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ANTIOXIDANT AND IRON-CHELATING ACTIVITIES OF THE FLAVONOIDS CATECHIN, QUERCETIN AND DIOSMETIN ON IRON-LOADED RAT HEPATOCYTE CULTURES

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Abstract—The cytoprotective effect of three flavonoids, catechin, quercetin and diosmetin, was investigated on iron-loaded hepatocyte cultures, considering two parameters: the prevention of iron-increased lipid peroxidation and the inhibition of intracellular enzyme release. These two criteria of cytoprotection allowed the calculation of mean inhibitory concentrations (IC_{50}) which revealed that the effectiveness of these flavonoids could be classified as follows: catechin > quercetin > diosmetin. These IC_{50} values have been related to structural characteristics of the flavonoids tested. Moreover, the investigation of the capacity of these flavonoids to remove iron from iron-loaded hepatocytes revealed a good relationship between this iron-chelating ability and the cytoprotective effect. The cytoprotective activity of catechin, quercetin and diosmetin could thus be ascribed to their widely known antiradical property but also to their iron-chelating effectiveness. These findings increase further the prospects for the development and clinical application of these potent antioxidants.

Flavonoids are phenolic compounds widely distributed in plants. They have been reported to exert multiple biological effects, including antioxidant and free radical-scavenging abilities [1–3]. Their antiradical property is directed towards $^{\cdot}OH$ [4–5] and $O_2^{\cdot-}$ [6–10], highly reactive species implicated in the initiation of lipid peroxidation. Moreover, flavonoids are soluble chain-breaking inhibitors of the peroxidation process, scavenging intermediate peroxyl and alkoxyl radicals [11–13]. Phenolic compounds also have been suggested to present a strong affinity for iron ions [14–17] which are known to catalyse many processes leading to the appearance of free radicals. Thus, the antiperoxidative capability of flavonoids could be ascribed to concomitant activities of scavenging free radicals and of chelating iron.

Since the iron-chelating activity of flavonoids has not yet been established in a biological model, we have undertaken a study of three flavonoids (catechin, quercetin and diosmetin) in iron-loaded hepatocyte cultures.

Primary hepatocyte cultures represent a good *in vitro* experimental model which preserves its differentiated characteristics for many days [18]. Moreover, it is capable of accumulating ferric iron complexed to nitrilotriacetic acid (Fe–NTA)‡ [19–21], resembling *in vivo* iron overload observed in experimental situations [22] or in a disease such as genetic hemochromatosis [23]. The three tested flavonoids, catechin, quercetin and diosmetin, have been chosen according to their structural characteristics (Fig. 1). Catechin, quercetin and diosmetin belong to the flavanol, flavonol and flavone groups, respectively. In comparison with quercetin, catechin is deprived of the double bond in conjugation with an oxo function on the C ring, whereas diosmetin is devoid of the 3-hydroxyl structure on the C ring and presents a methylated-hydroxyl function on the B ring.

The first aim of the present study was therefore to compare the antioxidant activity of these flavonoids in our iron-loaded hepatocyte culture model; for this purpose, we evaluated free malondialdehyde (MDA) production which indicates the extent of lipid peroxidation. In addition, the assessment of intracellular enzyme release (LDH), a currently used marker of cellular injury, confirmed the hepatoprotective action of these flavonoids.

The second aim of the work was to evaluate the removal of iron by the flavonoids from iron-loaded hepatocytes. A relationship between this iron-chelating ability and the cytoprotective activity was discussed.

MATERIALS AND METHODS

Fe–*NTA*

Nitrilotriacetic acid (NTA) was used to maintain ferric iron in a soluble state; it is a low affinity iron chelator. The Fe–NTA solution was prepared

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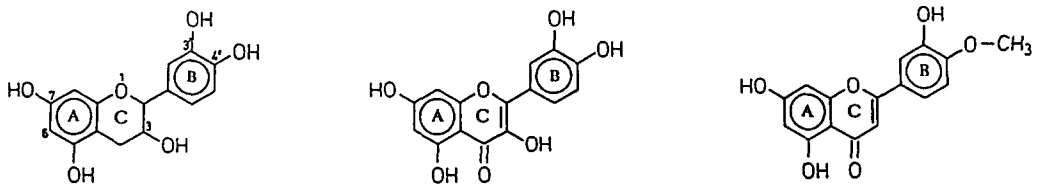
‡ Abbreviations: Fe–NTA, ferric iron nitrilotriacetate; NTA, nitrilotriacetic acid; MDA, malondialdehyde; LDH, lactate dehydrogenase; DMSO, dimethyl sulfoxide.



CATECHIN QUERCETIN DIOSMETIN

Fig. 1. Structure of flavonoids.

according to the method of White and Jacobs [24]. To 47 mg of NTA disodium salt (Sigma Chimie, St. Quentin Fallavier, France), dissolved in 10 mL of sterile water, were added 20 mg of ferric ammonium citrate (Merck, Darmstadt, Germany). The final concentration of ferric iron was 10 mM and the molar ratio ferric iron/NTA was 1:2. The solution was sterilized before use by filtration through a 0.22 μm filter.

Flavonoids

Quercetin was purchased from Ciba-Geigy (Basel, Switzerland). Catechin and diosmetin were provided by Sarsynthese (Merignac, France). Catechin was dissolved in water; quercetin and diosmetin were added in dimethyl sulfoxide (DMSO) which was maintained at a constant concentration in control samples (2%), a dose that did not affect control rates of lipid peroxidation, enzyme release or iron mobilization.

Cell isolation and culture

Adult rat hepatocytes were isolated from 2-month-old Sprague–Dawley male rats by cannulating the portal vein and perfusing the liver with a collagenase solution, as described previously [25]. The cells were collected in Leibovitz medium containing per mL: 2 mg bovine serum albumin and 5 μg bovine insulin. Cell suspension was filtered through gauze and allowed to sediment for 20 min in order to eliminate cell debris, blood and sinusoidal cells. The cells were then washed three times by centrifugation at 50 g, tested for viability and counted. The hepatocytes were then suspended in a mixture of 75% Eagle's minimum essential medium and 25% medium 199, supplemented with 10% fetal calf serum and containing per mL: streptomycin (50 μg), penicillin (7.5 IU), bovine insulin (5μg), bovine serum albumin (1 mg) and $NaHCO_3$ (2.2 mg). Usually, according to the experimental procedure, either 2.5×10^6 hepatocytes were plated in 25 cm^2 Nunclon flasks or 2.5×10^5 hepatocytes were suspended in 1 mL of medium in Multiwell tissue culture plates. The medium was changed 3–4 hr later, renewed the day after with the same medium as above but deprived of serum and supplemented with 10^{-7} M dexamethasone.

Lipid peroxidation

HPLC Procedure. Free MDA quantification was performed according to a method described previously [26]. The HPLC system (LDC Milton Roy, Orsay, France) was equipped with a Spherogel-TSK G1000 PW size exclusion column (7.5 mm i.d. × 30 cm, Cluzeau, Ste Foy, France). The eluant was composed of 0.1 M disodium phosphate buffer, pH 8 at a flow rate of 1 mL/min. The absorbance was monitored at 267 nm. The injections (250 μL) were performed by an autosampling injector (Promis, LDC) and the data were recorded and treated using a chromatography software (Thermochrom, LDC).

Preparation of free MDA standard. Five microlitres of 1,1,3,3-tetramethoxypropane (Sigma) were hydrolysed in 5 mL of 0.1 N HCl during 5 min in boiling water. This solution was then diluted 1000 times in 0.01 M Na_2HPO_4 buffer, pH 7.45, corresponding to a 6 μM MDA solution. The concentration of MDA in samples was calculated using a standard curve of free MDA.

Preparation of the samples for MDA analysis. During the first day of experimentation (J_1), the cultures were maintained in the presence of Fe–NTA in order to obtain final iron concentrations of 100 μM. More precisely, each sample with Fe–NTA was compared to control cultures without any supplementation and to cultures supplemented with 200 μM NTA, which is the amount of NTA added to the cultures in the experimental procedure. After these 24 hours of incubation, the medium was renewed with the same medium supplemented or not with iron ± DMSO, and the flavonoids were added at final concentrations of 50, 100, 150 μM for catechin and quercetin, and of 150, 200, 400 μM for diosmetin. After another 24 hr period of incubation (J_2), culture media were collected and hepatocytes were washed twice with 0.01 M phosphate buffer, pH 7.45. They were resuspended in 1 mL of the same buffer. The cells were lysed using an ultrasonic homogenizer. An aliquot was stored at −17° until protein content was estimated. The samples (culture media and cell homogenates) were filtered through a 500 Da membrane ultrafilter (Amicon, U.S.A.) in a 10 mL Amicon cell pressurized at 4 bars with nitrogen gas. The filtrate was used for HPLC procedure. Free MDA was quantified separately in culture media and in cell homogenates, and was then expressed as total MDA present in each culture sample (MDA in the cells + MDA in the culture medium) [27]. Protein content was determined on thawed cell homogenates according to the Bradford reaction [28], performed on a Cobas-Bio automatic

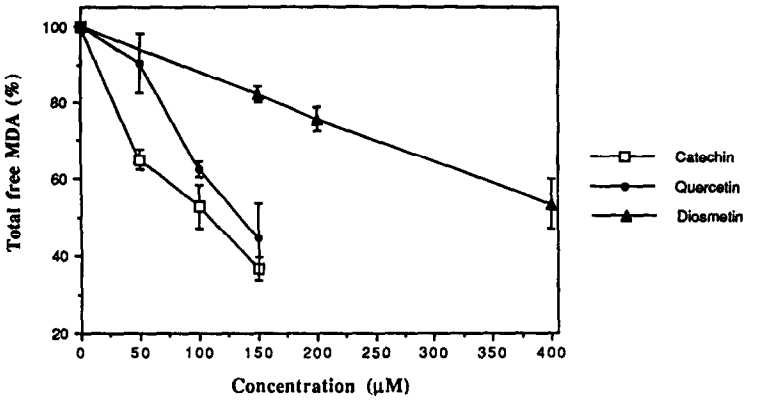


Fig. 2. Antiperoxidative activities of increasing concentrations of catechin, quercetin and diosmetin on free MDA production in hepatocyte cultures loaded for 24 hr with Fe–NTA (100 μM) and incubated for another 24 hr period with Fe–NTA in the presence or absence of flavonoid. Results are expressed as means ± SEM of three experiments, where 100% MDA recovery corresponded to the MDA level in cultures supplemented with iron only. Absolute values for total free MDA levels are 1724 ± 32 nM in Fe–NTA-supplemented controls and 37.3 ± 3.6 nM in controls without iron.

analyser, using the Bio-Rad reagent and bovine serum albumin as a standard.

Enzyme assay

The experimental conditions were the same as for lipid peroxidation assessment, except that the samples were cultured in Multiwell plates instead of Nunclon flasks. LDH was measured in the culture medium and in the hepatocytes after sonication in 1 mL of phosphate buffer. This assay was performed using a LDH kit (Roche) adapted to a Cobas-Bio automatic analyser. Inter- and intra-assay variations did not exceed 10%.

^{55}Fe mobilization from iron-loaded hepatocyte cultures

In order to compare the chelating activity of the flavonoids in cell cultures, the hepatocytes were loaded during 24 hr with 1 μM ^{55}Fe ferric chloride (sp. act. 1.5 mCi/mg Fe; Radiochemical center, Amersham, U.K.) [29]. Culture medium was then renewed (J_1) by the same medium but deprived of iron and supplemented or not with 100 μM of flavonoid or of desferrioxamine B (Desferal®) serving as a reference for iron chelation. After two days of incubation (J_3), the concentrations of ^{55}Fe in the flavonoid-supplemented samples were determined extracellularly and intracellularly after washing the cells three times with phosphate-buffered saline. The results were compared with those of control cultures corresponding to cultures without any addition of iron or flavonoid and to cultures supplemented during 24 hr with iron and the following two days with or without DMSO.

Statistics

Experimental values are means ± SEM of the number of experiments indicated in the legends. Significance was assessed using the Student's *t*-test at the level of 0.05.

Table 1. Mean inhibitory concentrations (IC_{50}) of catechin, quercetin and diosmetin required for 50% inhibition of lipid peroxidation and of enzyme release

	IC_{50} (μM)	
	Inhibition of lipid peroxidation	Inhibition of enzyme release
Catechin	108	104
Quercetin	137	139
Diosmetin	426	468

IC_{50} values were calculated from concentration–activity curves corresponding to the inhibition of free MDA production and of LDH release in hepatocyte cultures loaded for 24 hr with Fe–NTA (100 μM) and incubated for another 24 hr period with Fe–NTA in the presence or absence of flavonoid.

RESULTS

Cytoprotective activity of catechin, quercetin and diosmetin

Inhibition of lipid peroxidation. Maintenance in the presence of Fe–NTA (100 μM) of hepatocyte cultures for 48 hr induced a large increase in the MDA level. This level was taken as the 100% reference of free MDA production (Fig. 2). In the presence of a flavonoid, the amount of MDA was reduced in a dose-dependent manner. Control cultures did not exhibit a level of MDA higher than 3% (data not shown).

Mean inhibitory concentrations (IC_{50}) have been calculated from the concentration–activity curves (Fig. 2) and are reported in Table 1. In view of these values, the antilipoperoxidant activity of these flavonoids could be classified as follows: catechin > quercetin > diosmetin.

Inhibition of enzyme release. The damaging effect

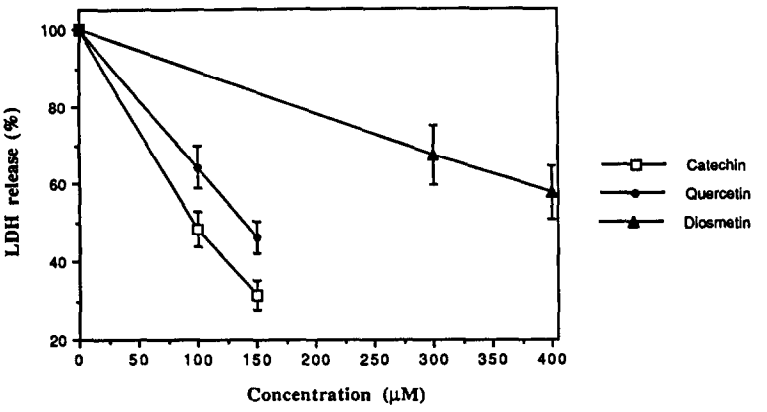


Fig. 3. Release of LDH in the medium of hepatocyte cultures maintained for 24 hr in the presence of Fe–NTA (100 μM) and for another 24 hr period with Fe–NTA in the presence or absence of various concentrations of flavonoid. Under these conditions 37.4 ± 5.7% of total LDH present in the control cultures was found in the culture medium whereas Fe–NTA alone led to 90 ± 7.6% of release of total LDH. Results are expressed as means ± SEM of three experiments, where 100% enzyme release corresponded to the maximum release by Fe–NTA.

of Fe–NTA on hepatocytes resulted in a large increase in LDH in the culture medium, correlated with a decrease in intracellular LDH levels (data not shown). This leakage of enzyme was prevented in a dose-dependent manner by the addition of a flavonoid (Fig. 3) which allowed IC_{50} calculation (Table 1). These values are in accordance with those relative to the inhibition of lipid peroxidation, which indicates that catechin was the most effective agent, quercetin exhibited a lower effectiveness and diosmetin was the least cytoprotective.

^{55}Fe *mobilization from iron-loaded hepatocyte cultures*

After the cultures have been maintained for 1 day in the presence of 1 μM of ^{55}Fe, the hepatocytes were treated for 2 days more, either with control medium ± DMSO (control ^{55}Fe), or with medium supplemented with 100 μM of flavonoid or desferrioxamine (Desferal). These 2 days of incubation in the presence of flavonoid corresponded to the minimal period of time required to observe significant iron mobilization from the cells. A high level of intracellular iron was observed in the cultures incubated with ^{55}Fe (Fig. 4a). The addition of a flavonoid or of desferrioxamine was followed by a decrease in intracellular iron levels concomitantly to an increase in iron concentrations in the culture medium (Fig. 4a and b). This phenomemon could be accountable to a mobilization of iron from the hepatocytes by the flavonoids and desferrioxamine. This iron-chelating ability could be classified as follows: catechin > quercetin > diosmetin (which was quite devoid of effect), whereas desferrioxamine, the iron chelator taken in reference, remained the most effective agent.

DISCUSSION

Comparison of the hepatoprotective effects of the three flavonoids tested in iron-loaded hepatocyte cultures revealed that, according to the IC_{50} values, their effectiveness could be classified as follows: catechin > quercetin > diosmetin. These results are supported by previous observations showing that the inhibitory activity of catechin was higher than that of quercetin on lipid peroxidation induced in various experimental models such as *N*-ethyl maleimide-treated platelets [30], tert-butyl hydroperoxide-treated mouse liver homogenates [31], CCl_4-treated microsomes [32] and ascorbic acid-treated rat brain mitochondria [33]. Moreover, diosmetin has been shown to have a lower antioxidant activity than catechin and quercetin [32–35], which is in agreement with our results. However, other studies did not reveal the same order of effectiveness and reported that the antioxidant activity of quercetin was better than that of catechin on rat brain mitochondria treated with $FeSO_4$ [33] and on rat liver microsomes treated with ascorbic acid [7]. This discrepancy of classification could probably be explained by differences in experimental conditions (time of incubation, and nature of the prooxidant and the experimental model). Moreover, the IC_{50} values calculated in our experiments were often far higher than those reported in the literature, in which considerable variations are noted [35]. We, however, worked for long periods of time (i.e. 48 hr) and on a hepatocyte model in which intensive metabolism is likely to require high doses of flavonoids. Nevertheless, the aim of these experiments was not to establish an absolute IC_{50} value but to compare the antioxidant power of various flavonoids.

This antioxidant power has been related to the free radical-scavenging activity of these compounds [31, 34], and among the three tested flavonoids, quercetin was shown to exhibit the highest antiradical property towards hydroxyl radical [4], peroxyl radical [11] and superoxide anion [7–9]. This predominance of quercetin could be attributed to its structural

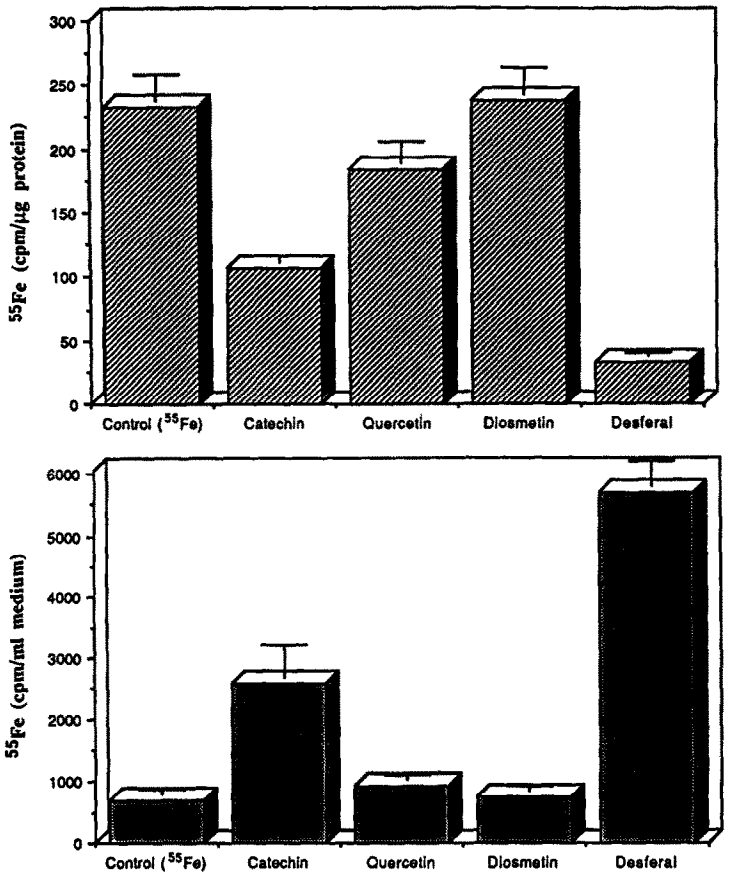


Fig. 4. Intracellular level (a) and release (b) of ^{55}Fe in hepatocyte cultures loaded for 1 day with ^{55}Fe (1 µM) and treated for 2 days more with or without 100 µM of a flavonoid or desferrioxamine (Desferal). Each point is the mean ± SEM of quadruplicate cultures.

characteristics (Fig. 1). Three structural groups are important determinants for radical-scavenging activity and/or antioxidant potential [6]: (a) the *o*-dihydroxy (catechol) structure of the B ring; (b) the 2,3-double bond in conjugation with a 4-oxo function [36]; (c) the additional presence of both 3- and 5-hydroxyl groups. Quercetin presents these three structural requirements, whereas catechin and diosmetin were devoid of the second and the first, respectively. Moreover, since catechin exhibited a higher antiradical property than flavone derivatives such as diosmetin [8–9], we can propose that the substitution of an OH group on the B ring markedly reduced the antioxidant effect of the flavonoid [8, 35–36], and that the 3-hydroxyl structure in the C ring is of major importance for its antioxidant effectiveness [8, 33].

In view of the absence of correlation between the classification of the antioxidant activity of the flavonoids in our model (catechin > quercetin > diosmetin) and the classification of their free radical-scavenging capability reported in the literature (quercetin > catechin > diosmetin), an additional mechanism could be suggested [10]. The protection of hepatocyte cultures by flavonoids during iron-induced lipid peroxidation could thus be partly ascribed to a mechanism of iron chelation. Our results revealed that these phenolic compounds had a good chelating activity in iron-loaded culture models and that they were capable of removing iron already inside the hepatocytes. These observations are supported by other findings which showed that the two flavonoids, quercetin and rutin, were effective inhibitors of iron-dependent lipid peroxidation due to the formation of inert complexes with iron, as shown by modifications of the spectra of quercetin and rutin in the presence of $FeSO_4$ [16]. Moreover, some flavonoids have been reported to be able to mobilize iron from ferritin [14] and to be capable of reducing Fe^{3+} to Fe^{2+} ions [12–13]. These considerations are of importance although some authors ruled out the possibility that the antiperoxidative action was related to an interaction of the flavonoids with iron ions [17, 37]. These authors worked, as we did, on iron-loaded systems where the peroxidative process had already started [17], but they found it unlikely that the flavonoid silymarin could chelate ferrous iron. It should however be noted that this flavonoid has not been tested in our model and that the

iron-chelating process was not fully established in their experiment.

The direct demonstration of iron removal from iron-loaded hepatocyte cultures by catechin, quercetin and diosmetin represents a significant contribution for the clarification of their anti-peroxidative mechanism observed *in vitro*. This will also help in the understanding of some studies which reported a protective activity of flavonoids *in vitro* as well as *in vivo*, acting as antioxidants but where their iron-chelating property was not carefully examined [3, 38–39]. Since in our experiment the iron-chelating activity follows the same order as the cytoprotective effect (catechin > quercetin > diosmetin), these two properties seem to be closely linked to each other. This relationship has to be taken into consideration in further developments of these protective flavonoids which could have important applications in human diseases, like the widely known silymarin used in liver dysfunction and diosmin acting as vascular protectant.

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