mono-alkylated derivatives of quercetin (Figure 2). Figure 2 shows clearly the involvement of superoxide in hydrogen peroxide production. Hydrogen peroxide can then generate hydroxyl radicals by iron traces present in the buffer solution.

The pro-oxidant activity of diHEQ is less marked than that of monoHEQ, probably because of its single B-ring

hydroxyl. However, the involvement of superoxide radicals and hydrogen peroxide suggests that its hydroxyl radical production involves a mechanism similar to that described for monoHEQ.

For triHEQ, as neither superoxide radicals nor hydrogen peroxide are implicated in the pro-oxidant activity, a quite different mechanism must be in place. Since the catechol moiety is substituted, the mechanism may involve the C3 hydroxyl and the 4-oxo function. For such compounds, no pro-oxidant mechanism has as yet been proposed in the literature. Nevertheless, the reactivity of the hydroxyl at the C3 position has previously been pointed out by several different authors (El-Sukkary & Speier 1981; Brown et al 1982; Takahama 1987) as an important element in the oxygenation of 3-hydroxyflavones.

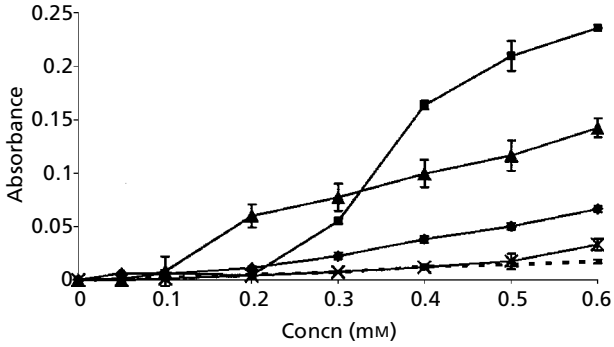


Figure 1 Degradation of deoxyribose by quercetin and its hydroxyethyl derivatives in the phosphate buffer, pH 7.4. ■, MonoHEQ; ▲, triHEQ; ◆, diHEQ; ×, quercetin; ---, pentaHEQ.

Hydroxyflavones, such as quercetin, may be oxidatively decarbonylated to the corresponding phenylglyoxylic acids, using the enzyme quercetinase. This can also be achieved in-vitro by treatment with a strong base in oxygenated aprotic solvents, or by the action of superoxide radicals generated by electrochemical reduction of oxygen. Brown et al (1982) have suggested that this oxygenation could involve the concerted addition of oxygen to the enol system, with the formation of an oxohydroperoxy intermediate. A mechanism can be proposed based on the supposition that the production of hydroxyl radicals from triHEQ is a consequence of oxygenation by the addition of oxygen reactive species, involving an oxohydroperoxy intermediate and transition metals (contamination from the substrate or from the buffer) (Figure 3).

Despite these explanations, the structural requirements for pro-oxidant activity can not be proposed. The activity complies with the redox properties of this series of compounds. Surprisingly, quercetin generates as many radicals as pentaEthQ, but clearly less than monoHEQ. This is perhaps because monoHEQ is a more powerful superoxide scavenger, leading to an enhanced production of hydroxyl radicals (by the mechanism describe in Figure 2).

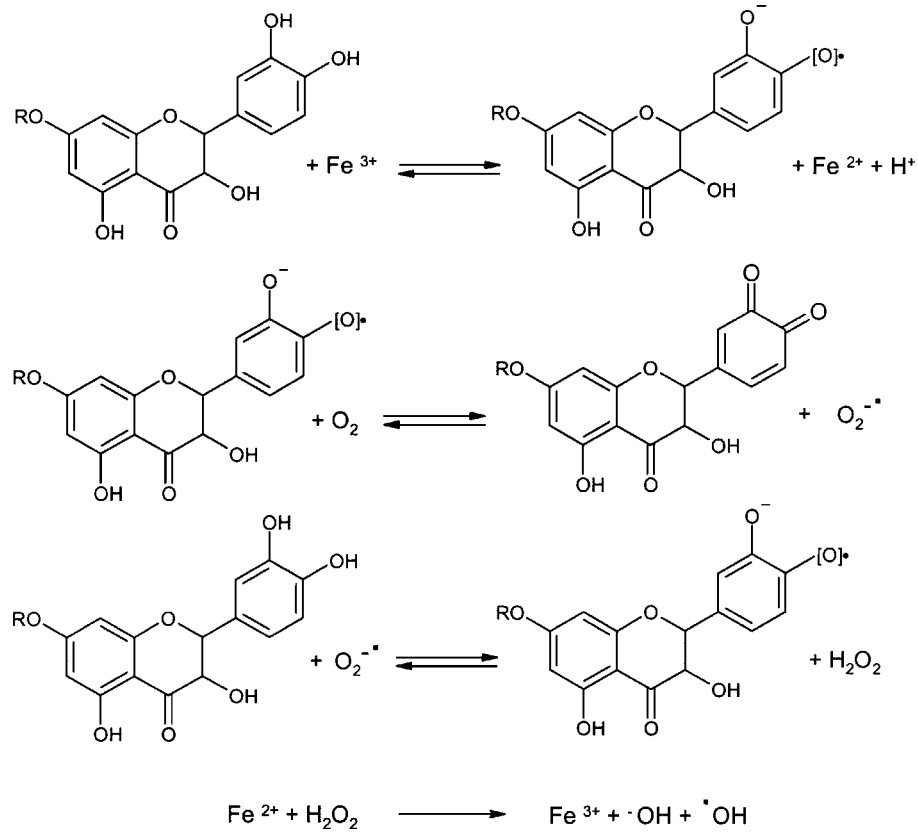


Figure 2 Postulated mechanism for auto-oxidation of monoHEQ.

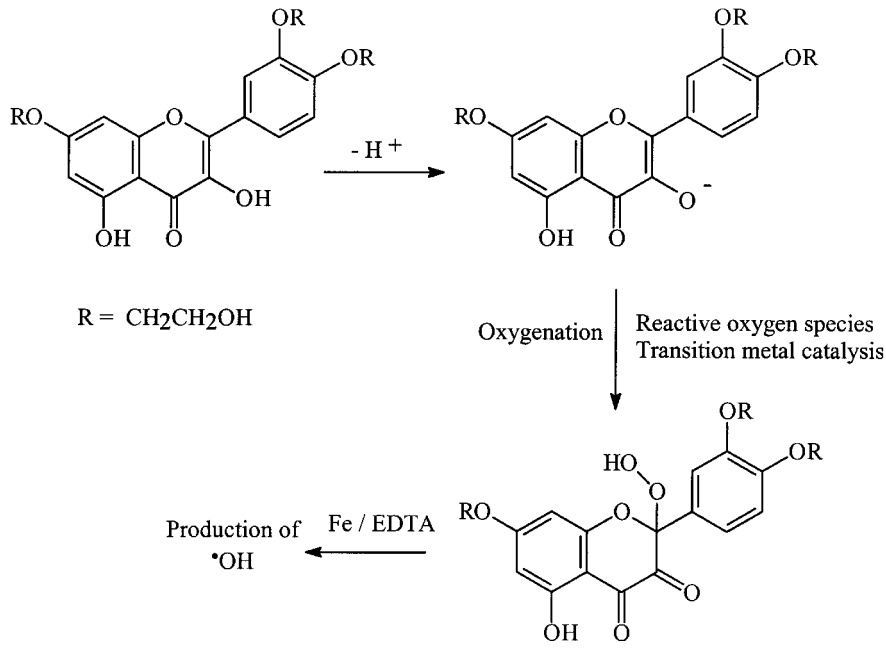


Figure 3 Postulated mechanism for hydroxyl radical production by triHEQ.

As mentioned by Canada et al (1990), the B ortho-dihydroxy configuration is not in itself a predictor of oxidation potential, nor is it a predictor of radical generation properties. Rather than simply the structure, we should take into account the stability of the phenoxyl radical formed after scavenging. Here the C2–C3 double bond and the hydroxyl on position 3 are of importance, giving the flavonoids the ability to propagate chain reactions.

The auto-oxidation of flavonoids at physiological pH seems to be the key process in their in-vivo toxicity, not only by promoting free radical production, but also by their possible conversion to electrophilic pro-oxidant via quinone/quinone methides formation (Boersma et al 2000; Awad et al 2000, 2001). Consequently, flavonoids should not be considered systematically as antioxidants, even if under in-vivo conditions, hydroxyl radicals generation by Fenton reaction seems of minor importance (Saran et al 2000).

Inhibition of lipid peroxidation

Based on their IC50 values (Table 4), the most active compounds are those having free hydroxyl in the B-ring (except diHEQ and triHEQ). This highlights, as has been previously described by other authors (Van Acker et al 1998; Van den Berg et al 2000), the high reactivity of the C3 hydroxyl when the catechol group is substituted.

The blockage of one or two of the B-ring hydroxyls of the compounds of groups I and II strongly reduces their scavenging activity.

For group V and VI compounds, it appears that the presence of the C2–C3 double bond is not essential for compounds lacking the hydroxyl at position C3 (luteolin vs eriodictyol). However, when this hydroxyl is present (quercetin vs taxifolin), the saturation of the double bond causes a reduction in scavenging activity. In other words, the presence of the C3 hydroxyl enhances the scavenging activity when the C2–C3 bond is unsaturated, but has no influence when the bond is saturated.

Substitution of the hydroxyl at the C3 position by a glycosyl group (rutin vs quercetin) or by a hydroxyethyl group (triHEQ vs tetraHEQ) caused a decrease in peroxyl radical scavenging ability.

For mono-alkylated rutin and quercetin derivatives, the C7 hydroxyl is not essential for activity and can be substituted by different alkyl groups confirming previous observations (van Acker et al 2000). Methyl and hydroxyethyl groups give the same activity, but an ethyl group seems to enhance peroxyl scavenging. This may be because the compound is thus more lipophilic and can penetrate deeper in the membrane layer to scavenge radicals.

Conclusion

Many studies have been conducted to elucidate the structure–antioxidant activity relationship for natural flavonoids. Despite some contradictory results, a general agreement has been reach on structural requirements for free radical scavenging.

The results obtained in this study on semi-synthetic flavonoids are in agreement with previous findings.

Luteolin and quercetin possess all the structural requirements for potent antioxidant activity. Furthermore, we have shown that the C7 hydroxyl is not essential for superoxide and peroxyl radical scavenging and that it can be substituted by lipophilic alkyl groups, which indeed actually enhance quercetin's scavenging activity.

However, this study highlighted the pro-oxidant behaviour of some flavonoids, in a Fenton reaction system,

Table 4 IC50 values for ABAB-induced lipid peroxidation.

Compounds	IC50 (μM)				
	Our results	Rekka & Kourounakis (1991)	Van Acker et al (1998)	Van den Berg et al (2000)	Robak & Gryglewski (1988)
Trolox C	21.10±0.10				
Group I					
Rutin	33±3		12.88		71.9±0.5
MonoHER	27.7±0.2	32	12.58	15.0±1.8	
MonoEthR	15.7±0.7				
DiHER	870±50	700	316.22	210±12	
TriHER	>1000	>1000		290±24	123.2±0.9
TetraHER	>1000	>1000		6200±200	
Group II					
Quercetin	14±2	17	7.58		9.4±0.6
MonoHEQ	13±2				
DiHEQ	34.5±0.6				
TriHEQ	42.3±0.9	26		13.0±1.4	
TetraHEQ	>1000				
PentaHEQ	>1000				
Group III					
MonoMeR	25±4				
MonoMeQ	13±2				
Group IV					
PentaMeQ	>1000				
PentaEthQ	>1000				
Group V					
Luteolin	11±1		11.78		
Group VI					
Taxifolin	30±1		13.80		
Leucocyanidol	23±1				
Eriodictyol	16.53±0.06				

IC50 values are expressed as the mean values±s.e.m. of three separate experiments (each experiment was carried out in triplicate).

involving superoxide radicals or hydrogen peroxide. This is probably a consequence of auto-oxidation catalysed by the iron–EDTA complex, through superoxide radical and hydrogen peroxide production.

Although the structural features conferring pro-oxidant activity are not quite clear, it is evident that, to avoid pro-oxidant activity, the 3 OH should be blocked to prevent its auto-oxidation and to prevent the possible formation of reactive alkylating product.

While the rutin derivatives (HER) were at first prepared simply to enhance the water solubility of rutin, this study has shown that the choice of the C7 and C3 positions for substitution is an excellent one, giving potent compounds. Such flavonoids are more lipophilic, allowing both a maximum peroxyl scavenging activity and an easy uptake into the membrane.

This study highlighted the fact that flavonoids cannot be considered solely as anti-oxidants. They can act as both antioxidant and pro-oxidant, depending on their concentration and on reaction conditions. Moreover, as the toxic effects of some flavonoids are thought to be the result of their pro-oxidant behaviour, careful examination of their antioxidant activity should be undertaken in different reaction conditions before planning any therapeutic use, even though other antioxidants (vitamin E and vitamin C) have shown pro-oxidant properties in-vitro and undoubtedly have favourable biological activity.

References

Awad, H. M., Boersma, M. G., Vervoort, J., Rietjens, I. M. C. M. (2000) Peroxidase-catalyzed formation of quercetin quinone methide-glutathioneadducts. *Arch. Biochem. Biophys.* **378**: 224–233

Awad, H. M., Boersma, M. G., Boeren, S., van Bladeren, P. J., Vervoort, J., Rietjens, I. M. C. M. (2001) Structure-activity study on the quinone/quinone methide chemistry of flavonoids. *Chem. Res. Toxicol.* **14**: 398–408

Boersma, M. G., Vervoort, J., Szymusiak, H., Lemanska, K., Tyrakowska, B., Cenas, N., Segura-Aguilar, J., Rietjens, I. (2000) Regioselectivity and reversibility of glutathione conjugation of quercetin quinone methide. *Chem. Res. Toxicol.* **13**: 185–191

Bors, W., Heller, W., Michel, C., Saran, M. (1990) Flavonoids as antioxidants: determination of radical-scavenging efficiencies. In:

Packer, L., Glazer, A. N. (eds) *Methods in enzymology.* Academic Press, San Diego, pp 343–355

Bradford, M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **72**: 248–254

Brown, S. B., Rajananda, V., Holroyd, J. A., Evans, E. G. V. (1982) A study of the mechanism of quercetin oxygenation by ^{18}O labelling. *Biochem. J.* **205**: 239–244

Buxton, G. V., Greenstock, C. L., Helman, W. P., Ross, A. B. (1988) Critical review of rate constants for reactions of hydrated electrons hydrogen atoms and hydroxyl radicals in aqueous solution. *J. Phys. Chem. Ref. Data* **17**: 513–904

Canada, A. T., Giannella, E., Nguyen, T. D., Mason, R. P. (1990) The production of reactive oxygen species by dietary flavonols. *Free Radic. Biol. Med.* **9**: 441–449

Cao, G., Sofic, E., Prior, R. (1997) Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships. *Free Radic. Biol. Med.* **22**: 749–760

Chang, W., Lee, Y., Lu, F., Chieng, H. (1993) Inhibitory effects of flavonoids on xanthine oxydase. *Anticancer Res.* **13**: 2165–2170

Cos, P., Ying, L., Calomme, M., Hu, J. P., Cimanga, K., Van Poel, B., Pieters, L., Vlietinck, A. J., Vanden Berghe, D. (1998) Structure-activity relationship and classification of flavonoids as inhibitors of xanthine oxidase and superoxide scavengers. *J. Nat. Prod.* **61**: 71–76

Costantino, L., Rastelli, G., Albasini, A. (1992) Inhibitory activity of flavonols towards the xanthine oxidase enzyme. *Int. J. Pharmaceutics* **86**: 17–23

Cotelle, N., Bernier, J. L., Catteau, J. P., Pommery, J., Wallet, J. C., Gaydou, E. M. (1996) Antioxidant properties of hydroxy-flavones. *Free Radic. Biol. Med.* **20**: 35–43

Courbat, P., Albert, A. (1975) Patent: DE 2515084 C2

Courbat, P., Valenza, A. (1973) Contribution à l'étude de dérivés O-β-hydroxyéthylés de la quercétine. *Helv. Chim. Acta* **56**: 1604–1614

El-Sukkary, M. M. A., Speier, G. (1981) Oxygenation of 3-hydroxyflavones by superoxide anion. *J.C.S. Chem. Comm.*: 745

Goldstein, S., Czapski, G. (1984) Mannitol as an hydroxyl radical scavenger in aqueous solution and biological systems. *Int. J. Radiat. Biol.* **46**: 725–729

Haenen, G. R. M. M., Jansen, F. P., Bast, A. (1993) The antioxidant properties of five O-(β-hydroxyethyl)-rutosides of the flavonoid mixture Venoruton. *Phlebology* **S1**: 10–17

Halliwell, B., Gutteridge, J. M. C. (1999a) Reactive species as useful biomolecules. In: *Free radicals in biology and medicine.* Third edn, Oxford University Press Inc., Oxford, pp 430–484

Halliwell, B., Gutteridge, J. M. C. (1999b) Free radicals, other reactive species and disease. In: *Free radicals in biology and medicine.* Third edition, Oxford University Press Inc., Oxford, pp 617–783

Hanasaki, Y., Ogawa, S., Fukui, S. (1994) The correlation between active oxygens scavenging and antioxidative effects of flavonoids. *Free Radic. Biol. Med.* **16**: 845–850

Heitzer, T., Schlinzig, T., Krohn, K., Meinertz, T., Münzel, T. (2001) Endothelial dysfunction, oxidative stress, and risk of cardiovascular events in patients with coronary artery disease. *Circulation* **104**: 2638–2640

Hodnick, W. F., Kalyanaraman, B., Pritsos, C. A., Pardini, R. S. (1988) The production of hydroxyl and semiquinone free radicals during the autoxidation of redox active flavonoids. *Basic Life Sci.* **49**: 149–152

Hu, J. P., Calomme, M., Lasure, A., De Bruyne, T., Pieters, L., Vlietinck, A., Van den Berghe, D. A. (1995) Structure-activity relationship of flavonoids with superoxide scavenging activity. *Biol. Trace Elem. Res.* **47**: 327–331

Janssens, D., Michiels, C., Arnould, T., Remacle, J. (1996) Effects of hydroxyethylrutosides on hypoxia-induced activation of human endothelial cells *in vitro*. *Br. J. Pharmacol.* **118**: 599–604

Jovanovic, S. V., Steenken, S., Simic, M. G., Hara, Y. (1998) Antioxidant properties of flavonoids: reduction potentials and electron transfer reactions of flavonoid radicals. In: Rice-Evans, C. A., Packer, L. (eds) *Flavonoids in health and disease.* Marcel Dekker, New York, pp 137–163

Kachur, A. V., Tuttle, S. W., Biaglow, J. E. (1998) Autoxidation of ferrous ion complexes: a method for the generation of hydroxyl radicals. *Radiat. Res.* **150**: 475–482

Laughton, M. J., Halliwell, B., Evans, P. J., Hoult, J. R. S. (1989) Antioxidant and pro-oxidant actions of the plant phenolics quercetin gossypol and myricetin. *Biochem. Pharmacol.* **38**: 2859–2865

Michel, C. C., Kendall, S. (1993) β-Hydroxyethyl rutosides reverse the increased permeability induced in frog microvessels by protein-free perfusions. *Phlebology* (Suppl. 1): 18–21

Michiels, C., Arnould, T., Remacle, J. (1994) Rôle clé de l'hypoxie et des cellules endothéliales dans le développement des veines variqueuses. *Medecine et Science* **10**: 845–853

Miura, Y. H., Tomita, I., Watanabe, T., Hirayama, T., Fukui, S. (1998) Active oxygens generation by flavonoids. *Biol. Pharm. Bull.* **21**: 93–96

Mora, A., Payá, M., Rios, J. L., Alcaraz, M. J. (1990) Structure-activity relationships of polymethoxyflavones and other flavonoids as inhibitors of non-enzymic lipid peroxidation. *Biochem. Pharmacol.* **40**: 793–797

Picq, M., Prigent, A. F., Némoz, G., André, A. C., Pacheco, H. (1982) Penta-substituted quercetin analogues as selective inhibitors of particulate 3′-5′ cyclic AMP phosphodiesterase from rat brain. *J. Med. Chem.* **25**: 1192–1198

Puppo, A. (1992) Effect of flavonoids on hydroxyl radical formation by Fenton-type reactions; influence of the iron chelator. *Phytochemistry* **31**: 85–88

Rekka, E., Kourounakis, P. N. (1991) Effect of hydroxyethylrutosides and related compounds on lipid peroxidation and free radical scavenging activity. Some structural aspects. *J. Pharm. Pharmacol.* **43**: 486–491

Rice-Evans, C. A., Miller, N. J., Paganga, G. (1996) Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic. Biol. Med.* **20**: 933–956

Robak, J., Gryglewsky, R. J. (1988) Flavonoids are scavengers of superoxide anions. *Biochem. Pharmacol.* **37**: 837–841

Saran, M., Michel, C., Stettmaier, K., Bors, W. (2000) Arguments against the significance of the Fenton reaction contributing to signal pathways under *in vivo* conditions. *Free Rad. Res.* **33**: 567–579

Scheline, R. R. (1966) A rapid synthesis of 3Omethylgallic acid. *Acta Chem. Scand.* **20**: 1182

Sichel, G., Corsaro, C., Scalia, M., Di Bilio, A. J., Bonomo, R. P. (1991) *In vitro* scavenger activity of some flavonoids and melanins against O_2^-. *Free Radic. Biol. Med.* **11**: 1–8

Takahama, U. (1987) Oxidation products of kaempferol anion radical. *Plant Cell Physiol.* **28**: 953–957

Tang, J., Yuan, P. (1996) Preparation and structure elucidation of Troxerutin. *Zhongguo Yiyao Gongye Zazhi.* **27**: 291–292

Ubeaud, G., Schmitt, C., Jaeck, D., Lave, T., Coassolo, P. (1995) Bosentan a new endothelin receptor antagonist: prediction of systemic plasma clearance in man from combined *in vivo* and in vitro data. *Xenobiotica* **25**: 1381–1390

Van Acker, S. A. B. E., van den Berg, D., Tromp, M. N. J. L., Griffioen, D. H., van Bennekom, W. P., van der Vijgh, W. J. F., Bast, A. (1996) Structural aspects of antioxidant activity of flavonoids. *Free Radic. Biol. Med.* **20**: 331–342

Van Acker, S. A. B. E., Plemper van Balen, G., Van der Berg, D., Bast, A., Van der Vijgh, W. J. F. (1998) Influence of iron chelation on the antioxidant activity of flavonoids. *Biochem. Pharmacol.* **56**: 935–943

Van Acker, F. A. A., Hageman, J. A., Haenen, G. R. M. M., van der Vijgh, W. J. F., Bast, A., Menge, W. M. P. B. (2000) Synthesis of

novel 3,7-substituted-2-(3′,4′-dihydroxyphenyl)flavones with improved antioxidant activity. *J. Med. Chem.* **43**: 3752–3760

Van den Berg, R., Haenen, G. R. M. M., van den Berg, H., van der Vijgh, W., Bast, A. (2000) The predictive value of the antioxidant capacity of structurally related flavonoids using the Trolox equivalent antioxidant capacity (TEAC) assay. *Food Chem.* **70**: 391–395

Yuting, C., Rongliang, Z., Zhongjian, J., Yong, J. (1990) Flavonoids as superoxide scavengers and antioxidants. *Free Radic. Biol. Med.* **9**: 19–21

Zhao, M. J., Jung, L. (1995) Kinetics of the competitive degradation of deoxyribose and other molecules by hydroxyl radicals produced by the Fenton reaction in the presence of ascorbic acid. *Free Rad. Res.* **23**: 229–243