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Dual mechanism of mangiferin protection against iron-induced damage to 2-deoxyribose and ascorbate oxidation

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Abstract

We studied mangiferin effects on the degradation of 2-deoxyribose induced by Fe(III)–EDTA/citrate plus ascorbate, in relation to ascorbate oxidation (measured at 265 nm). Results revealed that mangiferin was equally effective in preventing degradation of both 15 and 1.5 mM 2-deoxyribose. At a fixed Fe(III) concentration, increasing the concentration of ligands (either EDTA or citrate) caused a significant reduction in the protective effects of mangiferin. Interestingly, mangiferin strongly stimulated Fe(III)–EDTA ascorbate oxidation, but inhibited it when citrate was used as iron co-chelator. Mangiferin stimulated O_2 consumption due to Fe(II) (formed by Fe(III) ascorbate reduction) autoxidation when the metal ligand was EDTA, but inhibited it when citrate was used. These results suggest that mangiferin removes iron from citrate, but not from EDTA, forming an iron–mangiferin complex that cannot induce ascorbate oxidation effectively, thus inhibiting iron-mediated oxyradical formation. Taken together, these results indicate that mangiferin works mainly by a mechanism different from the classical hydroxyl radical scavengers, keeping iron in its ferric form, by complexing Fe(III), or stimulating Fe(II) autoxidation.

Keywords: Mangiferin; Ascorbate; 2-Deoxyribose; Iron chelation

1. Introduction

Iron is vital in life because it is an important component of molecules that undergo intracellular redox reactions. However, this property also makes iron potentially toxic, since redox reactions may generate reactive oxygen species (ROS) [1]. In fact, ROS are known to damage proteins, lipids and DNA [2]. As a consequence, cells and organisms have developed several mechanisms to reduce toxicity by iron ions, which include a highly regulated transmembrane iron transport, intracellular iron chelators and enzymes that destroy ROS [3,4]. An imbalance of these systems increases susceptibility to oxidative damage, resulting in mutations, cancer, neurological diseases and iron-overload related diseases like hemochromatosis and β-thalassemia, where iron can accumulate at high hepatic levels [5].

Iron-chelating agents were introduced in the 1960s for the treatment of such diseases. Currently, deferoxamine (DFO) is the only iron chelator still clinically used. However, the cost of the treatment and lack of intestinal absorption of DFO have prompted research in the pursuit of alternatives [6].

Mangiferin,1,3,6,7-tetrahydroxyxanthone-C2-beta-D-glucoside is one of xanthone derivatives [7]. Many studies indicate that mangiferin has a wide range of pharmacological uses, including antidiabetic, anti-HIV, anticancer, immunomodulatory, and antioxidant activity [8–12].

We have recently shown that mangiferin causes rapid oxidation of Fe(II) and prevents Fe(III) reduction by ascorbate, diminishing the availability of Fe(II) for the Fenton reaction and thus preventing ferrous iron-induced lipid peroxidation in isolated rat liver mitochondria [13].

The antioxidant activity of several polyphenols, involving prevention of $^{\bullet}OH$ formation and ascorbate oxidation, has been correlated with their iron-chelating properties; however, as far as we know, this has not been established with mangiferin [14].

The aim of the present work is to further document the action of mangiferin as an antioxidant, mainly through its Fe(III)-

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chelating properties and its ability to induce Fe(II) oxidation and not merely due to •OH scavenging activity.

2. Materials and methods

2.1. Chemicals

Mangiferin, ascorbic acid, phosphate sodium salts, citrate, EDTA, nitrilotriacetic acid (NTA), dimethyl sulfoxide (DMSO), 2-Deoxyribose, salicylate, and thiobarbituric acid (TBA) were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of the highest commercially available grade.

Stock solutions of EDTA were neutralized with HCL/NaOH. Ferric chloride stock solutions (1 mM) were prepared daily in 10 mM HCL. Solutions of Fe(III)–EDTA (1:1 and 1:5 ratio), Fe(III)–citrate (1:2 ratio) and Fe(III)–NTA (1:2 and 1:10 ratio) were also freshly prepared in HCl 10 mM. Stock solutions of mangiferin (1, 5, 10, 25 and 50 mM) in DMSO were used with 1/1000 dilution in phosphate buffer 10 mM (pH 7.2) to minimize solvent interference. Stock solutions of 1% TBA were freshly prepared in 50 mM NaOH. All solutions were made with milli-Q deionised water.

2.2. 2-Deoxyribose assay

The formation of •OH radicals was measured using 2-deoxyribose oxidative degradation. The principle of the assay is the quantification of the main 2-deoxyribose degradation product, malonaldehyde (MDA), by its condensation with TBA [15]. Typical reactions were started by addition of ascorbate (2 mM final concentration) to 1 ml of solution containing 10 mM phosphate buffer (pH 7.2), 15 or 1.5 mM 2-deoxyribose, 50 or 250 μM EDTA or 100 μM citrate, 10 or 50 μM Fe(III) and 5, 10, 25, 50 or 100 μM mangiferin (or no mangiferin). Ascorbate was added approximately 10 min after the addition of mangiferin. Reactions were carried out for 30 min at 37 °C in a shaking bath to ensure continuous flow of O_2 into the tubes and terminated by the addition of 1 ml of 4% phosphoric acid (v/v) followed by 1 ml 1% TBA solution. After boiling for 15 min, the absorbance of solutions at 532 nm was recorded. 'Zero time' absorbance values (where ascorbate is added to solutions after the addition of phosphoric acid and TBA) were subtracted from the values obtained for each experimental condition. Mangiferin (100 μM) did not interfere with the reaction of MDA with TBA (data not shown).

The results shown as 'Damage to 2-deoxyribose (% of control)' were calculated as: [(sample A_{532} − 'zero time' A_{532})/(Control A_{532} − 'zero time')] × 100.

2.3. Determination of ascorbate oxidation rate

The rate of ascorbate oxidation was followed at 265 nm [16] in a Hitachi U-2001 spectrophotometer at 28 °C. Iron-complexing agent solutions (EDTA or citrate) were added to 10 mM phosphate buffer media (pH 7.2) followed by the addition of mangiferin (or DMSO for control) and ascorbate (100 μM final concentration) to 2 ml solutions. The reactions were recorded for 5 min and the linear slope (with $r^2 > 0.95$) was then calculated. Absorbance was read against buffer plus mangiferin as blank. The time interval between addition of mangiferin and ascorbate was the same (2 min) in all experiments.

2.4. Measurement of oxygen concentration

Oxygen concentration was polarographically determined with a Clark-type electrode (Yellow Springs Instruments Co.) in a 1.3 ml glass chamber equipped with a magnetic stirrer at 28 °C.

2.5. Spectroscopic determination of mangiferin–Fe(III) interaction

Absorption spectra from 300 to 700 nm were obtained using a Hitachi U-2001 spectrophotometer at 28 °C. Reaction mixtures (2 ml final volume) contained mangiferin, Fe(III), citrate and EDTA, as outlined in the figure legend, in 10 mM phosphate buffer pH 7.2. The incubation time between Fe(III) and mangiferin was 2 min. This allowed the observation of changes in the characteristic peak of mangiferin at nearly 380 nm due to formation of iron–mangiferin complexes.

2.6. Statistical analysis

The results are presented as the mean ± S.D. and statistical significance between the groups was determined by means of one-way analysis of variance (ANOVA) followed by unpaired Student's *t*-test to determine statistical significance. *P* values less than 0.05 ($P < 0.05$) were considered as indicative of significance.

3. Results

Competition studies were performed in order to evaluate the effectiveness of mangiferin and two •OH scavengers (DMSO and salicylate) in protecting 1.5 or 15 mM 2-deoxyribose from iron-mediated oxidative damage (Fig. 1). The •OH scavengers at 20 mM protected 15 mM 2-deoxyribose significantly less than 1.5 mM 2-deoxyribose ($P < 0.01$).

Mangiferin was equally effective in preventing oxidative degradation of both 1.5 and 15 mM 2-deoxyribose. Although the mean mangiferin protection results were slightly superior at 1.5 mM 2-deoxyribose, indicating some •OH scavenging ability, they did not show statistical differences versus 15 mM 2-deoxyribose.

These results suggest that mangiferin, in contrast to typical •OH scavengers, does not interfere with the reaction between 2-deoxyribose and •OH radicals and supports the proposal that mangiferin acts by preventing •OH formation from Fe(III)–EDTA plus ascorbate rather than by trapping •OH radicals.

Mangiferin at 5 μM allowed only 14–17% of iron-induced damage to 1.5/15 mM 2-deoxyribose and higher concentrations of the xanthone did not reduce the levels of damage. Dimethyl

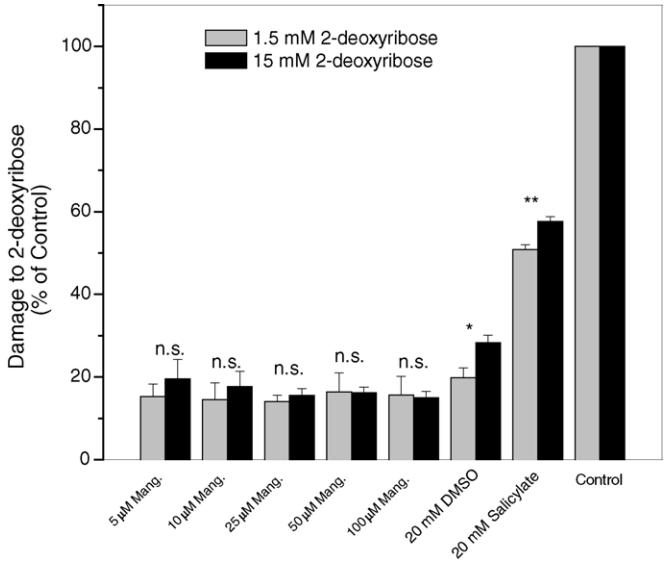

Fig. 1. Titration graphics for the effect of mangiferin (Mang.) and •OH scavengers dimethyl sulfoxide (DMSO) and salicylate on oxidative damage to 1.5 or 15 mM 2-deoxyribose induced by Fe(III)–EDTA plus ascorbate. Solutions were incubated for 30 min at 37 °C and contained 10 mM Phosphate buffer (pH 7.2), 2-deoxyribose (1.5 or 15 mM), 50 μM EDTA and 10 μM Fe(III). The concentration of mangiferin, DMSO and salicylate are as indicated on the *x*-axis. Reactions were started by addition of ascorbate to a final concentration of 2 mM. The bars show means ± S.D. ($n = 3$). Controls contain only DMSO (14. 3 μM), which is the solvent concentration in the mangiferin samples. A_{532} values for controls were 0.35 ± 0.01 and 1.06 ± 0.11 for 1.5 and 15 mM 2-deoxyribose, respectively. One tailed *t*-test was used for statistical analyses; $^{*}P < 0.05$, $^{**}P < 0.01$, n.s., not significant.

sulfoxide and salicylate at mM concentrations afforded less protection than mangiferin at μM levels. These results are consistent with the fact that classical antioxidants are effective only in mM concentrations in aqueous solutions and suggest that mangiferin works as an antioxidant by a different mechanism than the •OH scavengers.

The non-existence of a dose-dependent profile on mangiferin protection of 2-deoxyribose degradation at 10 μM Fe(III) could be due to a complete iron complexation by 5 μM mangiferin concentration; therefore a comparative study was performed to evaluate the effectiveness of mangiferin in preventing 10 or 50 μM iron-induced damage to 15 mM 2-deoxyribose. Fig. 2 shows that mangiferin was more effective in preventing 2-deoxyribose degradation at 10 than at 50 μM Fe(III) concentration. The graphic also shows that mangiferin reduced 50 μM iron-induced damage to 2-deoxyribose damage in a dose-dependent fashion up to 100 μM, suggesting an stoichiometry for mangiferin–Fe(III) complex of around 2:1.

Titration of mangiferin in the 2-deoxyribose assay was performed with two different concentrations of EDTA at a fixed Fe(III) concentration of 50 μM (Fig. 3). Mangiferin was less effective in preventing 2-deoxyribose degradation at higher concentrations of EDTA ($P < 0.05$). For example, the degradation of 2-deoxyribose at 5 μM mangiferin concentration increased from 14.1 to 29.7%, which represents more than 50% of protection reduction at higher EDTA concentrations. A similar experiment was performed with complexes of citrate and Fe(III) (Fig. 4).

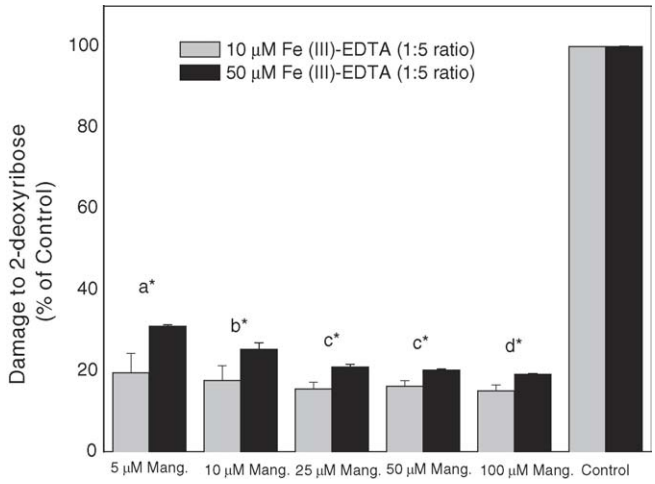

Fig. 2. Effect of mangiferin (Mang.) concentration on 2-deoxyribose degradation caused by Fe(III)–EDTA plus ascorbate. Experimental conditions are as described in the legend to Fig. 1, except that two concentrations of Fe(III) were used; 10 and 50 μM. Fe(III)–EDTA concentration ratios were kept at 1:5. The bars show means ± S.D. ($n = 3$). Controls are also as described in the legend of Fig. 1, except that the A_{532} were 1.06 ± 0.11 and 2.45 ± 0.12 for 10 and 50 μM Fe(III)–EDTA, respectively. One tailed *t*-test was used for statistical analysis; different letters indicate significant difference ($P < 0.05$) between mangiferin concentrations at 50 μM Fe(III). * Significance ($P < 0.05$) vs. respective reactions with 50 μM Fe(III).

These results show that citrate allows for greater protection of 2-deoxyribose by mangiferin than EDTA ($P < 0.05$). Since citrate forms a weaker complex with Fe(III) compared with EDTA, mangiferin more easily removes the metal from citrate resulting in more effective protection against iron-mediated damage to 2-deoxyribose.

Ascorbate oxidation experiments have shown interesting results; mangiferin at 50 μM caused a stimulatory effect on

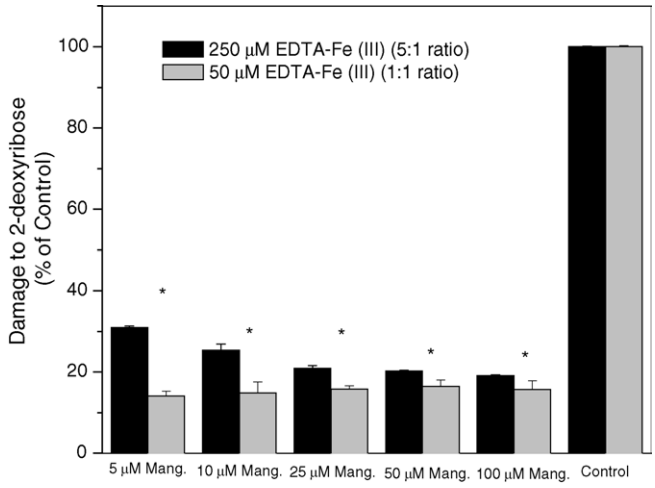

Fig. 3. Effect of mangiferin (Mang.) concentration on 2-deoxyribose degradation caused by Fe(III)–EDTA plus ascorbate. Solutions were incubated for 30 min at 37 °C and contained 10 mM Phosphate buffer (pH 7.2), 15 mM 2-deoxyribose, 50 μM Fe(III)–EDTA (with 50 or 250 μM EDTA), mangiferin (5 to 100 μM) and 2 mM ascorbate. The bars show means ± S.D. ($n = 3$). Controls are as described in the legend of Fig. 1, except that the A_{532} were 2.03 ± 0.26 and 2.45 ± 0.12 for 50 and 250 μM EDTA, respectively. Significance vs. respective reactions with 50 μM EDTA (one tailed *t*-test): $^{*}P < 0.05$.

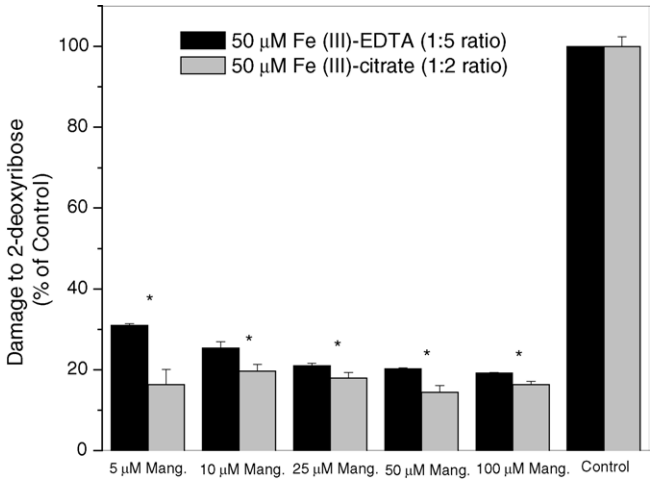

Fig. 4. Effect of mangiferin (Mang.) concentration on 2-deoxyribose degradation caused by Fe(III)–citrate (1:2) and Fe(III)–EDTA (1:5) plus ascorbate. Solutions were incubated for 30 min at 37 °C and contained 10 mM phosphate buffer (pH 7.2),15 mM 2-deoxyribose, 50 μM Fe(III), 100 μM citrate and 250 μM EDTA. The concentrations of mangiferin are as indicated on the *x*-axis. Reactions were started by addition of ascorbate to a final concentration of 2 mM. The bars show means ± S.D. ($n = 3$). Controls are as described in the legend of Fig. 1. A_{532} values for controls were 0.64 ± 0.02 and 2.45 ± 0.12 for citrate and EDTA, respectively. One tailed *t*-test was used for statistical analyses; significance vs. respective reactions: $^{*}P < 0.05$.

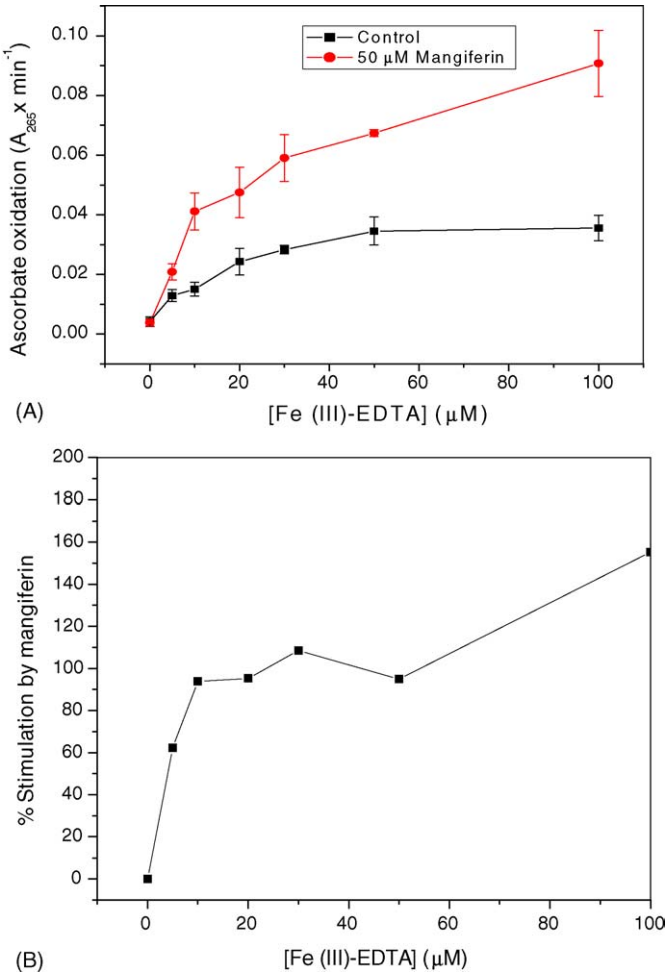

Fig. 5. Panel A: Dependence of Fe(III)–EDTA concentration on the rate of ascorbate oxidation. Reactions were carried out in the absence or presence of 50 μM mangiferin, containing the following final concentration of reagents: phosphate buffer (10 mM, pH 7.2), 100 μM ascorbate and varying Fe(III)–EDTA concentration (0–100 μM, with 1:1 ratio iron–EDTA). The rate of ascorbate oxidation (as loss of A_{265} per minute) was calculated as described in Materials and Methods. Values in Panel A are means ± S.D. ($n = 3$). Panel B is a replotting of data from Panel A showing the percentage of stimulation of ascorbate oxidation by mangiferin.

ascorbate oxidation, which increased with the elevation of iron–EDTA (1:1 ratio) concentration (Fig. 5A and B). Potentiation of ascorbate oxidation by mangiferin was around 100% with respect to control at almost all concentrations range of Fe(III)–EDTA, except for 100 μM Fe(III), where the absorbance disappearance at 265 nm was increased by more than 150%.

The high stability of Fe(III)–EDTA complexes prevents mangiferin access to iron, which is rapidly reduced to Fe(II) by ascorbate. The stimulatory effect of mangiferin on ascorbate disappearance could be due to an interaction between them or due to ferric ions regenerations in the reaction medium. Fig. 5, Panel A, at null concentration of Fe(III)–EDTA, shows the non-existence of such interaction between ascorbate and the xanthone. Recently we have shown that mangiferin induced Fe(II)–citrate oxidation to Fe(III) in a 10 mM Hepes buffer pH 7.2 [17], so it could be possible that mangiferin removes iron from the less stable complexes Fe(II)–EDTA, promoting its oxidation to Fe(III). In order to investigate this possibility we measured the effect of mangiferin on oxygen consumption mediated by Fe(II) autoxidation. Fig. 6 (line c) shows oxygen consumption rate due to autoxidation of Fe(II), generated by Fe(III) ascorbate reduction. Mangiferin strongly increased the oxygen consumption rate (line d), which suggests a stimulatory effect on Fe(II) autoxidation.

Changing the iron co-chelator from EDTA to citrate, a weaker iron ligand [15] resulted in an increased effectiveness of mangiferin in inhibiting ascorbate oxidation (Fig. 7). The presence of citrate instead of EDTA reduced oxygen consumption due to Fe(II) autoxidation (Fig. 6, line b) and allowed mangiferin to almost completely inhibit Fe(II) autoxidation (Fig. 6, line a).

The dependence of the ascorbate oxidation rate on mangiferin concentration was assayed in the presence of 100 μM Fe(III)–EDTA (1:1 ratio) (Fig. 8, Panel A) or 100 μM Fe(III)–citrate (1:2 ratio) and 100 μM ascorbate (Fig. 8, Panel B). Mangiferin increased ascorbate oxidation in a dose-dependent fashion when the iron-ligand used was EDTA, but inhibited it, when citrate was the iron co-chelator.

Fig. 9 (line a) shows the mangiferin spectrum with its characteristic peak at nearly 380 nm. The addition of 30 μM Fe(III) provoked a reduction of mangiferin absorption maxima of around 11% and induced a small, but measurable, shift in the position of this absorption maxima (from 380 to 384 nm) (Fig. 9, line b). The addition of 100 μM ascorbate did not modify the Fe(III)–citrate–mangiferin mixture spectrum. Addition of EDTA (200 μM final concentration) to Fe(III)–citrate–mangiferin mixture restarted mangiferin spectrum (line c), and finally, incorporating ascorbate (100 μM

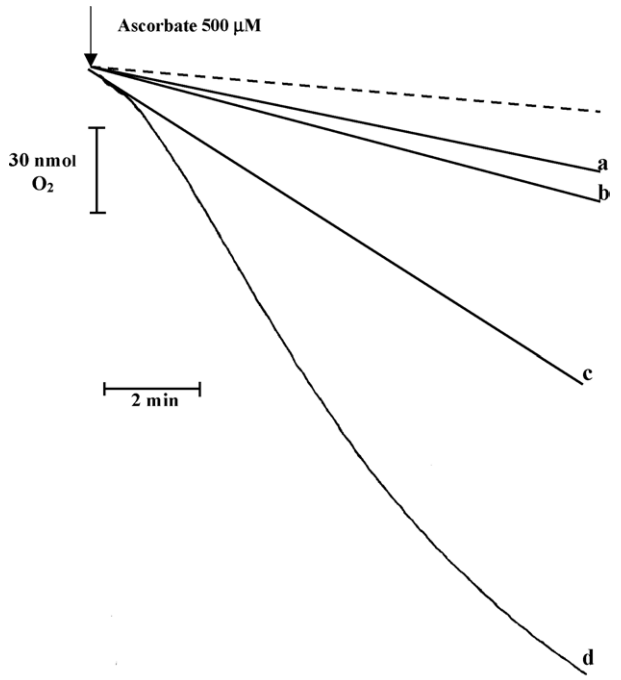

Fig. 6. Effect of mangiferin on O_2 consumption due to Fe(II) [formed by Fe(III) ascorbate reduction] autoxidation. Experimental conditions: 10 mM phosphate buffer (pH 7.2), 100 μM Fe(III), 200 μM citrate (a, b), 100 μM EDTA (c, d). Experiments were conducted at 28 °C; ascorbate (500 μM) was added where indicated by the arrow. (a) 50 μM mangiferin; (b) no mangiferin; (c) no mangiferin; (d) 50 μM mangiferin. Dashed line indicates O_2 consumption in phosphate buffer containing 50 μM mangiferin and 100 μM EDTA, without Fe(III) addition. The traces are representative of three experiments.

final concentration) to Fe(III)–EDTA–citrate–mangiferin mixture produced a spectrum similar to that obtained by mixing Fe(III)–citrate with mangiferin (line d). These spectroscopic analyses are in agreement with the results above mentioned and sustain the hypothesis of mangiferin–Fe(III) complex formation, and the mangiferin–Fe(II) interaction, stimulating Fe(II) autoxidation.

4. Discussion

We observed that mangiferin efficiently inhibits 2-deoxyribose degradation by Fe(III)–EDTA or Fe(III)–citrate plus ascorbate, but stimulates Fe(III)-dependent ascorbate oxidation when the iron co-chelator is EDTA, and inhibits it when citrate is used as iron-ligand. Although apparently contradictory, these results suggest a dual mechanism of mangiferin protection against iron-related oxidative damage to 2-deoxyribose and ascorbate, both of them leading to the maintenance of iron in its ferric form.

The process of $^{\bullet}OH$ formation and 2-deoxyribose damage may occur via the following sequence of reactions [18]:

$$\text{Fe(III)–EDTA} + \text{ascorbate} \rightarrow \text{Fe(II)–EDTA} + \text{ascorbyl} \quad (1)$$

$$\text{Fe(II)–EDTA} + O_2 \leftrightarrow \text{Fe(III)–EDTA} + O_2^{\bullet -} \quad (2)$$

$$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (3)$$

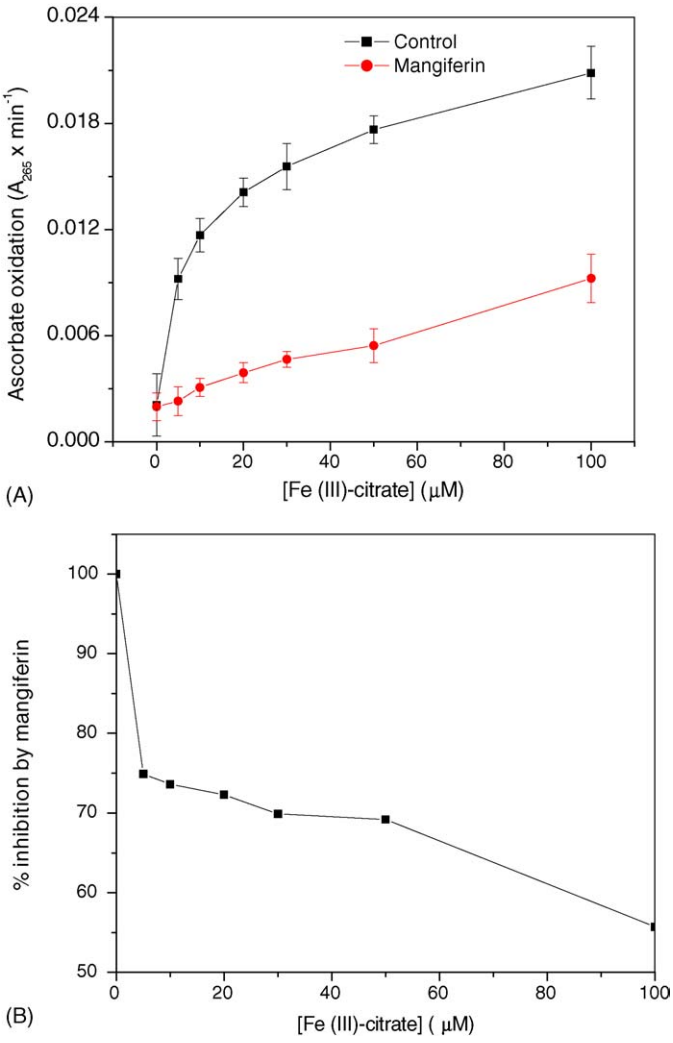

Fig. 7. Panel A: Dependence of Fe(III)–citrate concentration on the rate of ascorbate oxidation. Experimental conditions are as described in the legend of Fig. 5, except that the iron co-chelator was changed from EDTA to citrate, with 1:2 iron–citrate ratio. Data are the average of three experiments. Panel B is a replotting of data from Panel A showing the percentage of inhibition of ascorbate oxidation by mangiferin.

$$H_2O_2 + \text{Fe(II)–EDTA} \rightarrow \text{Fe(III)–EDTA} + OH^- + {}^{\bullet}OH \quad (4)$$

$$^{\bullet}OH + \text{2-deoxyribose} \rightarrow \text{degradation products} \quad (5)$$

It was reported that mangiferin rapidly scavenged 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals and inhibited lipid peroxidation, which was initiated enzymatically by reduced nicotinamide adenine dinucleotide phosphate (NADPH) or non-enzymatically by ascorbic acid or Fenton's reagent ($H_2O_2 + Fe^{2+}$) in rat liver microsomes [12]. These results did not exclude the possibility that mangiferin reacts directly with $^{\bullet}OH$. However, the present study provides evidence that 5–100 μM mangiferin does not prevent 2-deoxyribose degradation by trapping $^{\bullet}OH$. This is based on (i) the competition experiments using two concentrations of 2-deoxyribose and the quantitative difference in the antioxidants activities of mangiferin and classical $^{\bullet}OH$ scavengers (see Fig. 1), (ii) the mangiferin titration experiments using two different concentrations of EDTA, with a fixed

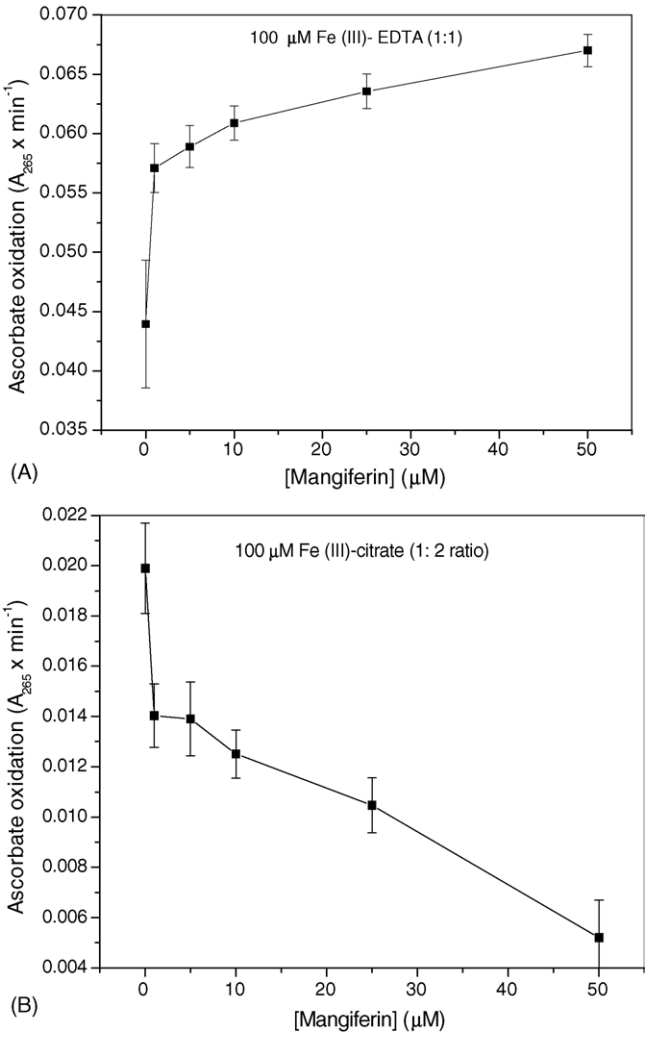

Fig. 8. Effect of mangiferin concentration on the rate of ascorbate oxidation, using two iron co-chelators; EDTA (Panel A) and citrate (Panel B). Reactions were carried out in the presence of 100 μM Fe(III) containing the following final concentration of reagents: phosphate buffer (10 mM, pH 7.2), 100 μM ascorbate, 100 μM EDTA or 200 μM citrate and varying mangiferin concentration (0–50 μM). The rate of ascorbate oxidation (as loss of A_{265} per minute) was calculated as described in Materials and Methods. Values in Panel A and B are means ± S.D. ($n = 3$).

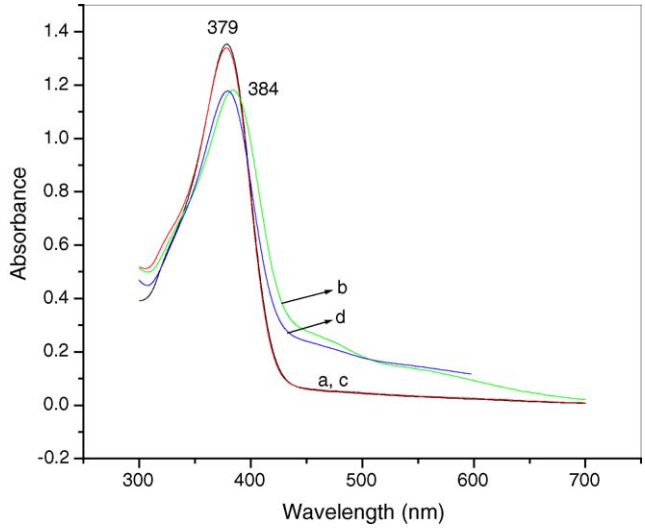

Fig. 9. Absorption spectra of 50 μM Mangiferin in the presence and absence of Fe(III), EDTA and ascorbate. Reaction medium contained 10 mM phosphate buffer, 50 μM mangiferin and 100 μM citrate. Experimental conditions were: (a) reaction medium only; (b) reaction medium plus 30 μM Fe(III) (2 min of incubation before spectrum acquisition); (c) conditions of b plus 200 μM EDTA; (d) condition of c plus 100 μM ascorbate. Addition of 100 μM ascorbate to b did not modify the spectrum profile.

amount of Fe(III) (see Fig. 3), and (iii) the same titration experiments, using two different iron co-chelators (see Fig. 4).

The effect of mangiferin on iron-mediated ascorbate oxidation shows that when citrate is used as iron chelator (forming a weaker complex with iron, when compared with EDTA), the xanthone removes Fe(III) from Fe(III)–citrate and forms $Fe(III)_x$–$mangiferin_y$ complex, that reacts with ascorbate with low efficiency, which prevents the first step (Eq. (1)) in the chain of reactions leading to $^{\bullet}OH$ formation (see Fig. 7). According to this, there is less Fe(II) concentration in the reaction medium, which consumes less oxygen for its autoxidation (Eq. (2), Fig. 6, lines a, b).

However, when the potent iron chelator EDTA is used, mangiferin strongly stimulates iron-mediated ascorbate oxidation. The increase in the rate of A_{265} disappearance occurs together with the apparently contradictory protection against 2-deoxyribose degradation. The high log formation constant for Fe(III)–EDTA ($\log \beta = 25.5$ [19]), impedes mangiferin removal of iron from the complex, facilitating ascorbate reduction work on Fe(III), which yields the Fenton reagent Fe(II)–EDTA. The log formation constant for this complex is around half of that of Fe(III)–EDTA [19] and sufficient for allowing mangiferin to stimulate Fe(II) autoxidation (see Fig. 6, line d), which increases Fe(III)–EDTA concentration in the reaction medium and also the rate of ascorbate oxidation. The lack of effects of SOD or CAT on oxygen consumption profiles (results not shown), suggests that the stimulation of Fe(II) autoxidation elicited by mangiferin is not mediated by reactive oxygen species generation (superoxide radicals and hydrogen peroxide).

It is well established that oxygen, nitrogen and sulphur molecule atoms are the most common iron ligands and that chelators in which oxygen atoms serve as the ligand of iron promote the oxidation of Fe(II) and tend to stabilize Fe(III), thus decreasing the reduction potential of the iron [20].

At a physiological pH, catechols readily form thermodynamically stable *bis* complexes with ferric iron as bidentate ligands favoured by low, more physiologically relevant, concentrations. Catechols are also biosynthesized and used as iron-sequestering agents by microorganisms [21,22]. The high hydroxylated mangiferin structure, including its catechol moiety, suggests such mechanism for iron interaction, which could explain the protection against iron-induced damage to 2-deoxyribose and the effects on ascorbate oxidation.

Spectroscopic data agree with the above results. Mangiferin–Fe(III) interaction modifies the mangiferin spectrum. The reversibility of such interaction was confirmed by EDTA addition, which restarts mangiferin spectrum. Ascorbate-derived spectra suggest the strength of mangiferin

interaction with Fe(III), which impairs ferric irons reduction to ferrous iron by endogenous reducers like ascorbate, sparing them and also preventing Fe(II) reloading of the biological system, which can readily participate in the Haber-Weiss-type reactions involved in $^{\bullet}$OH formation.

Most of the experiments show high efficiency of mangiferin in preventing iron-induced 2-deoxyribose damage that could also be explained by the formation of a redox active mangiferin–Fe(III) complex that mimics superoxide dismutase activity, which maintains the Fe-chelate within a continuous Fe(III)–Fe(II)–Fe(III) conversion with no significant accumulation of Fe(II) or superoxide. A recent report demonstrated that the 2:1 flavonoids–Fe(III) complexes were more effective than the parent compounds in scavenging superoxide radicals generated by xanthine oxidase/hypoxanthine [23].

The studies with citrate as iron co-chelator are of particular interest. Unlike EDTA, citrate is a physiological iron chelator, and it is believed to be an important constituent of the intracellular pool of iron complexes of low molecular weight [24]. Indeed, it is noteworthy that in the plasma of iron-overload patients non-transferrin-bound iron is present, apparently as complexes with citrate and acetate [25]. The effectiveness of mangiferin in the system using Fe(III)–citrate was much greater than with Fe(III)–EDTA (see Section 3). This is explained by the small log formation constant of Fe(III)–citrate ($\log \beta = 11$ [19]). Thus mangiferin can easily remove iron from the complex with citrate, preventing in vitro iron-mediated $^{\bullet}$OH formation (see Section 3).

We have recently shown the protecting ability of mangiferin enriched extract against mitochondrial Fe(II)–citrate induced lipoperoxidation. This protection occurred mainly through stimulation of Fe(II) autoxidation (insensible to superoxide dismutase and catalase activity) and Fe(III) complexation, which prevented its reduction to Fe(II) by ascorbate [13].

The fact that 5 μM mangiferin was able to produce more than 50% protection against iron-mediated 2-deoxyribose damage is of particular interest. Since in vivo levels of free or loosely bound iron are hardly higher than 1 μM, even in iron overload [26], less than 2 μM of mangiferin (taking into account the possibility of 2:1 of mangiferin–iron complexes) would afford protection against iron-mediated damage. This represents around 1 $\mu g\,ml^{-1}$ of mangiferin. A recent report shows that the administration of 10 $mg\,kg^{-1}$ of mangiferin to Wistar rats sustained mean free levels of mangiferin in rat blood over 1 $\mu g\,ml^{-1}$ during approximately 1 h [27]. Also, oral administration of mangiferin-containing natural extract to rats elicited plasma mangiferin concentrations higher than 1 $\mu g\,ml^{-1}$ [28]. These results suggest that the observed in vitro effects of this compound could be relevant to in vivo conditions of iron overload.

It is known that very few drugs (free radical scavengers) are present in vivo at concentrations that would allow them to compete with biological molecules for reaction with $^{\bullet}$OH or HOCL. In order to compete, a scavenger has to be present at no less than millimolar concentrations [29]. Mangiferin, like most drugs, never achieves this sort of concentration. Thus, an important antioxidant strategy in the human body is the safe sequestration of iron and copper ions into forms that will not catalyze this free radical formation. Mangiferin, in a concentration perfectly reachable in vivo, prevents 2-deoxyribose damage induced by Fe(III)–EDTA/citrate, and Fe(III)–citrate ascorbate oxidation, mainly through its iron-complexing ability. Further research on the putative beneficial effects of mangiferin with in vivo animal models of iron overload must be done to propose this xanthone and its naturally-contained extracts as a therapeutic intervention against tissue damage induced by iron overload.

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