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Mangifera indica L. extract (Vimang) inhibits Fe^{2+}-citrate-induced lipoperoxidation in isolated rat liver mitochondria

Gilberto Pardo Andreu[a,b,*], René Delgado[c], Jesus Velho[a], Natalia M. Inada[a], Carlos Curti[d], Anibal E. Vercesi[a]

[a] *Departamento de Patologia Clínica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas 13083-970 Campinas, SP, Brasil*
[b] *Departamento de Farmacia, Universidad de Camagüey, Carretera Circunvalación, Km 5½, Camagüey, CP 74650, Cuba*
[c] *Departamento de Investigaciones Biomédicas, Centro de Química Farmacéutica, Calle 200, Esq. 21, Playa, Ciudad de La Habana, Cuba*
[d] *Departamento de Física-Química, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, SP, Brasil*

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Abstract

The extract of *Mangifera indica* L. (Vimang) is able to prevent iron mediated mitochondrial damage by means of oxidation of reduced transition metals required for the production of superoxide and hydroxyl radicals and direct free radical scavenging activity. In this study we report for the first time the iron-complexing ability of Vimang as a primary mechanism for protection of rat liver mitochondria against Fe^{2+}-citrate-induced lipoperoxidation. Thiobarbituric acid reactive substances (TBARS) and antimycin A-insensitive oxygen consumption were used as quantitative measures of lipoperoxidation. Vimang at 10 μM mangiferin concentration equivalent 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 Vimang protection against Fe^{2+}-citrate-induced mitochondrial TBARS formation (7.89 ± 1.19 μM) was around 10 times lower than that for *tert*-butylhydroperoxide mitochondrial induction of TBARS formation. The extract 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. The extracted polyphenolic compound, mainly mangiferin, could form a complex with Fe^{2+}, accelerating Fe^{2+} oxidation and the formation of more stable Fe^{3+}-polyphenol complexes, unable to participate in Fenton-type reactions and lipoperoxidation propagation phase. The strong DPPH radical scavenging activity with an apparent IC_{50} of 2.45 ± 0.08 μM suggests that besides its iron-complexing capacity, Vimang could also protect mitochondria from Fe^{2+}-citrate lipoperoxidation through direct free radical scavenging ability, mainly lipoperoxyl and alcoxyl radicals, acting as both a chain-breaking and iron-complexing antioxidant. These results are of pharmacological relevance since Vimang could be a potential candidate for antioxidant therapy in diseases related to abnormal intracellular iron distribution or iron overload.

Keywords: *Mangifera indica*; Vimang; Lipid peroxidation; Mitochondria; Iron chelator; Antioxidant

1. Introduction

Iron is a vitally important element in mammalian metabolism because of its unsurpassed versatility as a biologic catalyst. However, when not appropriately shielded or when present in excess, iron plays a key role in the formation of extremely toxic oxygen radicals, which ultimately cause peroxidative damage to vital cell structures [1], particularly mitochondria. It was reported that the neurological and cardiac manifestations of Friedreich ataxia are associated with iron-mediated mitochondrial toxicity [2] and histopathologic and ultrastructural studies of the livers of patients with iron overload often reveal swollen mitochondria, having an electron dense matrix and ruptured mitochondrial membranes [3].

Although phlebotomy therapy is effective in removing excess iron in hereditary hemochromatosis, chelation therapy is required in the treatment of many patients who have combined secondary and transfusional iron overload due to

* Corresponding author. Tel.: +537 271 7822; fax: +537 336 471.
E-mail address: rdelgado@infomed.sld.cu (G. Pardo Andreu).

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disorders in erythropoiesis. In patients with beta-thalassemia who undergo regular transfusions, desferrioxamine treatment was found to be effective in preventing iron-induced tissue injury and in prolonging life expectancy. The use of the oral chelator desferrioxamine remains controversial, and work is continuing on the development of new orally effective iron chelators [4].

Polyphenolic compounds, including a large group of flavonoids, are abundant in vegetables, fruits, wine and tea. These compounds are generally known to possess potent antioxidant properties [5], which depend on their free radical scavenging capacity and on their iron chelating activity [6–9].

Recently, a standard aqueous stem bark extract from selected species of *Mangifera indica* L. (Anacardiaceae), which has been used as food supplement in Cuba under the brand name of Vimang, was reported to have potent in vivo and in vitro antioxidant and anti-inflammatory activities [10–13]. Chemical studies performed with this extract have enabled the isolation and identification of phenolic acids, phenolic esters, flavan-3-ols, mangiferin, which is the predominant component of this extract [14], and micronutrients such as selenium [15,16]. A recent report demonstrated that an aqueous extract of *M. indica* L. stem bark (Vimang), 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 phospholipids peroxidation, with a powerful effect on non-enzymic peroxidation (with Fe^{3+} ascorbate) [10]. Although this work suggests some iron-complexing ability as an antilipoperoxidative mechanism of the natural extract, exact mechanisms of iron interaction with Vimang have not yet been established.

Since Fe^{2+}-mediated liver and cardiac mitochondrial lipid peroxidation seems to be an early event in iron overload-related diseases [4,17], Fe^{2+}-citrate-induced lipoperoxidation on isolated liver mitochondria has become a suitable in vitro model for initial testing of potential drugs against diseases related to abnormal intracellular iron distribution and/or iron overload. The high polyphenolic content of *M. indica* extract (34%, g/g) could protect mitochondria from iron-induced lipoperoxidation following polyphenol-like patterns, i.e. by complexing iron and/or by direct free radicals scavenging activity. Therefore, in this paper we tried to assess the role of iron interaction with *M. indica* L. extract (Vimang) in the antioxidant action of this extract against mitochondrial Fe^{2+}-citrate-induced lipoperoxidation.

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 [15]. 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 [14] and represents 16% of powdered extract.

Stock solutions were prepared dissolving the solid extract in DMSO in a concentration range from 1 to 50 mM, equivalent to mangiferin concentration. Experiments were carried out with dilution of 1/1000 to prevent solvent influence on the results.

2.2. Reagents and solutions

1,1-Diphenyl-2-picrylhydrazyl radical (DPPH), 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 (FCCP) were purchased from Sigma (St. Louis, MO). Ferrous [Fe $(NH_4)_2(SO_4)_2{\cdot}6H_2O$] and ferric ($FeCl_3$ + 10 mM HCl) ion solutions were prepared in Milli-Q water and immediately used. Stock solutions of Vimang in DMSO were used with dilutions of 1/1000 in assay medium to minimize solvent interference. Controls experiments (without Vimang) contained 14.3 μM DMSO, which represent solvent concentration in all Vimang samples.

2.3. Isolation of rat liver mitochondria and standard incubation procedure

Mitochondria were isolated by conventional differential centrifugation from the liver of adult animals fasted overnight [18]. 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^{-1}, measured by the method of Biuret with BSA 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.

2.4. 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. Vimang 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.

2.5. Fe^{2+}-citrate and tert-butylhydroperoxide-mediated mitochondrial membrane lipid peroxidation (LPO) assay

LPO was estimated from malondialdehyde (MDA) generation [19]. The mitochondrial suspension (1 ml, 1 mg protein) was incubated in the standard medium with 4 mM *tert*-butylhydroperoxide or 50 μM $FeSO_4$ 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 MDA–TBA complex was extracted with 2 ml of *n*-butanol and absorbance was measured at 535 nm. MDA concentration was calculated from $\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

2.6. 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.

2.7. Measurements of mitochondrial transmembrane electrical potential ($\Delta\Psi$)

The mitochondrial membrane potential ($\Delta\Psi$) was monitored indirectly based on the activity of the lipophilic cation tetraphenylphosphonium (TPP^+) using a TPP^+ selective electrode built in our laboratory, in combination with a calomel reference electrode [20]. For calculation of the $\Delta\Psi$ value, the matrix volume of RLM was assumed as 1.4 μl mg^{-1} protein. The membrane potential was calculated assuming that the TPP^+ distribution between mitochondria and medium followed the Nernst equation. Corrections were made for the binding of TPP^+ to mitochondrial membrane as described by Jensen et al. [21]. The electrode was calibrated with additions of known concentrations of TPP^+. Signals were amplified and the output was registered with a potentiometric recorder (Kipp & Zonen, BD 121). Mitochondria (0.5 mg ml^{-1}) were incubated in 2 ml of standard medium supplemented with 3 μM TPP^+ (final concentration) and Vimang 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. Valinomicyn (1 μg ml^{-1}) was used to release mitochondrial potential.

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

Concentrations of Fe^{2+} were quantified in reaction medium (2 ml) without mitochondria using 5 mM 1,10-phenanthroline as previously described [20]. The red complex of Fe^{2+} with 1,10-phenanthroline was determined at 510 nm and compared with a Fe^{2+} standard curve [22].

2.9. DPPH reduction assay

Reduction of the 1,1-diphenyl-2-picrylhydrazyl radical (100 μM) by Vimang (0–50 μM) was monitored from the change in absorbance at 517 nm, 5 min after the drug was incubated with 40 mM sodium acetate, pH 5.5, and 1 ml ethanol (2.5 ml final volume) [23].

2.10. Statistical analyses

Data are presented as mean values ± S.D. of at least three independent experiments. Significant differences between means were determined using student one-tailed *t* test at level of $P < 0.05$. IC_{50} values were estimated using a non-linear regression algorithm.

3. Results

3.1. Effect of Vimang on iron-induced lipid peroxidation

Vimang inhibited thiobarbituric acid reactive substances (TBARS) formation induced by a 20-min incubation with 50 μM Fe^{2+}-citrate in rat liver mitochondria. Fig. 1 (Panel A) shows a titration curve for Vimang on TBARS, the IC_{50} value was 7.89 ± 1.19 μM. Full protection against peroxidation was observed with 50 μM Vimang and 10 μM butylated hydroxitoluene (result not shown), a well-known lipid peroxidation chain breaker. Panel B also shows Vimang effects against mitochondrial lipoperoxidation induced by 20 min incubation with 300 μM *tert*-butylhydroperoxide, with a larger IC_{50} value (>100 μM). These results show that Vimang is more effective in preventing iron-induced than peroxyl-induced lipoperoxidation, suggesting its iron-chelating ability as a primary mechanism in preventing iron-induced lipoperoxidation.

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

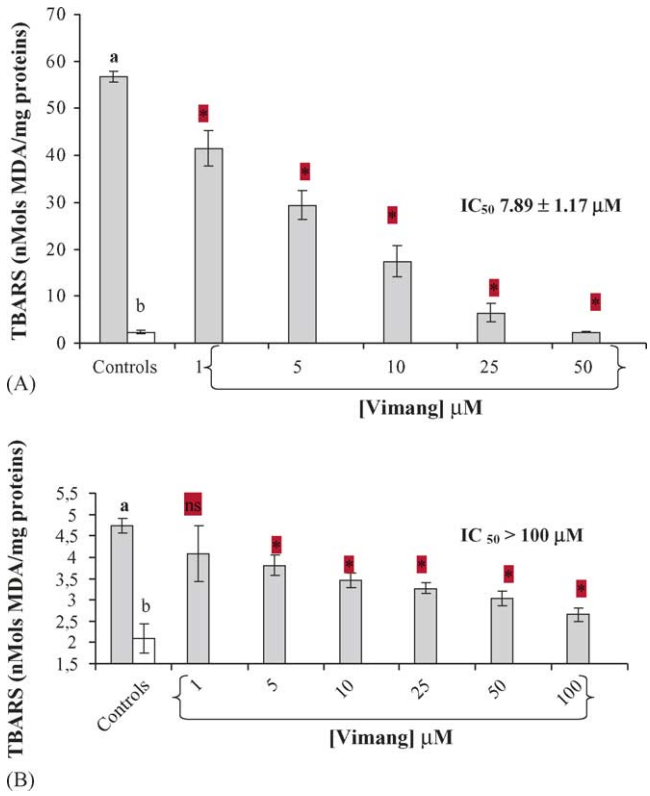

Fig. 1. Vimang inhibits MDA–TBA adducts formation induced by 50 μM Fe^{2+}-citrate (Panel A) or 300 μM *tert*-butylhydroperoxide (Panel B). Rat liver mitochondria (RLM) 0.5 mg ml^{-1} were incubated in reaction medium containing 125 mM sucrose, 65 mM KCL, 10 mM HEPES buffer (pH 7.2), 2 mM succinate and 2.5 μM rotenone, with or without Vimang. The experiments were initiated by addition of 50 μM Fe^{2+} (Panel A) or 300 μM *tert*-butylhydroperoxide (Panel B), except for the control b. Controls do not contain Vimang, they contain only DMSO (14.3 μM), which is the solvent concentration in the Vimang samples. Incubation period was 20 min at 28 °C. Values are the mean ± S.D. ($n = 6$). Significance vs. control (a) (one-tailed *t* test): $^{*}P < 0.05$; ns, not significant. IC_{50} values were estimated using a non-linear regression algorithm.

caused by lipid peroxidation [22]. 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

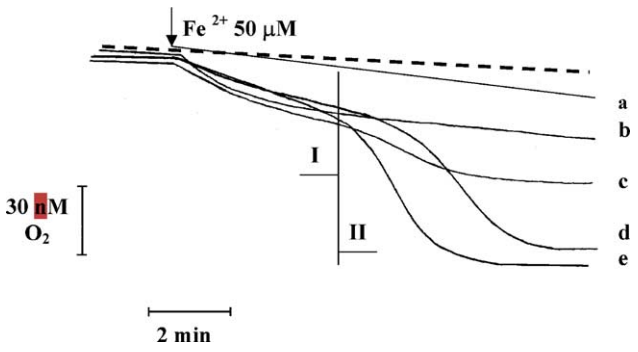

Fig. 2. Vimang inhibits mitochondrial lipid peroxidation measured as O_2 consumption. Experimental conditions are as in Fig. 1, except that 2.5 μM antimycin A was present. Iron was 50 μM. RLM (1 mg ml^{-1}) were incubated in reaction medium containing: (a) 10 μM Vimang; (b) 5 μM Vimang; (c) 2.5 μM Vimang; (d) 1 μM Vimang; and (e) no Vimang (14.3 μM DMSO only). Dashed line indicates O_2 consumption in mitochondrial suspension without Fe^{2+} addition. Results are representative of three experiments.

[22]. Addition of 50 μM Fe^{2+} to a mitochondrial suspension containing 2 mM citrate-induced extensive O_2 consumption (Fig. 2, line e) preceded by a lag phase [24]. This extensive O_2 consumption was inhibited by 300 mM desferrioxamine (result not shown) without prolonging the lag phase. Vimang was also able to inhibit iron-induced O_2 consumption in a concentration-dependent manner (lines a–e). At 10 μM Vimang, the rate of O_2 consumption was slightly higher than in controls, that is, in the absence of iron (dashed line). Vimang also prolonged the lag phase (I) observed between Fe $^{2+}$ addition and oxygen burst (II) due to lipoperoxidation, which indicate that this extract acts like a chain-breaking antioxidant. The small O_2 consumption observed just after addition of Fe^{2+} was due to Fe^{2+} autoxidation (see Fig. 6 for a detailed study).

3.2. *Effect of Vimang 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 non-specific increase in membrane

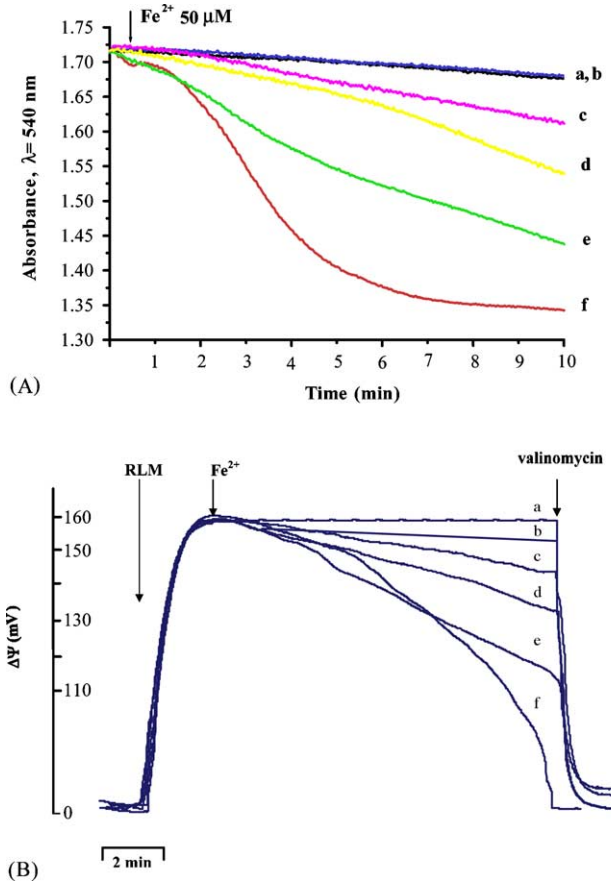

Fig. 3. (A) Vimang inhibits mitochondrial swelling induced by 50 μM Fe^{2+}-citrate. Experimental conditions are described in legend of Fig. 1. Reaction medium contains: (a) No Fe^{2+} addition; (b) 10 μM Vimang; (c) 5 μM Vimang; (d) 1 μM; (e) 0.5 μM Vimang; and (f) no Vimang (14.3 μM DMSO only). Fe^{2+} (50 μM) was added where indicated by the arrow (b–f). Results are representative of three experiments. (B) Vimang inhibits loss of mitochondrial potential ($\Delta\Psi$) induced by 50 μM Fe^{2+}-citrate. Experimental conditions are described in legend of Fig. 1, except that 3 μM TPP^+ were present. Reaction medium contains: (a) No Fe^{2+} addition; (b) 10 μM Vimang; (c) 5 μM Vimang; (d) 2.5 μM Vimang; (e) 1 μM Vimang; and (f) no Vimang (14.3 μM DMSO only). Rat liver mitochondria (RLM) (0.5 mg ml^{-1}), 50 μM Fe^{2+} and 1 μM Valinomycin were added where indicated by the arrows (a–f). Results are representative of three experiments.

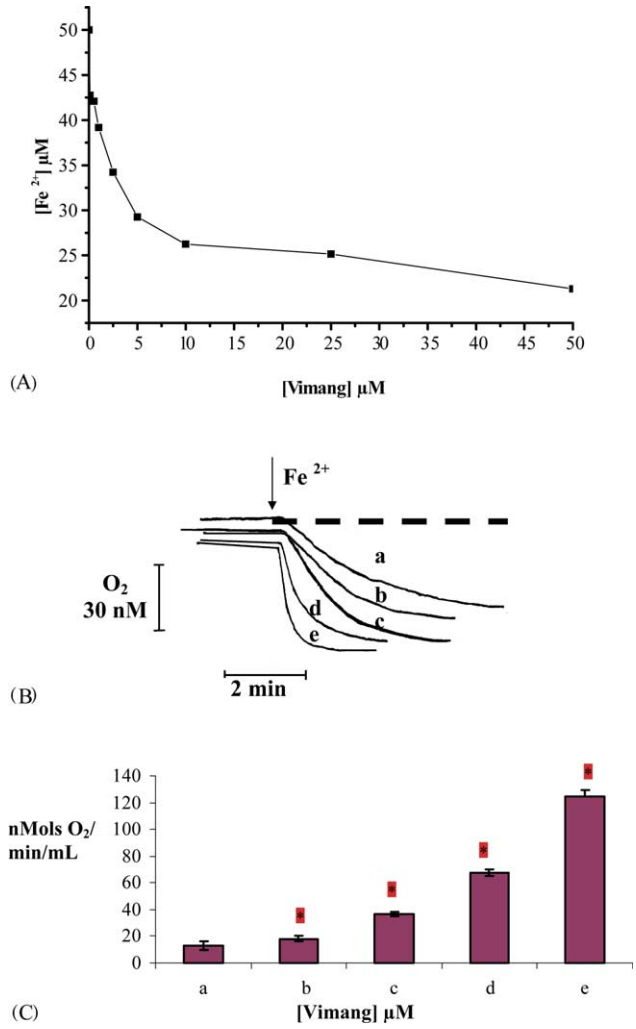

Fig. 4. (A) Vimang stimulates Fe^{2+} autoxidation in the absence of rat liver mitochondria. Experimental conditions: 125 mM sucrose, 65 mM KCl, 10 mM HEPES buffer (pH 7.2), 2 mM citrate, 5 mM 1,10-phenanthroline Experiments were conducted at 28 °C and were started by the addition of 50 μM Fe^{2+}. Sample with cero Vimang concentration contained 14.3 μM DMSO only. Values are the average of three determinations. (B) Effects of Vimang 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 Vimang (14.3 μM DMSO only); (b) 1 μM Vimang; (c) 5 μM Vimang; (d) 10 μM Vimang; and (e) 25 μM Vimang. Dashed line indicates O_2 consumption in reaction medium without Fe^{2+} addition. Results are representative of three experiments. (C) Oxygen consumption rates (nmol O_2/min/ml). Legends are the same as in (B). Significance vs. control (a) (one-tailed *t* test): $^*P < 0.05$.

permeability. It was previously shown that Fe^{2+}-citrate induces mitochondrial swelling, sensitive to butylated hydroxytoluene, due to lipid peroxidation [25]. Fig. 3A (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 ionosphere Valinomycin (Fig. 3B, line f).

Vimang inhibited the swelling process in a concentration-dependent manner (Fig. 3A, lines b–e). Near-full protection was attained at 10 μM.

Measurement of mitochondrial $\Delta\Psi$ using a TPP^+ selective electrode showed that Vimang prevented $\Delta\Psi$ dissipation induced by 50 μM Fe^{2+}-citrate in a dose-dependent fashion, also with near-full protection at 10 μM.

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

Vimang reduced Fe^{2+} concentration in reaction medium containing 2 mM citrate (without mitochondria) in a dose-dependent manner (Fig. 4A) and also increased the rate of O_2 consumption, possibly due to the stimulatory effect on oxidation of Fe^{2+} to Fe^{3+} (Fig. 4B and C). These results suggest

that Vimang 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, Vimang could be diminishing the amount of Fe^{2+} involved in the formation of $^{\bullet}OH$ radical through a Haber–Weiss-type reaction, which can initiate the peroxidation of mitochondrial membrane. Interestingly, the presence of catalytic amounts of superoxide dismutase enzyme (4 U ml^{-1}) and/or catalase diminished the amount of O_2 consumed by Fe^{2+} autoxidation but did not modify Vimang effects (results not shown), indicating some ferroxidase-like activity of the natural extract, which could catalyze Fe^{2+} oxidation without hydrogen peroxide (H_2O_2) or superoxide ($O_2^{\bullet -}$) production.

3.4. Vimang inhibits Fe^{3+} reduction by ascorbate

Since Vimang is a mixture of compounds, it might be possible that this extract not only stimulates Fe^{2+} autoxidation, but also Fe^{3+} reduction. This last proposal could reload biological systems with Fe^{2+}, which participates in Fenton–Haber–Weiss reactions, generating the extremely reactive $^{\bullet}OH$ radical. Vimang-Fe^{3+}complexes could also allow the oxidation of biological relevant reducers like ascorbate with subsequent Fe^{2+} regeneration. Ascorbic acid with higher iron-reducing activity shows antioxidant properties, but sometimes can act as a prooxidant under in vitro and in vivo conditions [6]. For example, lipid peroxidation is often initiated by ascorbate-mediated reduction of Fe^{3+}. Here we also analyzed the effect of Vimang on the reduction of iron by ascorbic acid.

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 Vimang concentrations, taking into account that 1,10-phenantroline forms a red complex with Fe^{2+}, easily detected at 500–520 nm, but not with Fe^{3+} [22,26]. Fig. 5 (line g) shows that 25 μM Vimang slightly increased Fe^{2+} concentration, possibly due to direct Fe $^{3+}$ reduction by some of its polyphenolic compounds. At the same time, this Vimang concentration effectively inhibits ascorbate-induced iron reduction (more than 50%) (Fig. 5, line f). These results strongly suggest that Vimang not only stimulates Fe^{2+} autoxidation, but also some of its components, possibly forming a stable complex with Fe^{3+}, inhibiting its reduction and redox cycling to Fe^{2+}.

3.5. DPPH reduction assay

Lag phase increases observed in Fig. 2 suggest that Vimang could be directly scavenging peroxyl and alcoxyl radicals formed after the initiation steps of Fe^{2+}-citrate-induced lipoperoxidation, acting not only as an iron chelator, but also as a chain-breaking antioxidant [24]. To confirm this, we searched the ability of Vimang to directly scavenge 1,1-diphenyl-2-picrylhydrazyl radicals. This molecule is characterized as a stable free radical by virtue of the delocalization

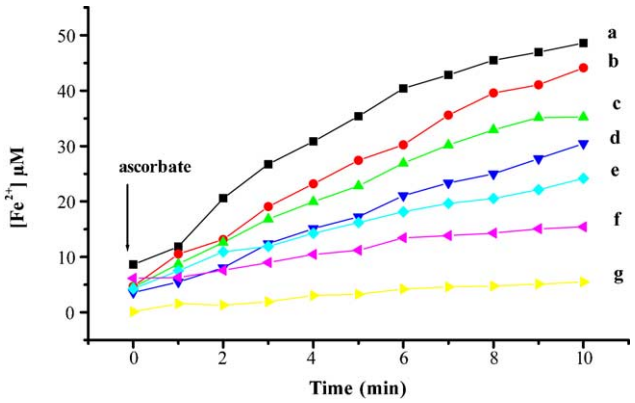

Fig. 5. Vimang inhibits Fe^{3+} reduction by ascorbate in the absence of rat liver mitochondria. Experimental conditions: 125 mM sucrose, 65 mM KCl, 10 mM HEPES buffer (pH 7.2), 2 mM citrate, Fe^{3+} 50 μM, 5 mM 1,10-phenanthroline. Experiments were conducted at 28 °C Ascorbate (4 mM) was added where indicated by the arrow (except line g). (a) No Vimang (14.3 μM DMSO only); (b) 0.1 μM Vimang; (c) 0.5 μM Vimang; (d) 5 μM Vimang; (e) 10 μM Vimang; (f) 25 μM Vimang; and (g) 25 μM Vimang, no ascorbate addition. Values are the average of three determinations.

of the spare electron over the molecule as a whole, so that the molecules do not dimerize, as would be the case with most other free radicals. The delocalization also gives rise to a deep violet color, characterized by an absorption band in ethanol solution centered at about 520 nm. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form with the loss of this violet color [27]. Fig. 6 shows a potent DPPH radical scavenging ability of Vimang with an $IC_{50} = 2.45 \pm 0.08$ μM. This result suggests that Vimang peroxyl and alcoxyl scavenging ability could be together wit iron-complexing capacity, an important mechanism to prevent Fe^{2+}-citrate-induced mitochondrial lipoperoxidation.

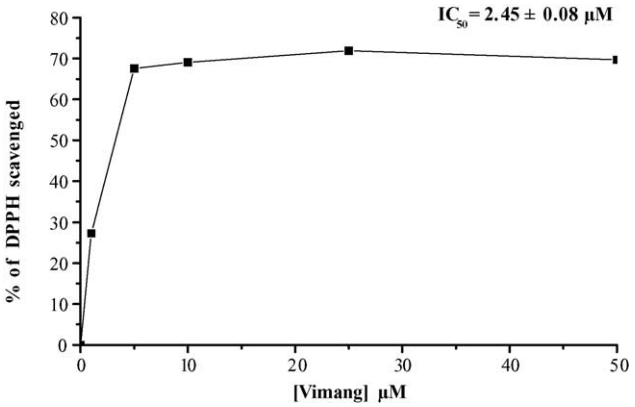

Fig. 6. Reduction of the 1,1-diphenyl-2-picrylhydrazyl radical (100 μM) by Vimang (0–50 μM) was monitored from the change in absorbance at 517 nm, 5 min after the drug was incubated with 40 mM sodium acetate, pH 5.5, and 1 ml ethanol (2.5 ml final volume). Sample with cero Vimang concentration contained only 14.3 μM DMSO. Values are the average of three experiments. IC_{50} value was estimated using a non-linear regression algorithm.

4. Discussion

The present study investigated the protective effect of *M. indica* L. extract (Vimang) against oxidative damage induced by iron. We observed that Vimang protects the mitochondrial membrane against lipid peroxidation and consequently protects against loss of membrane integrity. Fe^{2+}-citrate induces membrane lipid peroxidation with consequent mitochondrial depolarization and organelle swelling due to non-specific permeabilization of the inner mitochondrial membrane [25]. The presence of 1–10 μM of mangiferin equivalent of Vimang concentration (2.6–26 μg ml^{-1} powder extract) in the mitochondrial suspension inhibited Fe(II)-citrate-mediated mitochondrial swelling, loss of transmembrane potential, TBARS formation, and O_2 consumption during lipid peroxidation (Figs. 1–3).

Previous results have demonstrated that Vimang inhibits lipid peroxidation in rat brain phospholipids homogenate, showing a powerful effect on non-enzymic peroxidation ($FeCl_3$-ascorbate) compared to spontaneous autoxidation [10]. However, the explanations for the antioxidant mechanism of Vimang in iron-containing systems were only speculative. Transition metals such as iron and copper can participate in the generation of reactive oxygen species [28], which are associated with many pathological conditions [29,30]. One-electron reduction of oxygen by ferrous ion generates superoxide anion, which is itself an oxidant of many ferrous complexes (e.g. Fe^{2+}-citrate, Fe^{2+}-EDTA) causing formation of hydrogen peroxide. Hydroxyl radicals are further generated from hydrogen peroxide through the metal-catalyzed Fenton reaction.

The hydroxyl radical is one of the potent oxidants among reactive oxygen species, and is considered to be an initiator of lipid peroxidation [31]. However, some investigators have proposed the participation of perferryl ion in the initiation and propagation of lipid peroxidation [32]. Perferryl ion can be formed by the reaction of reduced iron with molecular oxygen [32]. Furthermore, transition metals are responsible for the formation of lipid alkoxyl radicals and peroxyl radicals, which may cause propagation of lipid peroxidation [33]. Considering the role of transition metals in the generation of free radicals, removal of iron or copper may inhibit the formation of oxygen radicals. Chelation of these materials will decrease the oxygen toxicity to cells. The structure–activity relationship of flavonoids to scavenge reactive oxygen species has been extensively studied [34] and metal-chelating activity of polyphenolics has been considered as a minor mechanism in the antioxidant action [35]. However, oxygen radicals, in particular hydroxyl radicals, are highly reactive and attack most molecules with extraordinarily high rate, constant under in vivo conditions [6]. Thus, suggestions that antioxidants act by scavenging oxygen radical in vivo are chemically unlikely. An antioxidant that affects oxygen radical-dependent damage in vivo is more likely to act by blocking radical formation, that is, by removing its precursors, superoxide and hydrogen peroxide, and then transition metals. Thus, metal coordination by polyphenolics may be most effective in antioxidant action. Recent studies showed that iron-chelating activity of some flavonoids is closely related to their antioxidant action [36]. Formation of inactive iron–rutin complex, which is unable to generate the reactive hydroxyl radical, plays an important role in the antioxidant action of flavonoids [37]. The subsequent assessment of the relationship between the antioxidant effect and the iron-chelating capacity of polyphenolics is of interest [36].

Fig. 4 shows that Vimang rapidly diminished Fe^{2+} concentration, associated with an increasing rate of O_2 consumption. These results suggest that the natural extract removes iron from the Fe^{2+}-citrate complex, facilitating Fe^{2+} oxidation to Fe^{3+}. The presence of gallic acid, propyl and methyl gallate in the extract justify the slightly reducing ability of the extract on Fe^{3+} (Fig. 5, line g). A 1:1 complex, in which the metal ion is coordinated to two adjacent hydroxy groups of the ligand subsequently decompose to form Fe^{2+} and the corresponding semiquinone, which reacts rapidly with another Fe^{3+} species to form the quinone [38,39]. The reduced iron could form non-flavonoid polyphenol complexes that could be inert to oxygen molecule or scavenge the hydroxyl radical generated in situ, acting as a "sacrificial" antioxidant which protects more relevant biological molecules from devastating $^{\bullet}OH$ reactivity [40]. Nevertheless, the higher proportion of mangiferin, catechin and epicatechin in relation to gallate derivatives in whole extract could explain the inhibitory effect on ascorbate-Fe^{3+} reduction elicited by Vimang (Fig. 5, lines a–f). These compounds could readily reoxidize the iron reduced by ascorbate [40] or could form a stable complex with Fe^{3+}, impairing its reduction by ascorbate. Indeed, we have recently shown (unpublished results) that mangiferin, the most abundant polyphenol in the extract, forms a complex with Fe^{3+}, which totally inhibits iron reduction by ascorbate. Binding of iron to the polyphenolic compound of the extract can suppress the accessibility of the iron to oxygen molecules by oxidizing ferrous ion to ferric state, resulting in the inhibition of the hydroxyl radical production or the perferryl ion formation. Formation of inert complex of baicalein with ferric iron was demonstrated to cause the inhibition of microsomal lipid peroxidation [41]. Our present results, in good agreement with these findings, indicate that the antioxidant action of the extract is ascribed to the oxidation of reduced transition metals required for the production of superoxide and hydroxyl radicals.

Fig. 6 shows that Vimang has a strong DPPH radical scavenging activity with an apparent IC_{50} of 2.45 ± 0.08 μM. These results suggest that besides its iron-complexing capacity, Vimang could also protect mitochondria from Fe^{2+}-citrate lipoperoxidation through direct free radical scavenging ability, mainly lipoperoxyl and alcoxyl radicals, acting as a chain-breaking antioxidant. The synergistic action of both mechanisms could explain the extremely low dose of Vimang (10 μM), which elicited protection against iron-citrate mitochondrial oxidative damage, although the $IC_{50} > 100$ μM obtained in protection of *tert*-butylhydroperoxide induced

mitochondrial lipoperoxidation (Fig. 1B) suggests the iron chelating capacity of Vimang as a primary and most important mechanism of its antiperoxidative ability.

The results presented in this manuscript are of pharmacological importance since the mitochondria are important targets of 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. Iron chelators such as Vimang could be an important approach to reduce iron-induced oxidative damages in these pathologies.

It is of particular interest the fact that 10 μM mangiferin-equivalent concentration of Vimang was able to produce significant protection against mitochondrial damage mediated by 50 μM iron. These are concentrations lower than those of classical iron chelators like pyridoxal isonicotinoyl hydrazone, which was able to induce more than 80% of protection against iron (50 μM) induced mitochondrial damage at 100 μM [42]. Since in vivo levels of free or loosely bound iron are hardly higher than 1 μM, even in iron overload [43], very low concentrations of the extract components would afford protection against iron-mediated mitochondrial damage. These results suggest that the observed in vitro effects of this compound could be relevant to in vivo conditions of iron overload.

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