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Influence of Iron Chelation on the Antioxidant Activity of Flavonoids

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ABSTRACT. The antioxidant activity of flavonoids is believed to be caused by a combination of iron chelation and free radical scavenging activities. Several authors have attempted to separate the iron chelation and scavenging activity of flavonoids in order to study these processes individually. There are, however, several contradictions in the literature, and the outcome largely depends on the experimental conditions and the type of assay used. In order to investigate the contribution of iron chelation to the antioxidant activity of flavonoids, we determined the antioxidant activity of a group of flavonoids from several subclasses in an iron-independent (azobisamidinopropane, [ABAP]) lipid peroxidation (LPO) process and compared them with data from an iron-dependent (Fe^{2+}/ascorbate) LPO process, which we determined earlier. These LPO data were compared with oxidation potentials, which were earlier found to have a good correlation with the scavenging activity of flavonoids. For most flavonoids, it was found that there was no difference between the LPO assays, indicating that iron chelation is either a constant factor among the flavonoids or is not significant in these types of assays. The IC_{50} values in the iron-independent LPO assay showed an excellent correlation with the oxidation potentials (Ep/2). Therefore, it can be concluded that for the majority of flavonoids tested, iron chelation does not play a role in the antioxidant activity in microsomal lipid peroxidation. BIOCHEM PHARMACOL **56**;8: 935–943, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. flavonoid; antioxidant; iron chelation; lipid peroxidation

Flavonoids are naturally occurring compounds which are widely distributed in vegetables, fruits and beverages such as tea and red wine. They have an important influence on the colour and flavour of these foods and have a broad pharmacological activity [1, 2]. Their beneficial effects have been described for diabetis mellitus, allergy, cancer, viral infections, headache, stomach and duodenal ulcer, parodentosis and inflammations [1, 3–6]. The molecular mechanism(s) underlying these effects are not completely understood as a consequence of the wide range of pharmacodynamic properties and the chemical heterogeneity of the flavonoids (Fig. 1 and Table 1). It is known that they can bind to biologic polymers such as enzymes, hormone carriers and DNA, chelate transition metal ions such as Fe^{2+}, Cu^{2+}, Zn^{2+} and Mg^{2+}, catalyse electron transport and scavenge free radicals. The ability to chelate Fe^{2+} and scavenge free radicals renders the flavonoids very good antioxidants, and many of the pharmacological actions are explained by their action as antioxidants [1, 2].

Generally, it is assumed that the ability of flavonoids to chelate Fe^{2+} is very important for their antioxidant activity, because "site-specific scavenging" may occur [7]. This means that if the Fe^{2+} is still catalytically active, the radicals are formed in the vicinity of the flavonoid, which surrounds the Fe^{2+}, and can be scavenged immediately. In that case, the flavonoid would have a double, synergistic action, which would make it an extremely powerful antioxidant.

We suggested earlier that chelation is the minor factor in the antioxidant activity of potent flavonoid antioxidants, but that it is of major importance in the antioxidant activity of flavonoids, which are less active [8]. In the present investigation, we attempted to unravel the role of Fe^{2+} chelation in the antioxidant activity of flavonoids in LPO by comparing the inhibition of ABAP-induced LPO by the flavonoids with that of iron/ascorbate-induced LPO from an earlier investigation [8] and by comparing these LPO data with the Ep/2, which was earlier found to be a good parameter to describe the scavenging activity of flavonoids [8].

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§ *Abbreviations*. ABAP, azobisamidinopropane; BHT, butylhydroxytoluene; Ep/2, half peak oxidation potential; F, lipophilicity parameter; LPO, lipid peroxidation; SAR, structure-activity relationship; TBA, thiobarbituric acid; and TBARS, thiobarbituric acid reactive substances.

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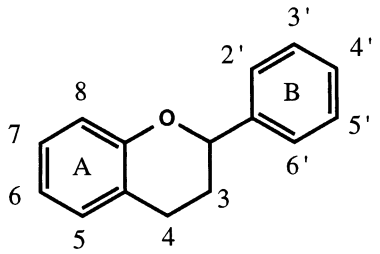
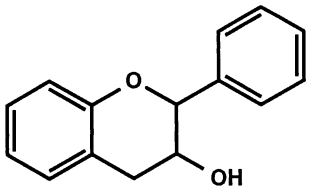


Flavonoid basic structure



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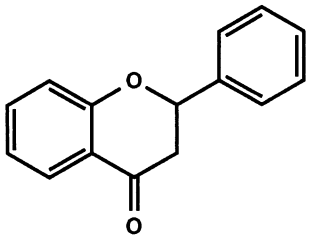
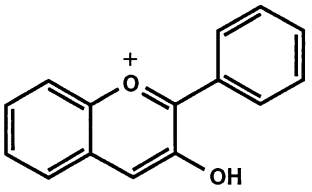


Flavanone



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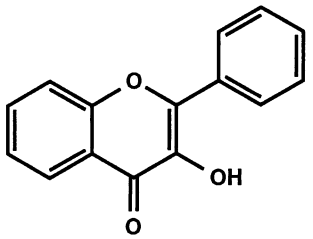
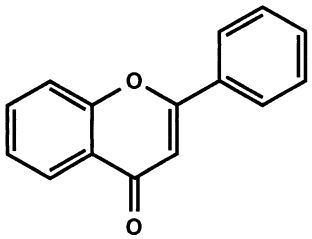
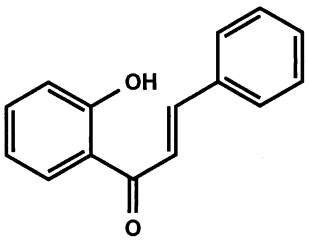


Flavon-3-ol



Flavone



Chalcone

FIG. 1. The subclasses of the flavonoid family.

MATERIALS AND METHODS

Chemicals

Hesperidin (97%), diosmin (95%), fisetin, naringin, phloridzin (99%), phloretin (98%) and galangin were obtained from Aldrich and hesperetin, naringenin (95%) and taxifolin were obtained from Sigma. Rutin was obtained from Merck and quercetin, myricetin (>97%), pelargonidin chloride, apigenin (98%) and kaempferol (96%) were purchased from Fluka. Cyanidin chloride and luteolin (90%) were purchased from Roth. The hydroxyethylrutosides, trihydroxyethyl quercetin and (+)-catechin (cyanidanol), were a generous gift from Novartis. ABAP was purchased from Polysciences. $FeSO_4$ and Na_2EDTA were obtained from Merck. All other chemicals were of the highest grade of purity available.

Iron Chelation Competition Experiments

To prevent the flavonoids from oxidising in the presence of the iron, oxygen was removed from the nanopure water by purging with nitrogen for at least 1.5 hr. The Tris/KCl (50 mM Tris, 150 mM KCl) and 50 mM Na/K phosphate incubation buffers, both pH 7.4, were continuously purged with nitrogen and thermostatted at 37° with a thermostat bath (Fisher Scientific Nederland). Stock solutions of monoHER and quercetin were prepared in 20% DMSO in

TABLE 1. Subclasses of naturally occurring flavonoids and some examples of each class

		Substituents					
Class	**Compound**	**3**	**5**	**7**	**3′**	**4′**	**5′**
Flavon(ol)es	Myricetin	OH	OH	OH	OH	OH	OH
	Quercetin	OH	OH	OH	OH	OH	H
	Fisetin	OH	H	OH	OH	OH	H
	Rutin	ORu	OH	OH	OH	OH	H
	Kaempferol	OH	OH	OH	H	OH	H
	Galangin	OH	OH	OH	H	H	H
	MonoHER	ORu	OH	OEtOH	OH	OH	H
	DiHER	ORu	OH	OEtOH	OH	OEtOH	H
	TriHER	ORu	OH	OEtOH	OEtOH	OEtOH	H
	TriHEQ	OH	OH	OEtOH	OEtOH	OEtOH	H
	TetraHER	ORu	OEtOH	OEtOH	OEtOH	OEtOH	H
Flavanon(ol)es	Naringenin	H	OH	OH	H	OH	H
	Naringin	H	OH	ORu	H	OH	H
	Hesperetin	H	OH	OH	OH	OMe	H
	Hesperidin	H	OH	ORu	OH	OMe	H
	Taxifolin	OH	OH	OH	OH	OH	H
Flavones	Apigenin	H	OH	OH	H	OH	H
	Diosmin	H	OH	ORu	OH	OMe	H
	Luteolin	H	OH	OH	OH	OH	H
Flavanoles	(+)-Catechin	OH	OH	OH	OH	OH	H
Chalcones	Phloretin	OH(2)	OH(4)	OH(6)	H	H	OH(6′)
	Phloridzin	OGl(2)	H(4)	OH(6)	H	H	OH(6′)
Antho-cyanidins	Cyanidin	OH	OH	OH	OH	OH	H
	Pelargonidin	OH	OH	OH	OH	H	H

Ru: rutinose (=Glu-Rha).

nanopure water (both "oxygen-free") in a concentration of 100 μM. The 2 mM Fe^{2+} solution was freshly prepared in oxygen-free nanopure water, just before use.

The sequence in which Fe^{2+}, EDTA, and the flavonoids were added was varied. The final concentrations were 10 μM, 10 μM and 30 μM, respectively, because the stoichiometry of the flavonoid-Fe^{2+} complex is thought to be about 3:1, although it is likely that a number of complexes with other stoichiometries are also formed.

The absorption spectra of flavonoid, flavonoid + Fe^{2+}, and flavonoid + Fe^{2+} + EDTA (added in different sequences) in both incubation buffers were compared with respect to the extent of iron chelation over 15.5 hr. The incubation buffers were used as a blank. The spectra were recorded from 250 to 700 nm on a Philips PU 8720 UV/Vis Scanning Spectrophotometer at 37°. The cuvettes were closed with a cap during measurements.

Preparation of Microsomes

Male Balb/c mice were sacrificed by decapitation. The livers were excised immediately and stored at −80° for later use. They were thawed at room temperature and homogenised in ice-cold phosphate buffer (50 mM, pH 7.4, 10 mL/g of tissue). The homogenate was centrifuged at 10,000 *g* (20 min at 4°). Subsequently, the supernatant was centrifuged at 10,000 *g* (20 min) and again at 105,000 *g* (60 min). The microsomal pellet was resuspended in phosphate buffer (0.5 g of liver/mL) and the last step was repeated. Finally the microsomes were stored at −80°. Before use, the microsomes were thawed and washed in ice-cold Tris/KCl buffer (50/150 mM, pH 7.4 at 37°) by centrifugation at 105,000 *g* (50 min). Finally, the pellet was heated at 100° for 90 sec to remove all enzymatic factors and resuspended in ice-cold Tris buffer just before use. The final protein concentration during the incubations was 2.1 mg/mL for ABAP-induced LPO.

ABAP-Induced LPO

Stock solutions of the flavonoids were freshly prepared in DMSO and nanopure water (1:1, both oxygen-free) just before use. The maximum DMSO concentration in the final incubation mixture was 2.5%, which was found to have no influence on the assay. Before adding the flavonoids, the microsomal suspension was incubated with Na_2EDTA for 5 min. The reaction was started by transferring the incubates to the water bath and adding freshly prepared ABAP (a hydrophilic azo-initiator, which generates a constant radical flow in the water phase of the LPO system) in ice-cold nanopure water (final concentration ABAP 10 mM, Na_2EDTA 100 μM). The mixture was incubated at 37° for 1.5 hr. LPO was assayed by measuring TBARS according to Haenen *et al.* [7]. In short, the following procedure was followed. At regular intervals during the incubation, an 0.3-mL aliquot of the incubation mixture was mixed with 2 mL of ice-cold TBA-trichloroacetic acid-HCl-BHT solution to stop the reaction. The

TBA-trichloroacetic acid-HCl-butylhydroxytoluene solution was prepared by dissolving 41.6 mg of TBA/10 mL of trichloroacetic acid (16.8% w/v in 0.125 N HCl). To 10 mL of TBA-trichloroacetic acid-HCl, 1 mL of BHT (1.5 mg/mL of ethanol) was added. After heating (15 min, 80°) and centrifugation (15 min), the absorbance at 540 nm was determined by use of an Argus 400 microplate reader (Packard). None of the flavonoids tested interfered with the TBA reaction. Only for the absorbance of the anthocyanidins was a correction at 540 nm made.

Determination of IC_{50} Values

The IC_{50} of both LPO assays was determined by measuring the inhibition obtained by several flavonoid concentrations and calculating the flavonoid concentration at which 50% inhibition was obtained by interpolation on a straight line fitted through the logarithm of the concentrations above and below 50% inhibition (between 10 and 90%). Each line was determined by at least two concentrations in triplicate.

Calculation of Parameters

The log *P* value is an experimental lipophilicity parameter, which is defined by the octanol-water partition coefficient. The *F* value is a theoretical parameter which has been defined by Rekker *et al.* to express the relative contribution of the substituents to the overall lipophilicity of the molecule [9]. The *F* values for all substituents are added to give one overall *F* value for the molecule (a calculated "log *P*"), which gives an indication as to lipophilicity of the flavonoid. The *F* values were calculated relative to the basic structure of the flavone according to Rekker *et al.* [9]. Each substituent has an *F* value, which also depends on the surroundings of the substituent, e.g. whether it is attached to an aliphatic or an aromatic system. This method of calculation only works within a series of structures with the same basic structure. Therefore, we only calculated the *F* values for the flavone/flavonol series.

RESULTS

Iron Chelation Competition Experiments

The aim of our study was to evaluate the role of iron-dependent processes in the antioxidant effect of flavonoids. To this end, the antioxidant potency of flavonoids was determined in an assay where Fe^{2+} does not play a role, i.e. ABAP-induced LPO. To ensure that Fe^{2+} was not involved, EDTA was added.

It was investigated whether flavonoid-iron complex formation was indeed prevented by EDTA, i.e. under the experimental conditions in the LPO assay. In phosphate buffer, quercetin showed two characteristic absorbance peaks at 270 and 380 nm. The presence of Fe^{2+} caused a shift of the last peak to 413 nm and an increase in absorbance from 400 to 700 nm, which is probably due to the formation of quercetin-iron complexes. When the iron was added as an iron-EDTA complex, the rise in absorbance was much smaller, which indicates that quercetin is able to compete with EDTA for the iron. However, some spectral changes were observed several minutes after the solution was mixed. These spectral changes were characterised by a decrease in absorbance at 380 nm and an increase in absorbance from 400–700 nm, which suggests oxidation of quercetin in the presence of Fe^{2+}-EDTA. Fe^{2+}-EDTA has no measurable absorbance in the UV/Vis spectrum.

The characteristic absorbance peaks of monoHER were at 260 and 358 nm, in both phosphate and Tris buffer. The presence of Fe^{2+} caused a shift of the two peaks, to 266 and 377 and to 274 and 414 nm, in the phosphate and Tris buffers, respectively. From Fig. 2, it appears that more monoHER-iron complexes were formed in Tris buffer. This difference between the two buffers could be explained by competition between monoHER and phosphate to chelate iron. When the iron was added as an iron-EDTA complex, the increase in absorbance was much smaller in both buffers. This indicates that EDTA is able to compete with monoHER for the iron.

Figure 2A shows the spectra of monoHER, Fe^{2+} and EDTA added to Tris buffer in different sequences. The spectra appeared to be dependent on the sequence with which the compounds were added. Immediately after the addition of the last compound, it was observed that: 1) when Fe^{2+} was added last, mainly monoHER-Fe^{2+} complex was found; 2) monoHER and monoHER-Fe^{2+} complexes were observed when EDTA was added last; and 3) when monoHER was added last, it seemed incapable of binding the Fe^{2+} immediately and thus mainly free monoHER was found.

After 1.5 hr (the time used in the LPO experiments), the shift in the spectra indicated that EDTA abstracts Fe^{2+} from the monoHER-Fe^{2+} complex (Fig. 2B). After 15.5 hr, an equilibrium was reached in which only a small amount of iron was still chelated by monoHER, most of the iron being bound to EDTA.

ABAP-induced LPO

EDTA was added to the assay, not only to prevent iron-flavonoid complex formation but also to ensure that the ABAP-induced LPO was independent of iron. Addition of EDTA also reduced the TBARS after 1.5-hr incubation in the spontaneous LPO, but a large amount of TBARS was still formed with ABAP as initiator. Therefore, it can be concluded that iron does not play an essential role in ABAP-induced LPO.

The anthocyanidins could not be tested due to their absorbance at 540 nm, which interferes with the assay. The IC_{50} values calculated for the scavenging activity of the other flavonoids with the ABAP-induced LPO test as well as with the Fe^{2+}/ascorbate-induced LPO test (from [8]) are listed in Table 2.

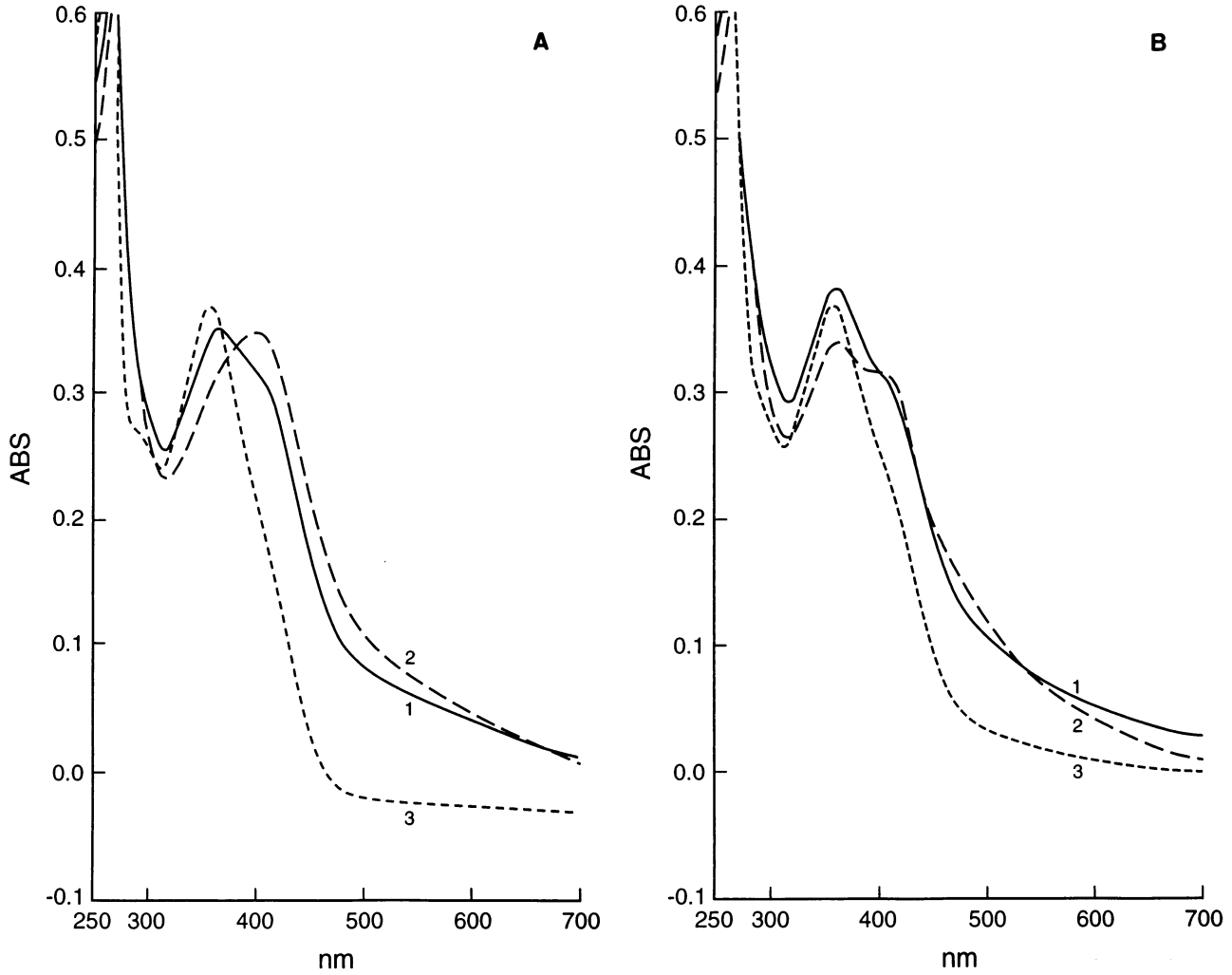


FIG. 2. Competition between 10 μM EDTA and 30 μM monoHER for 10 μM Fe^{2+} just after mixing the solution (A) and after 1.5 hr (B). Incubations with Fe^{2+} (1), EDTA (2) or monoHER (3) as final addition.

Overall, it appears that the differences between the iron-dependent and iron-independent LPO were very small, although sometimes statistically significant. Only in some cases (e.g. apigenin, hesperidin, and tetraHER) were there large differences between assays, indicating differences in mechanism.

In the flavonol series, triHER, rutin, myricetin, galangin and kaempferol were less active antioxidants in the ABAP-induced LPO assay, while triHQ and tetraHER were more active. Within this subclass, there was a large variation in IC_{50} (from 7.6 μM for quercetin to 3891 μM for tetraHER).

From the subclass of the flavanones, hesperetin (IC_{50} of 86.7 μM) was the best antioxidant. Its corresponding glycoside hesperidin showed a large decrease in IC_{50} (297.6 μM ABAP versus >3000 μM Fe^{2+}/asc).

From the flavones, luteolin was the most active antioxidant with an IC_{50} of 11.8 μM, which was in the range of the most powerful flavonoid antioxidants, the flavonols. Both apigenin and diosmin were inactive (IC_{50} > 3000 μM).

The only flavanol tested was catechin, which had an IC_{50} of 7.3 μM and was as active in the ABAP assay as in the Fe^{2+}/asc assay.

Finally, the chalcones phloretin and phloridzin were tested. Phloridzin was as active as in the Fe^{2+}/asc-induced LPO and phloretin seemed to be less active. Nevertheless, phloretin remained more potent than phloridzin.

Correlations

Correlations between the LPO-inhibiting capacity in the ABAP/EDTA assay with the LPO-inhibiting capacity in the Fe^{2+}/asc assay, the Ep/2 and *F* (Table 2) were examined. The results of these correlations are shown in Table 3. The pIC_{50} values of the Fe^{2+}/asc assay and the ABAP-induced LPO were well correlated ($r = 0.893$, Fig. 3). The correlation of the pIC_{50} values of ABAP-induced LPO with the Ep/2 values was even better, with an r of 0.912 (Fig. 4). However, from this equation triHER and apigenin appeared not to fit the equation. Upon removal of the values of

TABLE 2. The scavenging activity of the flavonoids

Flavonoid	pIC_{50}* (Fe^{2+}/Asc)†	pIC_{50} (ABAP)	p‡	Ep/2 (V)	F§
Myricetin	5.05 ± 0.09	4.91 ± 0.03	0.047	−0.03	−1.884
Quercetin	5.09 ± 0.09	5.12 ± 0.11	0.633	0.03	−1.570
Fisetin	4.74 ± 0.05¶	4.91 ± 0.07	0.056	0.12	−1.256
Rutin	5.02 ± 0.06	4.89 ± 0.05	0.030	0.18	−11.866
Kaempferol	5.18 ± 0.11	4.84 ± 0.12	0.011	0.12	−1.256
Galangin	5.13 ± 0.08¶	4.72 ± 0.10	0.007	0.32	−0.942
MonoHER	4.92 ± 0.06	4.90 ± 0.12	0.984	0.19	−16.091
DiHER	3.91 ± 0.39	3.50 ± 0.16	0.170	0.48	−17.804
TriHER	3.44 ± 0.04	2.19 ± 0.14	0.000	0.77	−19.517
TriHQ	4.09 ± 0.02	4.37 ± 0.05	0.000	0.55	−6.709
TetraHER	‖	2.41 ± 0.51	0.000	¶	−21.230
Naringenin	3.04 ± 0.57	3.31 ± 0.08	0.978	0.60	NC
Naringin	‖	‖		¶	NC
Hesperetin	3.95 ± 0.07	4.06 ± 0.07	0.133	0.40	NC
Hesperidin	‖	3.53 ± 0.05	0.000	0.44	NC
Taxifolin	4.60 ± 0.16#	4.86 ± 0.03	0.057	0.15	NC
Apigenin	2.76 ± 0.24	‖	0.000	¶	−0.942
Diosmin	‖	‖		¶	−12.232
Luteolin	4.81 ± 0.13	4.93 ± 0.01	0.315	0.18	−1.256
(+)-Catechin	5.42 ± 0.33	5.14 ± 0.13	0.092	0.16	NC
Phloretin	4.52**	4.38 ± 0.06		0.54	NC
Phloridzin	3.21 ± 0.13	3.30 ± 0.06	0.332	0.54	NC

*Negative logarithm of the IC_{50} (mean ± SD).
†Data from [8], 10 μM FeSO4, 50 μM ascorbate in boiled mouse liver microsomes, TBARS measured after 1 hr.
‡Significance of the difference between both LPO assays.
§Lipophilicity parameter, calculated according to Rekker.
‖IC_{50} > 3 mM.
¶Ep/2 > 1 V.
**Tested twice.
††Tested once.
NC: not calculated.

triHER from Eqn 1 (apigenin was not included in the first correlation, because it did not have an IC_{50} below 3 mM in ABAP-induced LPO), the constant lost its significance and had to be removed, as is shown in Eqn 5 of Table 3. The correlation with the *F* values is more a trend than a correlation (Fig. 5).

DISCUSSION

Flavonoids have gained interest because of their broad pharmacological activity. Putative therapeutic effects of many traditional medicines may be ascribed to the presence of flavonoids [3]. The pharmacological effect of flavonoids is due to their inhibition of certain enzymes and their antioxidant activity (for a review see [1] and [2]). Numerous authors have investigated the antioxidant activity of flavonoids, and several attempts have been made to elucidate the structure-activity relationships. Although there is general agreement that flavonoids possess both excellent iron-chelating and radical-scavenging properties [1, 7], there is much discussion and contradiction regarding their relative contribution and the effect of SAR on the antioxidant activity. The investigation of the SARs is hampered by a low solubility of the flavonoids in most assays and thus solvents such as DMSO and ethanol, which possess good radical-scavenging activities in themselves, are needed [10]. LPO is usually induced by a transition metal alone [11] or in combination with a reducing agent such as ascorbate [12]. In these assays, flavonoids can interfere not only with the propagation reactions of the free radical, but also with

TABLE 3. Correlations (pIC_{50} = AX + B)

pIC_{50}	X	A	p	B	p	r
1. Fe^{2+}/asc	pIC_{50}(ABAP)	0.79 ± 0.10	0.000	1.01 ± 0.46	0.044	0.893
2. ABAP	Ep/2	3.45 ± 0.40	0.000	5.37 ± 0.15	0.000	0.912
3. ABAP	F	0.09 ± 0.02	0.002	5.07 ± 0.27	0.000	0.786
4. ABAP	Ep/2	−3.66 ± 0.77	0.001	5.36 ± 0.17	0.000	
	F	0.001 ± 0.02	0.953			0.940
5. Fe^{2+}/asc*	pIC_{50}(ABAP)	1.01 ± 0.01	0.000			0.942

*After removal of triHER from the equation.

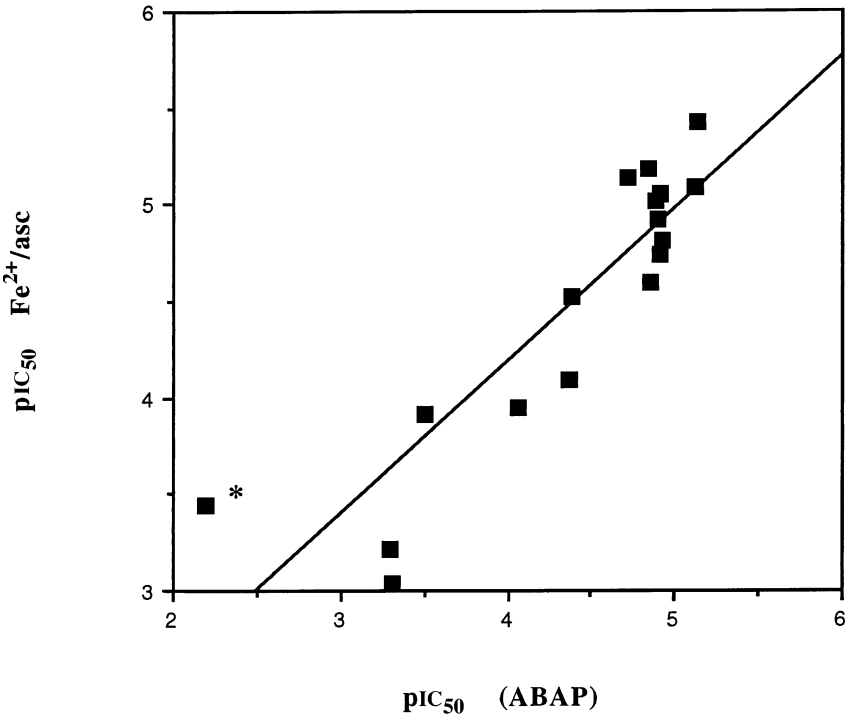


FIG. 3. Correlation of the IC_{50} values of Fe^{2+}/asc- and ABAP-induced LPO (r = 0.893). *Indicates the flavonoid triHER, for which iron chelation is suggested to play a role in the inhibition of LPO.

the formation of the radicals, by chelating the transition metal involved in the initiation reaction. Therefore, most LPO inhibition assays measure a combination of transition metal (usually iron) chelation and radical scavenging. In the present investigation, we have therefore compared the LPO-inhibiting capacity of the flavonoids in an ABAP-induced LPO system with the results from an earlier study, in which we investigated the LPO-inhibiting capacity in an Fe^{2+}/asc (10/50 μM)-induced LPO system [8]. In the ABAP assay, an excess of EDTA (100 μM) was added in order to prevent iron-dependent processes.

Both assays were optimised to give approximately equal amounts of TBARS at the end of the incubation, i.e. after 1.5 hr, when no inhibitors were used. This allowed us to compare the IC_{50} values of both assays directly. This assumption appears to be correct as can be seen from phloridzin, which was earlier shown not to chelate iron [8]. The IC_{50} values of phloridzin were equal in both LPO assays.

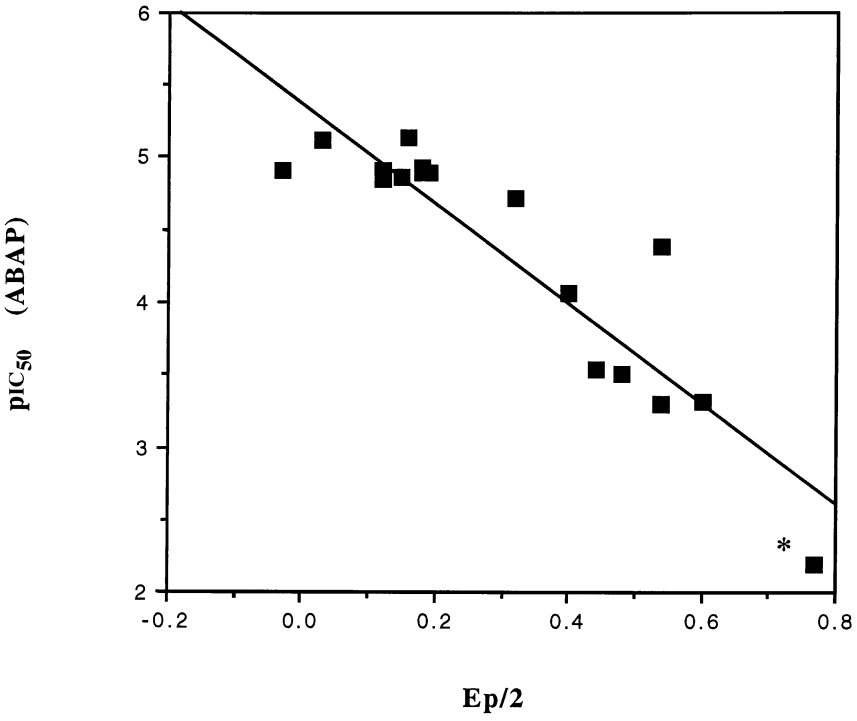


FIG. 4. Correlation of the IC_{50} values of ABAP-induced LPO with the oxidation potential Ep/2 (r = 0.912). *Indicates the flavonoid triHER, for which iron chelation is suggested to play a role in the inhibition of LPO.

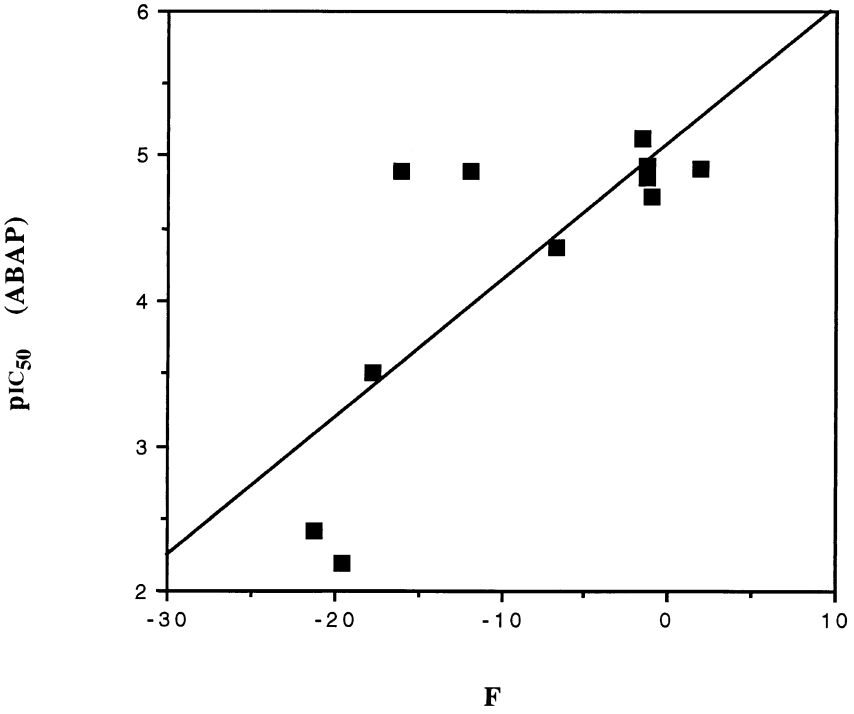


FIG. 5. Correlation of the IC_{50} values of ABAP-induced LPO with the calculated lipophilicity parameter *F* ($r = 0.786$).

Compounds that did not have an antioxidant activity in the ABAP assay did not show any antioxidant activity in any other assay, with the exception of apigenin, which had some activity in the Fe^{2+}/asc-induced LPO. This might be explained by the iron-chelating ability of apigenin. The inactive compounds cannot be included in the equation, but the parameters such as inhibition of both LPO assays and Ep/2 appear to be in good agreement with each other.

TetraHER, hesperidin and naringin did not have an IC_{50} below 3 mM in the Fe^{2+}/asc-induced LPO assay, whereas the first two compounds did have an IC_{50} below 3 mM in the ABAP-induced LPO assay. This might be an indication that the radical reactions induced by ABAP are more easily attained by relatively hydrophilic flavonoids than the radicals induced by Fe^{2+}/asc. TetraHER is a very hydrophilic flavonoid and hesperidin is much more hydrophilic than its aglycone hesperetin, and neither hydrophilic compound showed antioxidant activity in the Fe^{2+}/asc assay.

Even though there were some significant differences between the LPO assays for some compounds, e.g. kaempferol, galangin, triHQ, myricetin and rutin, the effects were usually very small. This is also clear from the good correlation between the pIC_{50} values of both Fe^{2+}/asc- and ABAP-induced LPO ($r = 0.893$, equation 1 in Table 3 and Fig. 3). Thus, iron chelation can give an additional effect, but it is not synergistic, as was described for $HO^{\cdot}$ scavenging in the deoxyribose assay [7]. In that case, the differences in IC_{50} would have been much larger. Only in the case of apigenin and triHER does iron chelation appear to play an important role. These compounds have a very low scavenging activity, based on their Ep/2 values (as can be seen from Table 2). However, they were more active in the Fe^{2+}/asc-induced LPO assay then could be expected from these Ep/2 values, probably because they are able to chelate iron. Iron chelation is a way of increasing the antioxidant activity of compounds with low scavenging activity, whereas the contribution of iron chelation to the antioxidant activity of good scavengers is almost negligible.

After the flavonoids for which iron chelation does appear to play a role (triHER and apigenin) are removed from the correlation of $pIC_{50}(Fe^{2+}/asc)$ with $pIC_{50}(ABAP)$, the coefficient for $pIC_{50}(ABAP)$ becomes nearly 1 (Eqn 5). Also, the correlation coefficient *r* increases from 0.893 to 0.942. It can therefore be concluded that for the compounds in Eqn 5 iron chelation does not play an additional role in inhibiting Fe^{2+}/asc-induced LPO.

F values were calculated relative to the flavone basic structure according to Rekker *et al.* [9]. They express the relative contribution of the substituents to the log P value, which is a lipophilicity parameter and is defined by the octanol-water partition coefficient. These *F* values might, however, be partly biassed. In the HER series, for example, increasing hydrophilicity parallels blocking of phenolic hydroxyl moieties, which accounts for the scavenging of radicals. The introduction of hydroxyl moieties in other flavonoids also causes an increase in scavenging activity as well as an increase in hydrophilicity. Another complication is that *F* values can only be calculated for compounds with identical basic structures.

The correlation of $pIC_{50}(ABAP)$ with the *F* values is more a trend than a correlation (Fig. 5). This may, however, be an indication that there is indeed some bias. Furthermore, only *F* values for the flavon(ol)s are available. Therefore, the *F* values only slightly improve the already excellent correlation of $pIC_{50}(ABAP)$ with Ep/2 ($r = 0.912$ vs 0.940 for a correlation which also includes *F* values).

Because iron chelation is not important in most of the cases, the structure-activity relationships for ABAP-induced LPO are the same as previously reported for Fe^{2+}/asc-induced LPO, and are described adequately by the SAR for the Ep/2 [8]. In short, the main antioxidant activity resides in ring B, where a catechol moiety is the optimal configuration. The degree of saturation of ring C does not have a large influence, except when a 3-OH is present. The combination of a 2,3 double bond with a 3-OH moiety renders a high antioxidant activity, especially in combination with a catechol moiety in ring B (e.g. quercetin), which results in the most active flavonoid antioxidants. The influence of the 4-keto moiety is not clear, as only one compound (catechin) was included without this moiety. Catechin, however, is very active in all assays, which might indicate that the 4-keto function is not necessary for good antioxidant activity.

Until now, only a limited number of authors have tried to investigate the role of iron chelation in the antioxidant activity of flavonoids, and they are not always in agreement with each other. Afanas'ev *et al.* studied the effect of iron chelation in LPO inhibition for rutin and quercetin [13]. In contrast to our findings, they found that iron chelation is important for rutin but not for quercetin. However, they attributed the antioxidant activity in CCl_4/EDTA-induced LPO exclusively to free-radical-scavenging activity. In our opinion, difference in lipophilicity between the compounds may also play an important role. Rutin is much less lipophilic than quercetin and this will influence which sites the flavonoid can reach within the membrane. CCl_4 is more likely to induce lipophilic radicals than NADPH, and this may explain why rutin is less potent in CCl_4-induced LPO. Morrazoni *et al.* found a good correlation between 1,1-diphenyl-2-picryl-hydrazyl, free radical (DPPH) interaction and CCl_4-induced LPO inhibition of anthocyanins and anthocyanidins, except for two glycosides, in which the sugar moiety induces a structural change which decreases antioxidant activity [14]. DPPH is a stable radical and interaction with it demonstrates a good radical-scavenging activity, which is independent of iron. Haenen *et al.* found a large effect of iron chelation on the antioxidant activity of monoHER in the $HO^{\cdot}$ scavenging assay [7]. They introduced the term 'site specific scavenging', which was found for the catechol-containing monoHER, but not for the other flavonoids from the HER series. This is, however, a purely chemical assay with completely different processes than the much more complex LPO assay. Apparently, these assays cannot be compared in this respect. Morel *et al.* loaded hepatocytes with iron-nitrilotriacetic acid (NTA) complex, and determined the malondialdehyde (a breakdown product of fatty acids) content and lactate dehydrogenase leakage [11]. The order of activity was catechin > quercetin ≫ diosmetin. This order was also found for iron mobilisation from the iron-preloaded hepatocytes. The authors explain their discrepancy with the order of free-radical-scavenging activity by the fact that they use hepatocytes in a 48-hr experiment and extensive metabolism can take place during this time. They suggest that it is an indication that iron chelation is important under their conditions. In our opinion there might also be a third explanation. As was observed in the competition experiments, quercetin oxidises rapidly in the presence of Fe^{2+}-EDTA. In the presence of Fe^{2+}-NTA, quercetin may also oxidise more rapidly than the other flavonoids tested, especially over 48 hr. Therefore, the concentration of quercetin might be lower than that of the other two flavonoids, which are less likely to oxidise.

It can be concluded that the role of iron in the antioxidant activity of flavonoids largely depends on the assay and the conditions used, and even then interpretation of the results may be complicated. In microsomal lipid peroxidation, iron chelation does not appear to play a role for most of the flavonoids tested here, and only flavonoids with a low radical-scavenging activity may benefit from its ability to chelate iron.

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