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Interaction of Vimang (*Mangifera indica* L. extract) with Fe(III) improves its antioxidant and cytoprotecting activity

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Abstract

A standard aqueous stem bark extract from selected species of *Mangifera indica* L. (Anacardiaceae)—Vimang, whose major polyphenolic component is mangiferin, displays potent *in vitro* and *in vivo* antioxidant activity. The present study provides evidence that the Vimang–Fe(III) mixture is more effective at scavenging 2,2-diphenyl-1-picrylhydrazyl (DPPH) and superoxide radicals, as well as in protecting against *t*-butyl hydroperoxide-induced mitochondrial lipid peroxidation and hypoxia/reoxygenation-induced hepatocytes injury, compared to Vimang alone. Voltammetric assays demonstrated that Vimang, in line with the high mangiferin content of the extract, behaves electrochemically like mangiferin, as well as interacts with Fe(III) in close similarity with mangiferin's interaction with the cation. These results justify the high efficiency of Vimang as an agent protecting from iron-induced oxidative damage. We propose Vimang as a potential therapy against the deleterious action of reactive oxygen species generated during iron-overload, such as that occurring in diseases like β-thalassemia, Friedreich's ataxia and haemochromatosis.

Keywords: *Mangifera indica* L.; Vimang; Mangiferin; Iron; Reactive oxygen species; Antioxidants; Cytoprotection

1. Introduction

It is generally assumed that the capacity of polyphenols to chelate iron is important for their antioxidant activity caused by "site-specific scavenging" [1]. This means that if the iron is still catalytically active, the radicals are formed in the vicinity of the polyphenol, which surrounds the iron, and can be scavenged immediately. Polyphenols would thus exert a double synergistic action that would make them possible powerful antioxidants against pathological conditions of iron overload like β-thalassemia, Friedreich's ataxia and haemochromatosis.

Recently a standard aqueous stem bark extract from selected species of *Mangifera indica* L. (Anacardiaceae), used as a food supplement in Cuba under the brand name of Vimang, has been reported to display potent *in vitro* and *in vivo* antioxidant activities [2,3] and a strong anti-inflammatory action [4]. This extract contains phenolic acids, phenolic esters, flavan-3-ols and as the major catechol polyphenolic component, mangiferin [5]; it has been proposed that its interaction with iron is responsible for its high antioxidant activity [2,6–9]. Indeed, catechol molecules complexes with iron would show an increased free radical scavenging and cytoprotecting actions compared to those of not-complexed compounds [10–13]. Considering the high mangiferin content of Vimang, this compound probably accounts for most of the chelating/antioxidant capacities of the whole extract.

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In the present study we have evaluated the interaction of Vimang with Fe(III), as well as its antioxidant activity in the presence or not, of the cation. The Vimang–Fe(III) mixture showed to be more effective than the extract alone, both at scavenging superoxide radicals generated by pyrogallol autoxidation and at protecting against ROS-mediated hypoxia/reoxygenation-induced hepatocytes injury.

2. Materials and methods

2.1. Plant material

M. indica was collected from a cultivated field located in the region of Pinar del Rio, Cuba. Voucher specimens of the plant (Code: 41722) were deposited at the Herbarium of the Academy of Sciences, guarded by the Institute of Ecology and Systematic, Ministry of Science, Technology and Environment, La Habana, Cuba. Stem bark extract of *M. indica* was prepared by decoction for 1 h. The extract was concentrated by evaporation and spray-dried to obtain a fine brown powder, which was used as the standardized active ingredient of Vimang formulations. It melts at 210–215 °C with decomposition.

The chemical composition of this extract has been characterized by chromatographic (planar, liquid and gas) methods, mass spectrometry and UV–vis spectrophotometry [5]. The total polyphenol content of Vimang determined by the Folin–Ciocalteu method, and expressed as gallic acid equivalents, was 34 g/100 g dry weight. Mangiferin content was determined by HPLC methods and represents 16% (w/w) of powdered extract [5].

2.2. Reagents and solutions

Mangiferin, tris(hydroxymethyl)aminomethane (Tris), dimethyl sulfoxide (DMSO), *tert*-butyl hydroperoxide, antimycin A, ethylene glycol-bis (β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), *N*-(2-hydroxyethyl) piperazine-*N*′-2-ethanesulfonic acid (HEPES), rotenone, succinate and ferric chloride anhydrous were purchase from Sigma (St. Louis, MO). All other reagents were products of the highest purity grade available. Ferric ($FeCl_3$ + 10 mM HCl) ion solutions were prepared in Milli-Q water and immediately used. Stock solutions of Vimang were prepared dissolving the solid extract in DMSO, in concentrations from 1 to 100 mM equivalents to mangiferin concentration (2.6–260 mg ml^{-1} powder extract). Pure mangiferin was also dissolved in DMSO (10 mM final concentration). Experiments were carried out with dilution of 1/1000 (1–10 μM) to prevent solvent influence on the results. Vimang–iron mixture were daily prepared by mixing Vimang solutions in DMSO with acidic ferric solutions, at proportions that correspond to 2:1 (mangiferin–Fe(III)) ratio. Adjustment was made considering 16% of mangiferin into the whole extract. Controls experiments (without Vimang) contained 14.3 μM DMSO, which represents the solvent concentration in all Vimang samples. Other chemicals were dissolved in Millipore filtered water.

2.3. Electrochemical assays

Electrochemical assays were carried out with a BAS CV-27 potentiostat. Data were recorded on an Omnigraphic XY recorder. Conventional electrochemical cells with three electrodes were employed. A glassy carbon electrode geometric area 0.0314 cm^2 was used as working electrode. It was polished prior to use with an alumina water suspension and rinsed thoroughly with water and acetone. A platinum wire was used as counter electrode and all potentials are referenced to a sodium saturated silver/silver chloride electrode ($Ag/AgCl/NaCl_{(sat)}$) without regard for the liquid junction potential. Cyclic voltammetric studies were carried out at sweep rates 10 and 100 mV s^{-1} in 5 ml of phosphate buffer solution pH 8.2, where 50 μl of 100 mM dimethylsulfoxide solution of mangiferin was added. Since the cyclic voltammograms were recorded at a window potential including negative ranges (−0.4 to 1.0 V), oxygen-free solution was obtained by bubbling argon through it. When the potential was scanned, the inert gas was kept in the solution to ensure that any oxidation reaction was begun by oxygen. For the electrochemical oxidation of the extract in presence of Fe(III) ion, aliquots of a fresh acidic aqueous solution of $FeCl_3 \cdot 6H_2O$, were subsequently added to give final concentrations of 0.25, 0.50, and 2 mM. The solution was stirred for 30 s and allowed to rest for 30 s for equilibration. The potential was scanned at 10 and 100 mV s^{-1}.

2.4. Superoxide scavenging assay

Superoxide scavenging was measured based on the inhibitory action of superoxide dismutase on the rate of the base-catalyzed auto-oxidation of pyrogallol [14]. The assay medium contained 0.05 M Tris buffer, pH 8.2, and the reactions were started by the addition of pyrogallol (100 μM final concentration), and recorded during 1 min at 420 nm. The linear slope (with $r^2 > 0.99$) was calculated, and the increase in absorbance after this time was taken as the initial rate of auto-oxidation.

2.5. DPPH scavenging assay

Reduction of the 1,1-diphenyl-2-picrylhydrazyl radical (100 μM) by Vimang (1–5 μM mangiferin equivalents)/Vimang–Fe(III) mixture was monitored from the change in absorbance at 517 nm, 5 min after compounds were incubated with 40 mM sodium acetate, pH 5.5, and 1 ml ethanol (2.5 ml final volume) [15].

2.6. Measurement of oxygen consumption in antimycin-treated mitochondria

Oxygen consumption in mitochondrial suspension 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 30 °C. Mitochondria were isolated by conventional differential centrifugation from the liver of adult animals fasted overnight [16]. The experiment was performed in a standard medium containing 125 mM sucrose, 65 mM KCl,

2 μM rotenone and 2 μM antimycin A in 10 mM HEPES buffer, pH 7.2.

2.7. Cytotoxicity assays in rat hepatocytes

Adult male Wistar rats, 250–300 g, were fed *ad libitum* and allowed to acclimatize for 1 week on clay chip bedding. Hepatocytes were isolated from these animals by collagenase perfusion of the liver [17]. Isolated hepatocytes (10^6 cells ml^{-1}, 10 ml) were suspended in Krebs–Henseleit buffer containing 12.5 mM HEPES, pH 7.4, in a continuously rotating round-bottomed 50 ml flasks, under an atmosphere of 95% O_2 and 5% CO_2 in water bath of 37 °C, for 30 min. The Vimang or its Fe(III) mixture were then added to the flasks and the hepatocytes incubated under an atmosphere of 95% N_2 and 5% CO_2 for 90 min, prior to re-oxygenation in an 1% O_2, 94% N_2, and 5% CO_2 atmosphere. Cell viability was assessed by determining Trypan blue (0.1%, w/v) exclusion from the hepatocytes after 90 min of re-oxygenation. The superoxide radical scavenging activity of Vimang (40 μM mangiferin equivalents) was compared with its 2:1 Fe(III) mixture. A 10 min premixed Vimang:Fe(III) mixture (20:10 mM) was prepared prior addition to the hepatocytes.

3. Results

3.1. Evidence for a Vimang–Fe(III) interaction by electrochemical analysis

The influence of Fe(III) on the voltammetric behaviour of Vimang (1 mM mangiferin equivalents), is shown on Fig. 1. An irreversible oxidation at ca +0.58 V versus Ag/AgCl/$NaCl_{sat}$ in the first anodic scan, whose amplitude decreased after subsequent scanning, was observed for the extract alone. We ascribed it as a catechol-localized process in mangiferin [18], the main component of Vimang, since a similar oxidation process was observed for the compound. Growing concentrations of Fe(III) yielded a concentration-dependent reduction on the anodic current peak height, apparently resulting from the coordination of the catechol moiety of mangiferin with iron (Fig. 1, panel B, lines b and c).

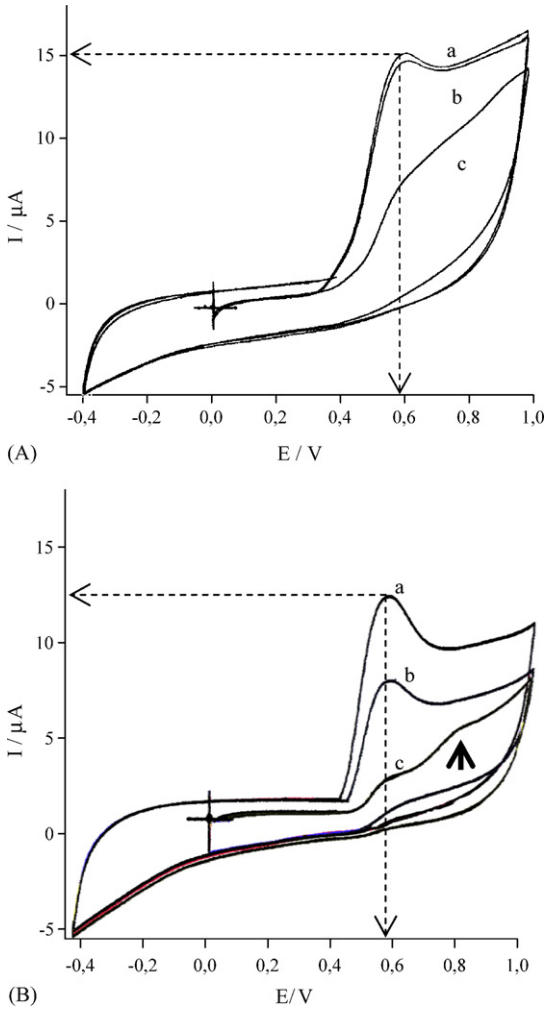

Fig. 1. Cyclic voltammograms of: (A) 1 mM Vimang–mangiferin equivalents (2.6 mg ml^{-1} powder extract) and (B) 1 mM pure mangiferin (trace a), in 0.1 M phosphate buffer pH 8.2; traces (b) and (c): Vimang or pure mangiferin plus 0.25 and 2 mM Fe(III), respectively. The scan rate was 100 mV s^{-1}; a glassy carbon electrode (area 0.0314 cm^2) was used. Experimental conditions are described in Section 2. The addition of 2 mM EDTA to Vimang or mangiferin iron mixtures (lines c conditions), restored the voltammograms of Vimang or mangiferin alone (lines a).

3.2. Effects of Vimang/Vimang–Fe(III) on mitochondrial lipid peroxidation monitored as O_2 consumption by antimycin A-treated organelles

Lipid peroxidation was monitored by measuring O_2 consumption of mitochondria in the presence of 2 μM antimycin A. The arrest of mitochondrial respiration by this respiratory chain inhibitor, suggests that O_2 consumption under this condition, is due to lipid peroxidation [19]. Fig. 2(line a) shows that the addition of 300 μM *t*-butyl hydroperoxide to the mitochondrial suspension, induced antimycin A-insensitive O_2 consumption preceded by a lag phase, that was partially inhibited by Vimang at 50 μM concentration of mangiferin equivalents (line c), and that was almost completely inhibited by a 50 μM Vimang–Fe(III) mixture. This demonstrates that Fe(III) increases the antioxidant activity of Vimang against mitochondrial membrane lipid peroxidation.

3.3. DPPH-scavenging capacity of Vimang/Vimang–Fe(III) mixture

Fig. 3 shows the scavenging activity of Vimang on DPPH, a stable free radical potentially reactive with all compounds capable of donating a hydrogen atom. The interaction with Fe(III) caused an expressive increase in the scavenging activity of Vimang against this radical, in particular when the extract was tested for relative low concentrations.

3.4. Superoxide-scavenging capacity of Vimang/Vimang–Fe(III) mixture

The superoxide radical-scavenging activities of Vimang in absence or presence of Fe(III), were compared using a non-enzymatic superoxide-generating system, namely, the base-

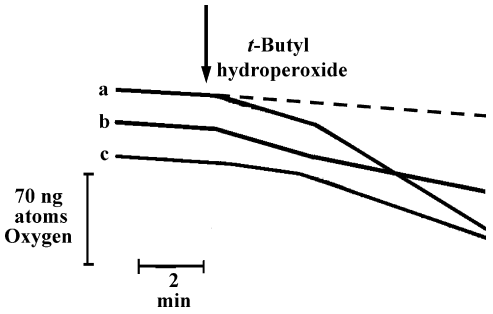

Fig. 2. Effects of Vimang, in absence or presence of Fe(III), on mitochondrial lipid peroxidation, measured as O_2 consumption. Experimental conditions are described in Section 2. *tert*-Butyl hydroperoxide (300 μM) was added in a suspension of rat liver mitochondria (1 mg ml^{-1}) in the reaction medium containing: (a) no Vimang or Fe(III); (b) 50 μM Vimang (130 μg ml^{-1} powder extract); (c) 50 μM Vimang plus 25 μM Fe(III). Dashed line indicates O_2 consumption in mitochondrial suspension without *t*-butyl hydroperoxide addition in the presence of either Fe(III), Vimang or Vimang–Fe(III). Results are representative of three experiments. The values of oxygen consumption rates were: 14.2 ± 1.01 ng atoms Oxygen min^{-1} mg protein^{-1} (line a); 5.25 ± 1.85 ng atoms Oxygen min^{-1} mg protein^{-1} (line b); 9.63 ± 1.27 ng atoms Oxygen min^{-1} mg protein^{-1} (line c); 2.75 ± 0.65 ng atoms Oxygen min^{-1} mg protein^{-1} (dashed line). Dashed line is significantly different at $P < 0.05$ from others (a and c), except for line b ($P > 0.05$). Line b (Vimang–iron) differs significantly at $P < 0.05$ from line c (Vimang).

catalyzed pyrogallol auto-oxidation. As shown on Fig. 4(panels A and B), in agreement with its well established antioxidant effect, Vimang inhibited pyrogallol autoxidation in a dose dependent manner (IC_{50} 136 ± 7 μM). Following incubation with Fe(III), the superoxide scavenging activity of the extract presented an approximately two-fold increase (IC_{50} 76.5 ± 5 μM). Neither deferoxamine–Fe(III) mixture nor Fe(III) alone exhibited such activity.

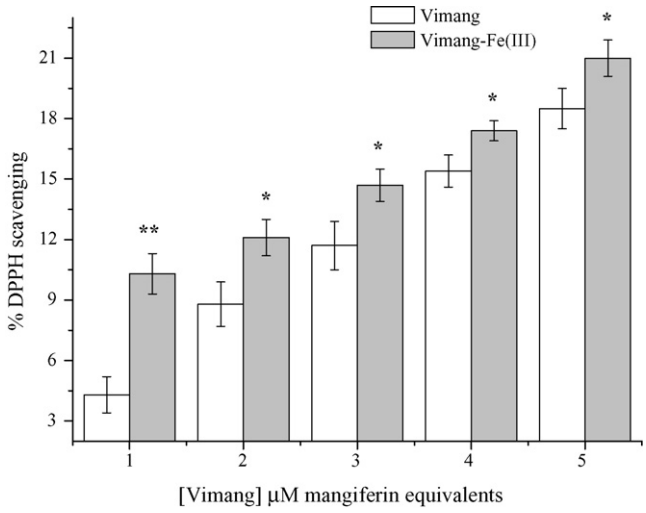

Fig. 3. Effects of Vimang, in absence (□) or presence (■) of Fe(III), on DPPH scavenging, determined as described in Section 2. Vimang concentrations corresponding to 1–5 μM mangiferin equivalents, represents 2.6–13 μg ml^{-1} powder extract, respectively. Iron was incubated 10 min with Vimang solutions at concentrations that represented the half of those from mangiferin in each sample (mangiferin:iron, 2:1). Values are means ± S.D. ($n = 3$) relative to a control in absence of Vimang or Vimang–iron mixture. Controls contained only 14.3 μM DMSO, which is the solvent concentration in the Vimang/Vimang–iron mixture samples and 2.5 μM Fe(III). Fe(III) in the absence of ligand did not present DPPH scavenging activity. A_{517} value for control was 0.422 ± 0.01. One tailed *t*-test was used for statistical analyses; $^{*}P < 0.05$, $^{**}P < 0.01$ Vimang vs. Vimang–iron mixture DPPH scavenging abilities.

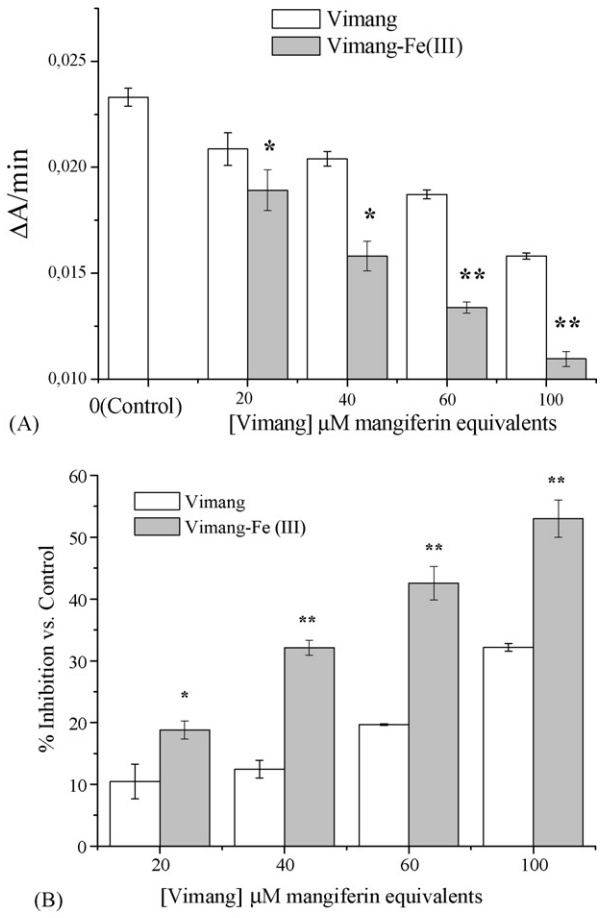

Fig. 4. (A) Evaluation of pyrogallol-generated superoxide radical scavenging activity of Vimang, in absence (□) or presence (■) of Fe(III) (A). The reaction mixture contained either Vimang (20–100 μM mangiferin equivalent, that represents 52–260 μg ml^{-1} powder extract, respectively) or Vimang–Fe(III) at 2:1 ratio, premixed during 10 min in 50 mM Tris–HCl buffer, pH 8.2. The reactions were initiated by the addition of pyrogallol (100 μM final concentration). The absorbance was monitored at 420 nm during 60 s. (B) Replot of data from A, showing the percent of protection elicited by Vimang and Vimang–Fe(III) mixture. Bars represent means ± S.D. ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ respective to the assays in absence of Fe(III). Control contained buffer plus 20 μM $FeCl_3$ and 14.3 μM DMSO. Fe(III) in the absence of ligand had no superoxide scavenging activity. The half inhibitory concentrations values (IC_{50}) were 136 ± 7 μM for Vimang and 76.5 ± 5 μM for Vimang–iron mixture.

3.5. Cytoprotective effects of Vimang/Vimang–Fe(III) mixture

As shown on Fig. 5, the superoxide dismutase protected against hypoxia/reoxygenation-induced hepatocytes injury. The Vimang–Fe(III) mixture was approximately 2.3-fold more effective than the extract alone in such protection; its level was even higher than that of 100 U ml^{-1} superoxide dismutase. The deferoxamine–Fe(III) mixture did not exhibit any protective effect in this system.

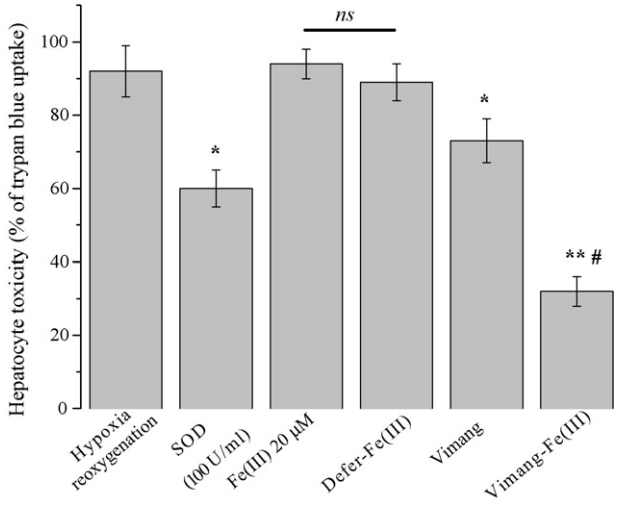

Fig. 5. Prevention of ROS-mediated hypoxia/reoxygenation-induced hepatocytes injury by Vimang, in absence or presence of 20 μM Fe(III), evaluated by Trypan blue uptake, as described in Section 2. The concentration of ligand (Vimang or deferoxamine) used was 40 μM. A 10 min premixed ligand–Fe(III) complex was prepared prior hepatocytes addition. Bars represent means ± S.D. ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ respective to hypoxia–reoxygenated cells. $^{\#}$Statistical difference ($P < 0.05$) between complexed and uncomplexed Vimang.

4. Discussion

The present study provides evidence that the Vimang–Fe(III) mixture (2:1—using mangiferin content of the extract to set the ligand proportion in the mixture) is more effective at scavenging DPPH and superoxide radicals, as well as in protecting against *t*-butyl hydroperoxide-induced mitochondrial lipid peroxidation and hypoxia/reoxygenation-induced hepatocytes injury, compared to Vimang alone. Voltammetric assays demonstrated that Vimang, in line with the high mangiferin content of the extract, behaves electrochemically like mangiferin, as well as interacts with Fe(III) in close similarity with mangiferin's interaction with the cation.

Superoxide dismutase acting at external sites demonstrated a capacity to protect hepatocytes from hypoxia/reoxygenation-induced injury; in contrast, deferoxamine–ferric complex failed to protect these cells. The reason for this lack of effect would to be the strong sequestering property of deferoxamine, a hexadentate ligand, towards Fe(III); in contrast, mangiferin, the major (16%), polyphenolic component of Vimang, behaves as a bidentate ligand [20]. The 2:1 bidentate–Fe(III) complexes possess at least two coordinate sites available to a superoxide radical, whereas in the 1:1 deferoxamine–Fe(III) complex all available coordinating sites are occupied by ligands capable in principle, to hinder a free electron transfer between the complex and a superoxide radical.

The increased free radical scavenging ability of Vimang after iron coordination could be explained by the redox cycling of the metal and the ligand, particularly mangiferin, the main catecholic polyphenol in the natural extract. Mangiferin binds tightly with ferric ion, probably on account of its catechol moiety, to form a complex [20] which could be oxidized to semiquinone ferric complexes by superoxide radicals. The catechol–iron complex has also been hypothesized to undergo an internal redox reaction to generate a semiquinone radical intermediate of catechol chelated to the ferrous iron [12,13], a process that is favoured by either low pH or low redox potential catechols. The semiquinone–ferrous complex could be then oxidized by $O_2^{\bullet -}$ to form a semiquinone ferric complex, that could be readily reduced to catecholic ferric complexes, while the superoxide radicals could be converted to oxygen. They may also form catecholic dimmers or trimers, or may undergo disproportionation to give the parent catechol and the *o*-quinone, which could also be reduced by $O_2^{\bullet -}$. The formation of a redox active mangiferin–Fe(III) complex that mimics superoxide dismutase activity, probably maintains the iron–chelate within a continuous Fe(III)–Fe(II)–Fe(III) conversion, with no significant accumulation of Fe(II) or superoxide. This statement increases the mangiferin antioxidant capability, i.e., in the absence of iron a much higher mangiferin concentration will be required to achieve the same level of cytoprotection. Interestingly, it was recently demonstrated that iron and cupper complexation by catechol-like flavonoids hinders oxidation of the ligand despite the increase in their antioxidant capacity, and that metal complexes with catechol antioxidants are able to undergo electron-transfer reactions comparable to those known for superoxide dismutase [21,22].

The evidence that also SOD protected against hypoxia/reoxygenation-induced hepatocytes injury, suggests that the targets of the mangiferin–Fe(III) complex are mainly ROS generated or accumulated outside the cell; this conclusion agrees with a recent report demonstrating a lack of differences in cytoprotection offered by several iron complexes of flavonoids, despite the differences in their partition coefficients [13]. Thus at least partly, the cytoprotective action of Vimang–Fe(III) could involve removal of extracellular H_2O_2. On the other hand, the extent of protection elicited by the extract-metal mixture, higher than SOD, suggests that they may prevent the oxidative damage to the hepatocytes not only at the extracellular, but also at the intracellular level. In this regard, recent studies suggest that Vimang or mangiferin are capable to reach mitochondria after a single oral dose administration [23,24].

Alongside mangiferin, Vimang contains other polyphenols, including phenolic acids (gallic, 3,4-dihydroxy benzoic, and benzoic acids), phenolic esters (methyl gallate, propyl gallate, propyl benzoate) and flavan-3-ols (catechin/epicatechin) [5], also showing potential capacity to interact with iron [25]. Like mangiferin, the catechin, the second major polyphenol of Vimang, is a catechol-containing compound also displaying important antioxidant activity; increase in its superoxide scavenging activity by iron has been demonstrated [10,13,21,26]. Therefore, the involvement of catechin in the improvement by Fe(III) of the whole antioxidant effect of Vimang, should be taken into consideration. Indeed, the levels of mangiferin–Fe(III) complex and superoxide scavenging activity observed in a parallel study (results not shown), were lower than those observed for the Vimang–Fe(III) mixture at equivalent concentrations of mangiferin; thus, a possible synergistic action between mangiferin–Fe(III) and catechin/other

polyphenols–Fe(III) complexes concerning the antioxidant activity of Vimang, also appears worth considering.

According to the present results Vimang, mainly *via* mangiferin, would afford protection against iron- and ROS-mediated pathological processes by means of respectively, its iron chelating and iron-stimulating superoxide scavenging activities. This means that the extract not only could diminish the amount of "free" or "loosely bound iron ions" through out its coordination capacity (rendering them inactive or poorly active in the Fenton reaction), but also could gain an extra antioxidant capacity upon metal complexation. Such property would be relevant in diseases involving primary or secondary iron overloading, like β-thalassemia, Friedreich's ataxia and haemochromatosis [27–29], as well as in several other pathological conditions including infection, neoplasia, cardiomyopathy, atherosclerosis, neurodegeneration, and also aging [30–33], in which iron is a possible mediator for ROS action. Iron excess is believed to generate oxidative stress, an increase in the steady state concentration of reactive oxygen and nitrogen species. The toxicity of superoxide anion and hydrogen peroxide arises from their iron dependent conversion into the extremely reactive hydroxyl radical ($^{\bullet}$OH) (Fenton–Haber–Weiss reaction) that causes severe damage to membranes, proteins, and DNA [34]. According with a recent report, many iron complexes can promote oxidative stress disrupting the mitochondrial energy metabolism, so that targeting mitochondrial iron with directed chelation therapy should be an attractive strategy for managing a number of clinical disorders of iron imbalance [35]. The hexadentate hydroxamic acid–deferoxamine, despite being very expensive and ineffective by the oral route [36], still remains the only clinically approved iron chelator used in diseases involving iron; Vimang could at present become a more convenient therapeutic alternative in these cases. Since *in vivo* levels of free or loosely bound iron even in iron overload, are hardly higher than 1 μM, [37], very low concentrations of the above-mentioned Vimang extract components, may afford protection against iron-mediated oxidative damage in such pathological conditions. Studies now in progress are aimed at the assay of the protective effects of Vimang/mangiferin on *in vivo* animal models of iron overload.

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