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Iron complexing activity of mangiferin, a naturally occurring glucosylxanthone, inhibits mitochondrial lipid peroxidation induced by Fe^{2+}-citrate

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Abstract

Mangiferin, a naturally occurring glucosylxanthone, has been described as having antidiabetic, antiproliferative, immunomodulatory and antioxidant activities. In this study we report for the first time the iron-complexing ability of mangiferin as a primary mechanism for protection of rat liver mitochondria against Fe^{2+}-citrate induced lipid peroxidation. Thiobarbituric acid reactive substances and antimycin A-insensitive oxygen consumption were used as quantitative measures of lipid peroxidation. Mangiferin at 10 μM induced near-full protection against 50 μM Fe^{2+}-citrate-induced mitochondrial swelling and loss of mitochondrial transmembrane potential ($\Delta\Psi$). The IC_{50} value for mangiferin protection against Fe^{2+}-citrate-induced mitochondrial thiobarbituric acid reactive substance formation (9.02 ± 1.12 μM) was around 10 times lower than that for *tert*-butylhydroperoxide mitochondrial induction of thiobarbituric acid reactive substance formation. The xanthone derivative also inhibited the iron citrate induction of mitochondrial antimycin A-insensitive oxygen consumption, stimulated oxygen consumption due to Fe^{2+} autoxidation and prevented Fe^{3+} ascorbate reduction. Absorption spectra of mangiferin–Fe^{2+}/Fe^{3+} complexes also suggest the formation of a transient charge transfer complex between Fe^{2+} and mangiferin, accelerating Fe^{2+} oxidation and the formation of a more stable Fe^{3+}–mangiferin complex unable to participate in Fenton-type reaction and lipid peroxidation propagation phase. In conclusion, these results show that in vitro antioxidant activity of mangiferin is related to its iron-chelating properties and not merely due to the scavenging activity of free radicals. These results are of pharmacological relevance since mangiferin and its naturally contained extracts could be potential candidates for chelation therapy in diseases related to abnormal intracellular iron distribution or iron overload.

Keywords: Mangiferin; Lipid peroxidation; Mitochondria; Iron chelator

1. Introduction

A major macronutrient in higher animals, iron is an essential element for all cellular forms of life. Reviews of iron biology consistently point out the chemical properties that make iron suitable for its multifaceted roles in biology (redox activity, ligand exchange kinetics, oxygen binding, etc.) and at the same time present a challenge concerning its acquisition, transport, and safe utilization (Aisen and Listowsky, 1980; Goswami et al., 2002). Iron, although crucial for life, is toxic if present in excessive amounts in the human body. Patients suffering from diseases such as hemochromatosis and thalassemia accumulate, either directly or through repeated blood transfusions, high levels of iron in their vital organs, which rapidly leads to death through cardiac failure if not treated. Moreover, iron is also a mediator for free radical effects in several other

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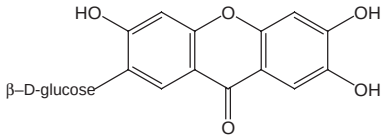

Fig. 1. Mangiferin (1,3,6,7-tetrahydroxyxanthone-C2-β-D-glucoside) structure.

pathological conditions, including ischemic heart disease and cancer (Halliwell and Gutteridge, 1990; Hershko, 1994; Toyokuni, 1996).

Currently, desferrioxamine, a hexadentate hydroxamic acid, is still the only clinically approved iron chelator. This drug is very expensive and orally ineffective so patients must endure long periods (12–24 h/day, 5–6 days/week) of subcutaneous infusion of desferrioxamine to excrete these excessive levels of iron (Richardson and Ponka, 1998). Such inherent limitations of desferrioxamine have prompted research directed to the pursuit of alternatives.

Mangiferin, 1,3,6,7-tetrahydroxyxanthone-C2-beta-d-glucoside (Fig .1), is one of xanthone derivatives and C-glucosylxanthones (Aritomi and Kawasaki, 1969). Many studies indicate that mangiferin has a wide range of pharmacological properties including antidiabetic, anti-HIV, anticancer, immunomodulatory and antioxidant activity (Ichiki et al., 1998; Guha et al., 1996; Yoshimi et al., 2001; Leiro et al., 2004; Sato et al., 1992).

The antioxidant activity of several polyphenols involving prevention of $^{\cdot}OH$ formation and lipid peroxidation has been correlated with their iron-chelating properties; however, as far as we know, this has not been established in the case of mangiferin (Yoshino and Murakami, 1998; Miller et al., 1996; Afanas'ev et al., 1989). Even though its antioxidant action in aqueous solutions has been well investigated, its effects against lipid peroxidation are still poorly documented.

Iron-mediated lipid peroxidation is a key event leading to cell membrane damage (including mitochondrial membranes), DNA alterations, carcinogenic processes and cell death (Castilho et al., 1999; Britton et al., 1994; Cardoso et al., 1999; Nair et al., 1998). Therefore, the aim of the present study was to investigate the antioxidant activity of mangiferin against lipid peroxidation of isolated rat liver mitochondria induced by Fe^{2+}-citrate. We also correlated the iron chelation properties of mangiferin with its activity against lipid peroxidation and mitochondrial damage.

2. Materials and methods

2.1. Reagents and solutions

Mangiferin, ADP, antimycin A, butylated hydroxytoluene, citrate, EGTA, ascorbic acid, *N*-(2-hydroxyethyl) piperazine-*N*′-2-ethanesulfonic acid (HEPES), 1,10-phenanthroline, rotenone, succinate, thiobarbituric acid and carbonyl cyanide *p*-trifluorophenylhydrazone were purchased from Sigma (St. Louis, MO). Ferrous $[Fe(NH_4)_2(SO_4)_2 \times 6H_2O]$ and ferric ($FeCl_3$ + 10 mM HCl) ion solutions were prepared in Milli-Q water and immediately used. Stock solutions of mangiferin in dimethyl sulfoxide were used with dilutions of 1/1000 in assay medium to minimize solvent interference.

2.2. Isolation of rat liver mitochondria and standard incubation procedure

Mitochondria were isolated by conventional differential centrifugation from the liver of adult animals fasted overnight (Kaplan and Pedersen, 1983). The livers were homogenized in 250 mM sucrose, 1 mM EGTA, and 10 mM Hepes buffer (pH 7.2). The mitochondrial suspension was washed twice in the same medium containing 0.1 mM EGTA and the final pellet was resuspended in 250 mM sucrose to a final protein concentration of 80–100 mg/ml, measured by the method of Biuret with bovine serum albumin as protein standard.

The experiments were carried out in standard medium containing 125 mM sucrose, 65 mM KCl, 5 mM potassium succinate, 2 mM inorganic phosphate, 1 mM magnesium chloride, 2 μM rotenone, 10 mM Hepes buffer (pH 7.2) and 5 mM succinate.

2.3. Measurement of oxygen concentration

Oxygen concentration in mitochondrial suspensions 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. The respiratory control ratio (state 3/state 4 respiratory rate) of mitochondrial preparations was more than 4.0, measured with 2 mM succinate as substrate. Mangiferin at 25 μM did not change the respiratory control of isolated rat liver mitochondria, or the respiratory rate of state 4 (data not shown). Determination of O_2 concentration in media without mitochondria was also performed with a Clark-type electrode.

2.4. Fe^{2+}/citrate and tert-butylhydroperoxide-mediated mitochondrial membrane lipid peroxidation assay

Lipid peroxidation was estimated from malondialdehyde generation (Buege and Aust, 1978). The mitochondrial suspension (1 ml, 1 mg protein) was incubated in the standard medium with 4 mM *tert*-butylhydroperoxide or 50 μM Fe^{2+} plus 2 mM sodium citrate at 37 °C. After 20 min, 1 ml of 1% thiobarbituric acid (TBA, prepared in 50 mM NaOH), 0.1 ml of 10 M NaOH and 0.5 ml of 20% H_3PO_4 were added, followed by incubation for 20 min at 85 °C. The malondialdehyde–thiobarbituric acid complex was extracted with 2 ml of *n*-butanol and absorbance was measured at 535 nm. Malondialdehyde concentration was calculated from $\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

2.5. Mitochondrial swelling

Mitochondrial swelling was estimated from the decrease in turbidity of the mitochondrial suspension measured at 520 nm in a Hitachi U-3000 spectrophotometer.

2.6. Measurement of mitochondrial transmembrane electrical potential ($\Delta\Psi$)

The mitochondrial membrane potential was estimated as fluorescence changes of safranin (Åkerman and Wikstrom, 1976), recorded on a model F-4010 Hitachi spectrofluorimeter operating at excitation and emission wavelengths of 495 and 586 nm, respectively, with a slit width of 5 nm. Mitochondria (0.5 mg/ml) were incubated in 2 ml of standard medium supplemented with 5 μM safranin and mangiferin at different concentrations. After 30 s, 5 mM succinate was added. When $\Delta\Psi$ was established, 50 μM Fe^{2+} was added to the medium and the times of $\Delta\Psi$ collapses were compared. Relative changes in membrane potential were expressed in arbitrary fluorescence units and were not converted to potential values.

2.7. Determination of Fe^{2+} oxidation and Fe^{3+} reduction by ascorbate

The concentration of Fe^{2+} was quantified in reaction medium (2 ml) without mitochondria using 5 mM 1,10-phenanthroline as previously described (Hermes-Lima et al., 1995). The red complex of Fe^{2+} with 1,10-phenanthroline was determined at 510 nm and compared with a Fe^{2+} standard curve.

2.8. Determination of the Fe^{2+} and Fe^{3+} complex with mangiferin

Fe^{2+} (5–50 μM, final concentration) and Fe^{3+} (50–150 μM, final concentration) were added to 2 ml reaction medium containing citrate 2 mM plus 50 μM mangiferin (final concentrations at 2 ml) and an immediate wavelength scan from 200 to 600 nm was performed in a Hitachi 2001 spectrophotometer at 28 °C. This allowed the observation of changes in the characteristic peak of mangiferin at wavelength near 380 nm due to formation of iron–mangiferin complexes.

3. Results

3.1. Mangiferin effect on iron-induced mitochondrial lipoperoxidation

Mangiferin inhibited thiobarbituric acid reactive substance formation induced by 20 min incubation with 50 μM Fe^{2+}-citrate in rat liver mitochondria. Fig. 2 (Panel A) shows a titration curve for mangiferin on thiobarbituric acid reactive substance, the IC_{50} values were 9.02 ± 1.12 μM. Panel B also shows mangiferin effects against mitochondrial lipid peroxidation induced by 20 min incubation with 300 μM *tert*-butylhydroperoxide, with a higher IC_{50} value (> 100 μM). These results show that mangiferin is more effective in preventing iron-induced lipid peroxidation than peroxyl induced lipid peroxidation, suggesting its iron-chelating ability.

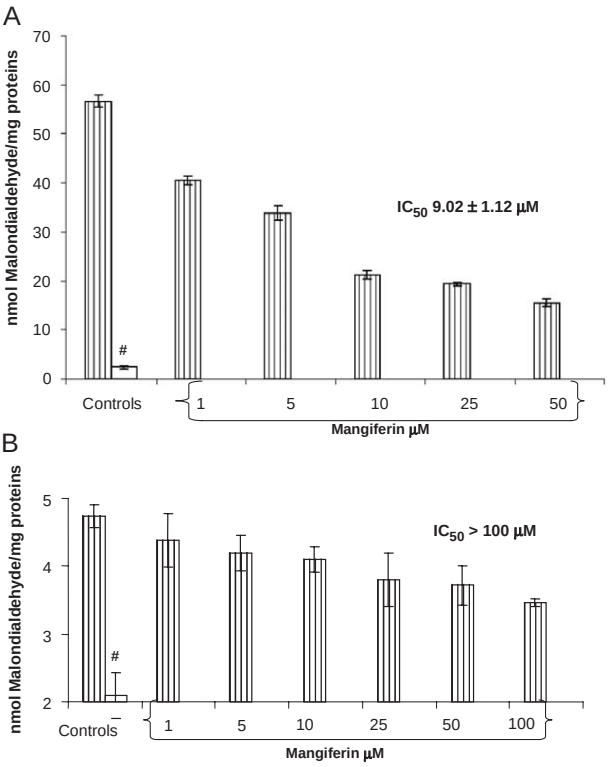

Fig. 2. Mangiferin inhibits malodialdehyde–thiobarbituric acid adducts formation induced by 50 μM Fe^{2+}-citrate (Panel A) or 300 μM *tert*-butylhydroperoxide (Panel B). Rat liver mitochondria (1 mg/ml) were incubated in reaction medium containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES buffer (pH 7.2), 2 mM succinate and 2.5 μM rotenone, with or without mangiferin (1–50 μM). The experiments were initiated by addition of 50 μM Fe^{2+} or 300 μM *tert*-butylhydroperoxide (except for the control #). Incubation period was 20 min at 28 °C. Values are the means ± S.D. (n = 6).

Lipid peroxidation was also determined by measuring O_2 consumption of mitochondria in the presence of 2 μM antimycin A. The arrest of mitochondrial respiration by this inhibitor suggests that O_2 uptake under these conditions is caused by lipid peroxidation (Hermes-Lima et al., 1995). Indeed, O_2 uptake induced by Fe^{2+}-citrate was almost totally prevented by butylated hydroxytoluene, a well-known chain-breaking antioxidant that stops the propagation of lipid peroxidation reaction (Castilho et al., 1994). Addition of 50 μM Fe^{2+} to a mitochondrial suspension containing 2 mM citrate induced extensive O_2 consumption (Fig. 3, line e) preceded by a lag phase (Cadenas and Sies, 1998). This extensive O_2 consumption was inhibited by 300 mM desferrioxamine (result not

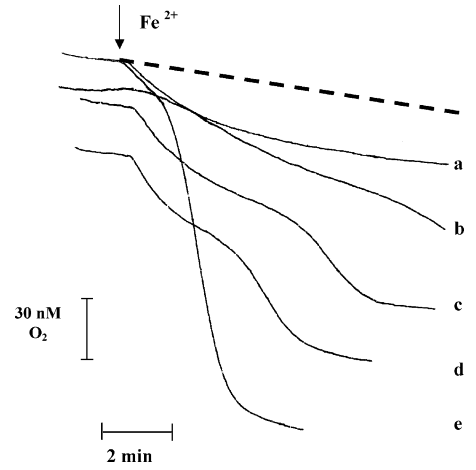

Fig. 3. Mangiferin inhibits mitochondrial lipid peroxidation measured as O_2 consumption. Experimental conditions are as in Fig. 2, except that 2.5 μM antimycin A was present. Iron was 50 μM. Rat liver mitochondria (0.5 mg/ml) were incubated in reaction medium containing: (a) 10 μM mangiferin; (b) 5 μM mangiferin; (c) 2.5 μM mangiferin; (d) 1 μM mangiferin; (e) no mangiferin. Dashed line indicates O_2 consumption in mitochondrial suspension without Fe^{2+} addition. Results are representative of three experiments.

shown). Mangiferin was also able to inhibit iron-induced O_2 consumption in a concentration-dependent manner (lines a–d). At 10 μM mangiferin, the rate of O_2 consumption was slightly higher than in controls, that is, in the absence of iron (dashed line). Mangiferin also prolonged the lag phase observed between Fe^{2+} addition and oxygen burst due to lipid peroxidation. The small O_2 consumption observed just after addition of Fe^{2+} was due to Fe^{2+} autoxidation (see Fig. 5B and C for a detailed study).

3.2. Effect of mangiferin on iron-mediated mitochondrial swelling and loss of $\Delta\Psi$

Iron-induced damage to the inner mitochondrial membrane can be assessed by the classic swelling techniques, which monitor the net influx of the osmotic support (sucrose, KCl) associated with a nonspecific increase in membrane permeability. It was previously shown that Fe^{2+}-citrate induces mitochondrial swelling, sensitive to butylated hydroxytoluene, due to lipid peroxidation (Castilho et al., 1994). Fig. 4A (line f) shows that Fe^{2+}-citrate complex induced mitochondrial swelling as revealed by the large decrease in absorbance of the mitochondrial suspension at 540 nm. It was associated with complete $\Delta\Psi$ depolarization as observed after addition of the mitochondrial uncoupler carbonyl cyanide *p*-trifluorophenylhydrazone (Fig. 4B, line f).

Mangiferin inhibited the swelling process in a concentration-dependent manner (Fig. 4A, lines b–e). Nearly full protection was attained at 10 μM. It also prevented $\Delta\Psi$ dissipation induced by 50 μM Fe^{2+}-citrate in a dose-dependent fashion, with near-full protection at 10 μM.

3.3. Fe^{2+}-citrate autoxidation and O_2 consumption induced by mangiferin

Mangiferin reduced Fe^{2+} concentration in reaction medium containing 2 mM citrate (without mitochondria) in a dose-dependent manner (Fig. 5A, line a). A 5-min preincubation period (Fig. 5A, line b) reduced Fe^{2+} concentration even more. Mangiferin also increased the rate of O_2 consumption, possibly due to the stimulatory effect on oxidation of Fe^{2+} to Fe^{3+} (Fig. 5B,C). These results suggest that mangiferin could be removing Fe^{2+} from citrate complex and oxidizing it to a ferric form in a process that requires O_2 as electron acceptor. According to this, mangiferin could be diminishing the amount of Fe^{2+} involved in the formation of $^{\bullet}OH$ radical through the Haber–Weiss-type reaction, which can initiate the perox-

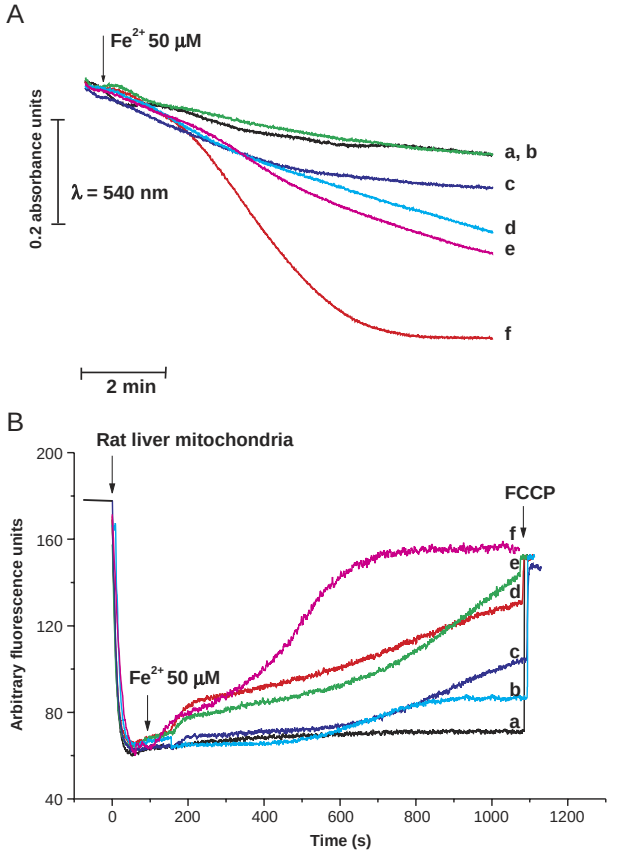

Fig. 4. (A) Mangiferin inhibits mitochondrial swelling induced by 50 μM Fe^{2+}-citrate. Experimental conditions are described in legend of Fig. 2. Reaction medium contains: (a) No Fe^{2+} addition, (b) 10 μM mangiferin, (c) 5 μM mangiferin, (d) 2.5 μM, (e) 1 μM mangiferin, (f) no mangiferin. Fe^{2+} (50 μM) was added where indicated by the arrow (b–f). Results are representative of three experiments. (B) Mangiferin inhibits loss of mitochondrial potential ($\Delta\Psi$) induced by 50 μM Fe^{2+}-citrate. Experimental conditions are described in legend of Fig. 2, except that 5 μM safranine was present. Reaction medium contains: (a) No Fe^{2+} addition, (b) 10 μM mangiferin, (c) 5 μM mangiferin, (d) 2.5 μM, (e) 1 μM mangiferin, (f) no mangiferin. Rat liver mitochondria (0.5 mg/ml), 50 μM Fe^{2+} and 1 μM carbonyl cyanide *p*-trifluorophenylhydrazone (FCCP) were added where indicated by the arrows (a–f). Results are representative of three experiments.

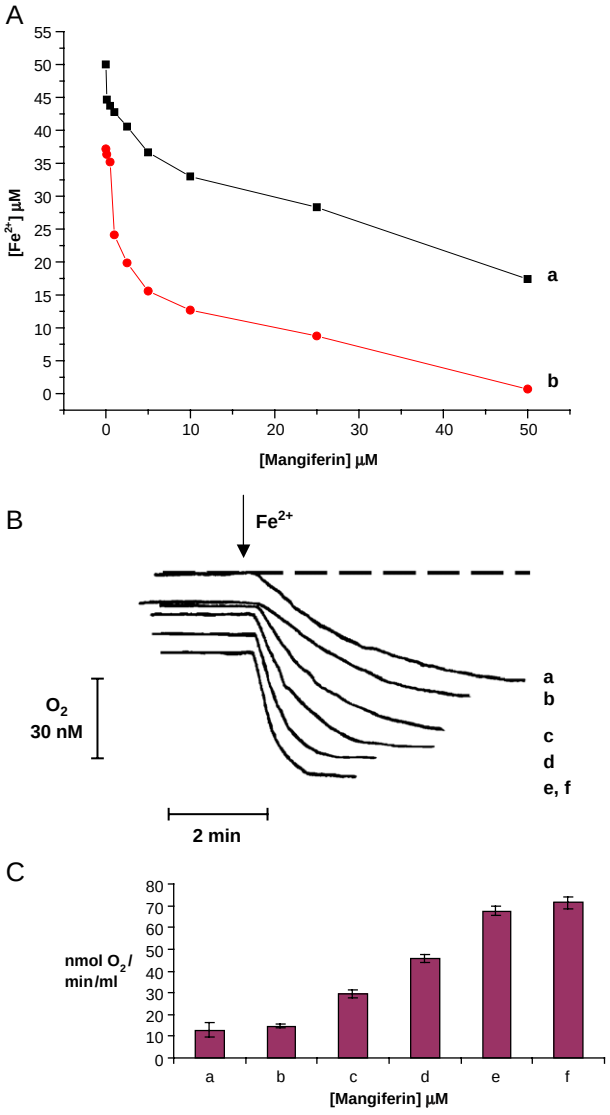

Fig. 5. (A) Mangiferin stimulates Fe^{2+} autoxidation in the absence of rat liver mitochondria. Experimental conditions: 125 mM sucrose, 65 mM KCl, 10 mM Hepes buffer (pH 7.2), 2 mM citrate, 5 mM 1,10-phenanthroline. (a) 1,10-phenantroline was added immediately after Fe^{2+} addition and absorbance was read at 510 nm, (b) 1,10 phenantroline was added after 5 min of mangiferin–Fe^{2+} incubation period. Experiments were conducted at 28 °C with agitation and were started by the addition of 50 µM Fe^{2+}. Values are the average of three determinations. (B) Effects of mangiferin on O_2 consumption mediated Fe^{2+} autoxidation in the absence of rat liver mitochondria. Experimental conditions: 125 mM sucrose, 65 mM KCl, 10 mM Hepes buffer (pH 7.2), 2 mM citrate. Experiments were conducted at 28 °C; Fe^{2+} (50 µM) was added where indicated by the arrow. (a) No mangiferin, (b) 1 µM mangiferin, (c) 5 µM mangiferin, (d) 10 µM mangiferin, (e) 25 µM mangiferin, (f) 50 µM mangiferin. Dashed line indicates O_2 consumption in reaction medium without Fe^{2+} addition. Results are representative of three experiments. (C) Oxygen consumption rate (nmol O_2/min/ml). Legends are the same as in (B).

idation of mitochondrial membrane. The stoichiometry for the autoxidation of Fe^{2+}–mangiferin complexes was 1.8 ± 0.2, suggesting the participation of reactive oxygen species in mangiferin-induced Fe^{2+} oxidation. Interestingly, the presence of catalytic amounts of superoxide dismutase enzyme and/or catalase diminished the amount of O_2 consumed by Fe^{2+} autoxidation but did not modify mangiferin effects (results not shown), indicating some ferroxidase-like activity of this xanthone, which could catalyze Fe^{2+} oxidation with subsequent O_2 reduction to water, without hydrogen peroxide (H_2O_2) or superoxide ($O_2^{\bullet -}$) anion radical formation.

3.4. Spectroscopic characteristics of $Fe^{2+/3+}$–mangiferin complexes

The oxidation of Fe^{2+} via direct electron abstraction by mangiferin seems unlikely under aerobic conditions. An alternative hypothesis is that this compound forms a charge transfer complex with ferrous iron, facilitating its oxidation by oxygen. This transient complex with Fe^{2+} could deliver its electrons more readily than Fe^{2+}-citrate complex and could form a more stable complex with Fe^{3+}. To search for these possibilities, absorption spectra of samples containing 50 µM mangiferin with growing concentrations of Fe^{2+} (5–20 µM) or Fe^{3+} (50–150 µM) were made in reaction medium containing 2 mM of citrate. Fig. 6A and B, line a, show a characteristic spectrum of mangiferin (reaction medium plus 2 mM citrate, pH 7.2) with maximum absorption at 275 and 380 nm. Addition of Fe^{2+} induced a small, but measurable, dose-dependent red shift in the position of absorption maxima of mangiferin (from 380 to 387 nm) (Fig. 6A, lines b, c, d). The occurrence of a new family of spectra originated from mangiferin spectrum was confirmed by the presence of an isosbestic point at wavelength near 338 nm with emerging absorption peaks at 338 and 455 nm. Addition of freshly prepared $FeCl_3$ (50 µM final concentration) to 50 µM mangiferin solution originated nearly the same spectrum as that with 50 µM Fe^{2+} (Fig. 5B, line b). Growing concentrations of Fe^{3+} increased absorbance at 338 nm and originated a new family of spectra, nonisosbestic with mangiferin spectrum (isosbestic point at 512 nm). It is likely that the formation of such complexes facilitates electrotransfer from electron-donating components (Fe^{2+}–mangiferin) to the electron-accepting components (oxygen) and that it could be responsible for the net Fe^{2+} oxidation observed in the presence of mangiferin.

3.5. Assays of inhibition of Fe^{3+} reduction by ascorbate

The lack of major spectral differences between the two complexes (mangiferin complexes to Fe^{2+} or to Fe^{3+}) may have been not only due to oxidation of Fe^{2+} to Fe^{3+} but also due to the reduction of Fe^{3+} to Fe^{2+} by mangiferin. This last proposal could reload biological systems with Fe^{2+}, which participates in Fenton–Haber–Weiss reactions, generating the extremely reactive $^{\cdot}OH$ radical. Mangiferin–Fe^{3+}complex could also allow the oxidation of biological relevant reducers like ascorbate with subsequent Fe^{2+} regeneration.

To examine these possibilities, we used 1,10-phenantroline to measure the levels of Fe^{2+} formation from Fe^{3+} solution (50 μM) treated with 4 mM ascorbate and different mangiferin concentrations. We also determined the rate of O_2 consumption due to autoxidation of Fe^{2+} originated from Fe^{3+} ascorbate reduction. Fig. 7A shows that mangiferin does not reduce Fe^{3+} (line f); indeed, this xanthone inhibits the reduction of ferric ions elicited by ascorbate in a dose-dependent fashion, reaching near full inhibition at 25 μM (lines a–e). These results suggest a composition of 1:2 for mangiferin–Fe^{3+} complex at pH 7.2, with excess iron. Fig. 7B confirms these results and also discards a possible mangiferin–ascorbate interaction. Line a shows null oxygen consumption in a ferric solution (50 μM) with 25 μM mangiferin (without ascorbate), indicating no reduction process in ferric ions. Line d shows oxygen consumption due to Fe^{2+} (formed by Fe^{3+} ascorbate reduction) autoxidation. Line b represents the inhibitory effects of 25 μM mangiferin on Fe^{2+} formation from Fe^{3+} ascorbate reduction. Oxygen consumption was almost completely inhibited,

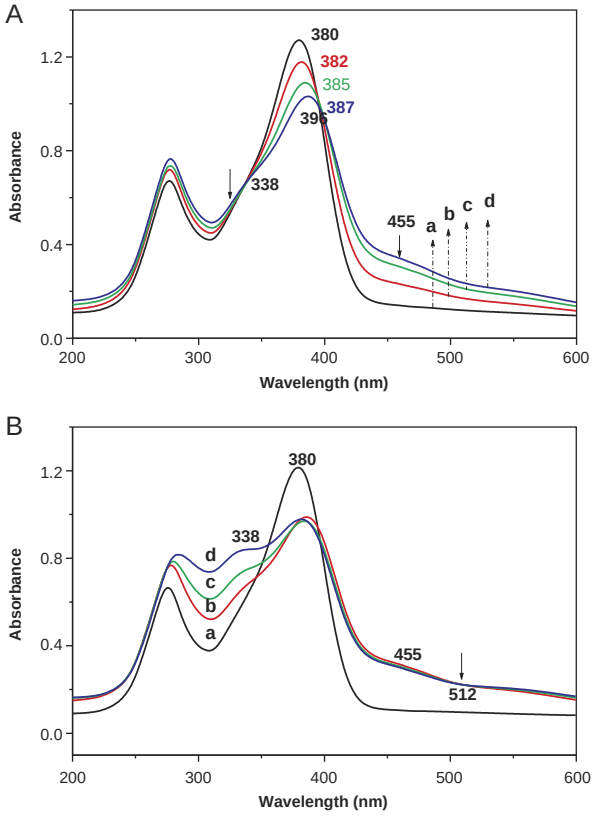

Fig. 6. Effects of Fe^{2+} and Fe^{3+} on mangiferin UV–VIS spectrum (200–600 nm). Incubation mixture containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES buffer (pH 7.2), 2 mM citrate, and 50 μM mangiferin. Panel A: (a) mangiferin 50 μM (no Fe^{2+} addition); (b) mangiferin 50 μM+5 μM Fe^{2+}; (c) mangiferin 50 μM+10 μM Fe^{2+}; (d) mangiferin 50 μM+20 μM Fe^{2+}. Panel B: (a) mangiferin 50 μM (no Fe^{3+} addition); (b) mangiferin 50 μM+50 μM Fe^{3+}; (c) mangiferin 50 μM+100 μM Fe^{3+}; (d) mangiferin 50 μM+150 μM Fe^{3+}. Experiments were conducted at 28 °C. Scan speed was 2 nm/s. A baseline was established with incubation mixture plus 50 μM Fe^{2+} (Panel A) and 150 mM Fe^{3+} (Panel B). Typical examples are shown.

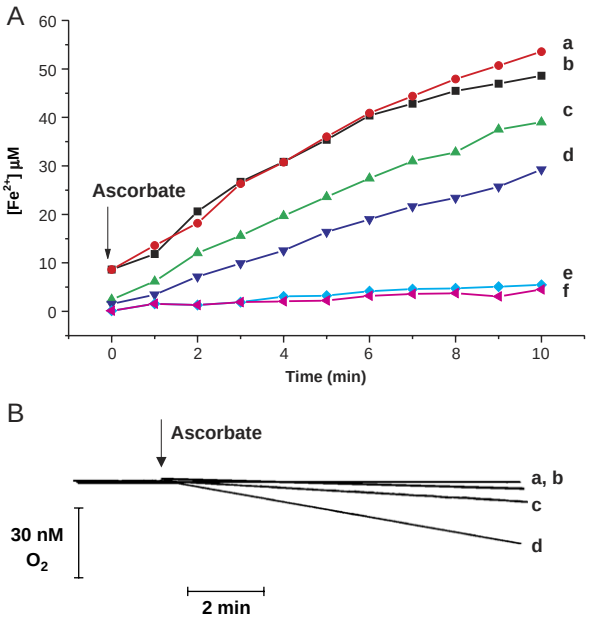

Fig. 7. (A) Mangiferin inhibits Fe^{3+}-reduction by ascorbate in the absence of rat liver mitochondria. Experimental conditions: 125 mM sucrose, 65 mM KCl, 10 mM HEPES buffer (pH 7.2), 2 mM citrate, Fe^{3+} 50 μM, 5 mM 1,10-phenanthroline. Experiments were conducted at 28 °C. Ascorbate (4 mM) was added where indicated by the arrow. (a) No mangiferin; (b) 1 μM mangiferin; (c) 5 μM mangiferin; (d) 10 μM mangiferin; (e) 25 μM mangiferin; (f) no ascorbate addition. Values are the average of three determinations. (B) Effect of mangiferin on O_2 consumption due to Fe^{2+} (formed by Fe^{3+} ascorbate reduction) autoxidation in the absence of rat liver mitochondria. Experimental conditions: 125 mM sucrose, 65 mM KCl, 10 mM HEPES buffer (pH 7.2), 50 μM Fe^{3+}, 2 mM citrate. Experiments were conducted at 28 °C. Ascorbate 4 mM was added where indicated by the arrow. (a) Mangiferin 25 μM, no ascorbate addition; (b) mangiferin 25 μM; (c) mangiferin 10 μM; (d) no mangiferin. Traces are representative of three assays.

which shows mangiferin capacity to inhibit ascorbate-mediated Fe^{3+} reduction to Fe^{2+}.

4. Discussion

The present study investigated the protective effect of mangiferin, a naturally occurring glucosylxanthone, against mitochondrial oxidative damage induced by iron. Citrate was chosen as an iron chelator due to its potential pathological relevance and ability of shifting the Fe^{3+}/Fe^{2+} coupled reduction potential from + 0.77 V to + 0.33 V, which causes high rates of Fe^{2+} autoxidation (Halliwell and Gutteridge, 1986; Hamm et al., 1954). Mangiferin protected the mitochondrial membrane against lipid peroxidation and preserved its integrity.

Fe^{2+}-citrate induces membrane protein oxidation and lipid peroxidation with consequent mitochondrial depolarization and swelling due to nonspecific permeabilization of the inner mitochondrial membrane (Castilho et al., 1994). Addition of 1–10 μM mangiferin to the mitochondrial suspensions inhibited Fe^{2+}-citrate-mediated mitochondrial

swelling, loss of transmembrane potential, thiobarbituric acid reactive substance formation, and O_2 consumption during lipid peroxidation (Figs. 1–4).

A recent report demonstrated that an aqueous extract of *Mangifera indica* L. stem bark with mangiferin as a major component (~ 20%) inhibited bleomycin-iron dependent and copper-phenantroline dependent DNA-damage; it also showed that the natural extract inhibited phospholipid peroxidation, showing a powerful effect on nonenzymic peroxidation (with Fe^{3+}/ascorbate) (Martinez et al., 2000). In this work, we show for the first time the iron-complexing ability of mangiferin, which could explain the results obtained with the xanthone-containing extract. The data of the present study suggest that mangiferin removes iron from the Fe^{2+}-citrate complex and forms an unstable complex with it, favoring Fe^{2+} oxidation to Fe^{3+} with subsequent formation of a more stable complex with Fe^{3+}, which is not able to initiate and/or propagate mitochondrial lipid peroxidation. This Fe^{3+}–mangiferin complex impairs ferric irons reduction to ferrous iron by endogenous reducers like ascorbate, sparing them and also preventing Fe^{2+} reloading of the biological system, which can readily participate in the Haber–Weiss-type reactions involved in $^{\bullet}OH$ formation.

Mangiferin may prevent both the initiation and the propagation of mitochondrial lipid peroxidation induced by Fe^{2+}-citrate (Fig. 3). Fe^{2+}-citrate autoxidation may generate reactive species such as $^{\bullet}OH$, which can initiate the peroxidation of mitochondrial phospholipids (Kachur et al., 1998). It was observed that liposomal peroxidation is initiated by Fe^{2+}-citrate autoxidation and that lipid peroxidation requires both Fe^{3+} and Fe^{2+}, perhaps with oxygen to form a Fe^{3+}–dioxygen–Fe^{2+} complex as another possible lipid peroxidation initiator species (Minotti and Aust, 1987). Moreover, Fe^{2+}-citrate and Fe^{3+}-citrate may catalyze preformed lipoperoxide decomposition, producing either peroxyl or alkoxyl species that feed the propagation process. Possibly, Fe^{2+}/Fe^{3+}–mangiferin complexes are not involved in the initiation or propagation steps of mitochondrial peroxidation, which may partially explain the antiperoxidative effect of this compound. It was reported that mangiferin rapidly scavenged 1,1-diphenyl-2-picrylhydrazyl radicals and inhibited lipid peroxidation, which was initiated enzymatically by reduced nicotinamide adenine dinucleotide phosphate (NADPH) or nonenzymatically by ascorbic acid or Fenton's reagent (H_2O_2+Fe^{2+}) in rat liver microsomes (Sato et al., 1992). It was also reported that in vivo mangiferin pretreatment for 7 consecutive days reduced 12-*O*-tetradecanoylphorbol-13-acetate-induced mitochondrial lipoperoxidation by 15% (Sánchez et al., 2000). These results suggest that besides its iron-chelating capacity, mangiferin could also protect mitochondria from Fe^{2+}-citrate lipid peroxidation through direct free radical scavenging ability, mainly lipoperoxyl and alkoxyl radicals, acting as a chain-breaking antioxidant or stimulating endogenous mitochondrial antioxidant system (enzymatic or not).

The synergistic action of these mechanisms could explain the extremely low dose of mangiferin (10–25 μM) which elicited protection against iron citrate mitochondrial oxidative damage, although the IC_{50}>100 μM obtained in protection of *tert*-butylhydroperoxide induced mitochondrial lipid peroxidation (Fig. 2B) suggests the iron chelating capacity of mangiferin as a primary and most important mechanism of its antiperoxidative ability.

The results presented in this manuscript are of pharmacological importance since mitochondria are significant targets for iron-promoted free radical formation and lipid peroxidation in diseases related to abnormal intracellular iron distribution and/or iron overload, such as hereditary hemochromatosis, β-thalassemia, Friedreich's ataxia and sideroblastic anemia (Britton et al., 2002; Eaton and Qian, 2002; Faa et al., 2002; Puccio and Koenig, 2002; Sheth and Brittenham, 2000). Iron chelators such as mangiferin could be an important approach to reduce iron-induced oxidative damage in these pathologies. Iron induced peroxidation of mitochondrial membrane lipids is associated with impairment of membrane-dependent functions of mitochondria and lysosomes. Iron overload impairs hepatic mitochondrial respiration primarily through a decrease in cytochrome *C* oxidase activity, and hepatocellular calcium homeostasis may be compromised through damage to mitochondrial and microsomal calcium sequestration mechanisms (Britton et al., 2002). Mitochondrial DNA has also been reported to be a target for iron-induced damage, and this may have consequences in regard to malignant transformation (Itoh et al., 1994). Mitochondrial membrane damage may also release different apoptogenic factors into the cytosol, including cytochrome *C*, apoptosis inducing factor and procaspases, which results in apoptosis (Green and Reed, 1998). Mitochondrial respiratory enzymes and plasma membrane enzymes such as sodium–potassium–adenosine triphosphatase (Na(+)+K(+)-ATPase) may be key targets of damage by non-transferrin-bound iron in cardiac myocytes. Reduced cellular levels of ATP, lysosomal fragility, impaired cellular calcium homeostasis, and damage to DNA all may contribute to cellular injury in iron overload and all may be prevented by mangiferin.

The fact that 10–25 μM mangiferin was able to produce more than 50% protection against mitochondrial damage mediated by 50 μM iron is of particular interest. These concentrations are lower than those of classical iron chelators like pyridoxal isonicotinoyl hydrazone, which was able to induce more than 80% protection against iron (50 μM) induced mitochondrial damage at 100 μM (Santos et al., 2001). Since in vivo levels of free or loosely bound iron are hardly higher than 1 μM, even in iron overload (Halliwell and Gutteridge, 1999), less than 2 μM of mangiferin (taking into account the possibility of 2:1 of mangiferin–iron complexes) would afford protection against iron-mediated mitochondrial damage. This represents around 1 μg/ml of mangiferin. A recent report shows that the administration of 10 mg/kg of mangiferin to Wistar rats

sustained mean free levels of mangiferin in rat blood over 1 μg/ml during approximately 1 h (Lai et al., 2003). Also, oral administration of mangiferin-containing natural extract to rats elicited plasma mangiferin concentrations higher than 1 μg/ml (Dai et al., 2004). 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 (Halliwell, 1991). 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, protects mitochondrial damage induced by Fe^{2+}-citrate, mainly through its iron-complexing ability. Further research on the putative beneficial effects of mangiferin on 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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