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Protective effects of *Mangifera indica* L extract (Vimang), and its major component mangiferin, on iron-induced oxidative damage to rat serum and liver

Gilberto L. Pardo-Andreu [a,*], Mariela Forrellat Barrios [b], Carlos Curti [c], Ivones Hernández [a], Nelson Merino [a], Yeny Lemus [a], Ioanna Martínez [a], Annia Riaño [a], René Delgado [a]

[a] *Departamento de Investigaciones Biomédicas, Centro de Química Farmacéutica, Calle 200, Esq. 21, Playa, Ciudad de La Habana, Cuba*

[b] *Instituto de Hematología e Inmunología. Apartado Postal 8070, Ciudad de La Habana, CP 10800, Cuba*

[c] *Departamento de Física e Química, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, 14040-903 Ribeirão Preto, SP, Brazil*

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Abstract

In vivo preventive effects of a *Mangifera indica* L extract (Vimang) or its major component mangiferin on iron overload injury have been studied in rats given respectively, 50, 100, 250 mg kg^{-1} body weight of Vimang, or 40 mg kg^{-1} body weight of mangiferin, for 7 days prior to, and for 7 days following the administration of toxic amounts of iron-dextran. Both Vimang or mangiferin treatment prevented iron overload in serum as well as liver oxidative stress, decreased serum and liver lipid peroxidation, serum GPx activity, and increased serum and liver GSH, serum SOD and the animals overall antioxidant condition. Serum iron concentration was decreased although at higher doses, Vimang tended to increase it; percent tranferrin saturation, liver weight/body mass ratios, liver iron content was decreased. Treatment increased serum iron-binding capacity and decreased serum levels of aspartate-amine transferase (ASAT) and alanine-amine transferase (ALAT), as well as the number of abnormal Kupffer cells in iron-loaded livers. It is suggested that besides acting as antioxidants, Vimang extract or its mangiferin component decrease liver iron by increasing its excretion. Complementing earlier *in vitro* results from our group, it appears possible to support the hypothesis that Vimang and mangiferin present therapeutically useful effects in iron overload related diseases.

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

1. Introduction

Iron overload in humans causes tissue damage and possible organ failure, liver being a primary target, and hepatic fibrosis and cirrhosis often having been observed in patients [1]. As demonstrated both *in vitro* and *in vivo* [2,3], lipid cell membrane peroxidation probably underlies such toxicity.

Deferoxamine and 1,2-dimethyl-3-hydroxypyrid-4-one (deferiprone, L1) are being currently prescribed for the treatment of iron overload diseases. However, such compounds show several limitations [4–6] that indicate the need of a search for more effective and less toxic drugs for patients that either not respond, or show toxic side-effects following their administration [7].

The ability of polyphenols to chelate iron is a very important part of their antioxidant activity. Their "site-specific scavenging" action can occur while iron is still being catalytically active forming free radicals, permitting it to be rapidly scavenged [8]. Polyphenols therefore, could have a double, synergistic action, making them into effective antioxidants, particularly active in pathological situations involving iron overload, e.g. ß-thalassemia, Friedreich's ataxia and hemochromatosis.

A standardized aqueous extract from the bark of selected species of *Mangifera indica* L. (Anacardiaceae) is used as a food supplement in Cuba, under the brand name of Vimang. It has potent *in vitro* and *in vivo* antioxidant activities [9,10], apparently due to phenolic acids, phenolic esters, flavan-3-ols components and in special, mangiferin [11]. The interaction of

* Corresponding author. Tel.: +537 2715067; fax: +537 2736471.
E-mail address: gilbertopardo@infomed.sld.cu (G.L. Pardo-Andreu).

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Vimang component(s) with iron has been proposed as being responsible for its antioxidant activity [9,12], and we have demonstrated that Vimang protects isolated mitochondria from lipid peroxidation induced by Fe (II)-citrate [13] and inhibits 2-deoxyribose damage induced by Fe(III)-EDTA-Ascorbate [14]. Vimang as well as mangiferin's interaction with iron exerts antioxidant and cytoprotective effects in situations of oxidative injury caused by anoxia/reoxygenation of isolated hepatocytes [15–19]. The present work confirms the *in vitro* results by characterizing the protective actions of Vimang and mangiferin in an *in vivo* experimental situation of iron overloading.

2. Material and methods

2.1. Reagents

Iron-dextran, bathophenanthroline disulfonic acid, 5,5′-dithiobis(2-nitrobenzoic)acid (DTNB), thiobarbituric acid, pyrogallol, reduced glutathione and diethyl ether were purchased from Sigma–Aldrich Chemical Co. All other reagents were of the highest purity available.

2.2. Drugs

Stem bark extracts of *Mangifera indica* L. were prepared by decoction in a polar solvent for 1 h, concentrated by evaporation and spray dried to obtain a fine brown powder, coded as 112, that melted at 210–215 °C with decomposition, and contained the active ingredient used in Vimang pharmaceutical formulations, following dissolution in distilled water saline. Planar, liquid and gas chromatographic methods, mass spectrometry and UV/VIS spectrophotometry, showed them to contain polyphenols as major (45%) fraction [11]. Chemical isolation procedures identified phenolic acids and esters, flavan-3-ols and showed mangiferin as the predominant (20%) component [11]. Mangiferin (2-β-D-glucopyranosyl-1,3,6,7-tetrahydroxy-9H-xanthen-9-one), supplied by the Centre of Pharmaceutical Chemistry (Cuba), had been purified from Vimang by extraction with methanol; HPLC showed it to be 95% pure [7].

2.3. Animals

Sixty female, 159 g Wistar rats, obtained from CENPALAB (Bejucal, Havana, Cuba), were housed in a controlled environment at 20 ± 2 °C (12 h light and 12 h dark cycle) and acclimatized for 7 days prior to experimentation, during which they were allowed free access to food (Standard diet for rodents, CENPALAB), and tap water. After random division into six groups of ten animals, each of four groups received respectively Vimang (50, 125 or 250 mg kg^{-1}), or mangiferin (40 mg kg^{-1}) daily, orally administered by a feeding needle for two consecutive 7 day periods. Two control groups received only 2 ml of saline. Treatments were given in the morning between 8:30 A.M. and 9:30 A.M. On the eighth day, Vimang or mangiferin-treated animals were intraperitonially given daily doses of 100 mg kg^{-1} each, of iron-dextran/saline for seven additional days, 2 h following antioxidant administration, that was maintained during the period of iron-dextran treatment, and had been previously shown to cause iron overload in mice [20,21]. One of the group of control animals already orally given saline, also received intraperitoneal injections of iron-dextran (antioxidant-untreated controls), while the other control group received only saline i.p (blanks, free of the iron overload). Animal body weights were measured daily. All procedures had been approved by the institutional Animals Care Committee and were in accordance with the European Union Guidelines for Animals Experimentation. Fifteen days after the beginning of the treatments, animals were sacrificed by placement into a diethyl ether chamber, blood samples were taken from abdominal aorta, and animal livers were rapidly removed and weighed.

2.4. Biochemical determinations

Components of serum and of liver homogenates, prepared as described below by a standard procedure, were assessed using a DU-640 spectrophotometer (Beckman Instruments, Inc., CA, USA). Protein contents were determined according to Lowry et al. [22], and total antioxidant capacity was measured using $ABTS^+$ (2,2′-azidodiethylbenzothiazolin sulfonate) (Randox Laboratories Ltd., Scotland) radical formation kinetics, at 600 nm [23]. Antioxidants present in serum suppress the bluish-green staining of $ABTS^+$, by an extent proportional to their concentration. Lipid peroxides (TBA reactants) were determined spectrophotometricaly [24]. Malonyldialdeyde (MDA) concentrations were calculated from $\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$. Superoxide radical scavenging was measured by the inhibition of superoxide dismutase activity on the rate of base-catalyzed auto-oxidation of pyrogallol [25]. Glutathione peroxidase (GPx) in serum samples was estimated using the kit supplied by Randox Laboratories Ltd., Scotland (Cat. No. RS505). In this method GPx activity is proportional to the velocity of NADPH oxidation at 340 nm, using cumene hydroperoxide as substrate. Glutathione was determined according to Sedlak and Lindsay, using 5,5′-dithiobis(2-nitrobenzoic) acid (Ellman's reagent) [26]; the standard curve employed was constructed using GSH. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in serum were measured using the commercial kits of Boehringer Mannheim (Germany).

2.5. Liver tissue preparation

Excised livers were perfused with saline at 4 °C to exclude blood cells, and blotted on filter paper. Liver halves were cut off, weighed and reserved for histopathological examination. A portion of the second half was homogenized with a glass homogenizer in nine volume of ice-cold 50 mM phosphate buffered saline, and 100 μl portions of the homogenate were immediately sampled by pippeting and prepared for measuring thiobarbituric acid reactive substance and GSH levels. Remains were also weighed and digested for 5–8 min over a low flame in an equal volume of a (1:1) mixture of sulfuric and nitric acids and their iron content estimated.

2.6. Histopathology

Freshly taken liver samples were fixed in 4% buffered formalin solution, embedded in paraffin, 5 micrometer sections cut and stained with hematoxylin–eosin and perl's prussian blue.

Iron-loaded granules of Kupffer cells were counted at a magnification of 400×, in 10 microscope fields taken at random per liver section of rats treated with Vimang or mangiferin.

2.7. Determination of serum and total liver iron contents

Serum non-heme iron concentration (SIC) was determined by the generation of an iron-bathophenantroline colored complex [27], using an external iron standard of 80 μmol l^{-1}. Total iron binding capacity of serum (TIBC) which estimates the degree of serum iron saturation, was determined as previously described [27,28]. Iron transferrin saturation (TS) was determined from the serum iron/TIBC ratio. Iron content in digested liver samples was spectrophotometrically measured at 535 nm, after reaction with 3 mM bathophenanthroline-disulfonic acid [29].

2.8. Statistical analysis

Results were expressed as means ± S.D., analyzed by one-way ANOVA, followed by Tukey's post-hoc test, by Stat-Xact-4.0.1. Parameters of two groups were compared using Student's *t*-test. $P < 0.05$ was the established level of significance.

3. Results

3.1. Effects of Vimang and mangiferin on the growth curve and liver weight/body mass ratios in iron-loaded rats

The growth curve of rats was not significantly modified after 7 days of iron-dextran administration (Fig. 1A), nor were conspicuous health abnormalities or effects on rat body weight gain observed. However, iron-dextran administration significantly increased by around 5%, animal liver/body weight ratios (Fig. 1B), an effect hindered by 125 or 250 mg of Vimang kg^{-1} treatments, and closely reproduced by 40 mg kg^{-1} mangiferin.

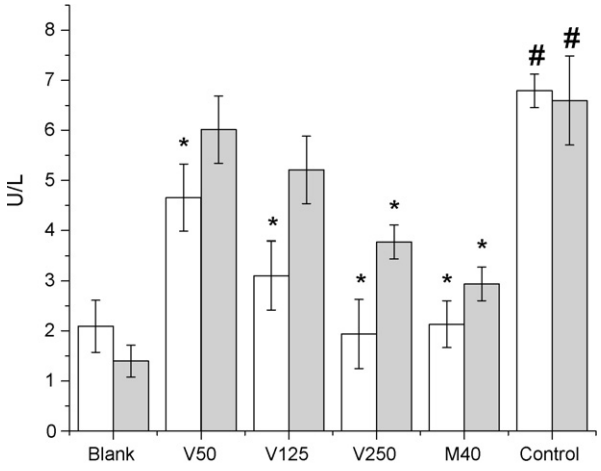

Fig. 2. Effect of Vimang or mangiferin on iron-induced hepatotoxicity. Rats were randomly chosen animals, divided into six groups, received respectively, Vimang extract, 50 mg/kg (V50), 125 mg/kg (V125) or 250 mg/kg (V250) or 40 mg/kg mangiferin (M40). Iron-dextran treatment and other experimental details were as described in Section 2.3. Serum aspartate-amine transferase (ASAT), (white bars) and alanine-amine transferase (ALAT), (light gray bars) activities were assayed using standard commercial kits (Boehringer, Mannheim, Germany). Bars represent means ± S.D. ($n = 10$). #$P < 0.05$ compared with blanks; values are means ± S.D. ($n = 10$).*$P < 0.05$ compared with controls.

3.2. Liver injury caused by Vimang and mangiferin in iron-loaded rats

Hepatotoxicity was evidenced by three-fold increases of ASAT and ALAT (Fig. 2), an effect markedly reduced by Vimang or mangiferin treatments (Fig. 2).

3.3. Effect of Vimang or mangiferin on rat serum iron, serum iron-binding capacity and liver iron content

Fig. 3 shows that serum iron concentration (SIC), serum iron-binding capacity (SIBC), transferrin saturation (TS) and

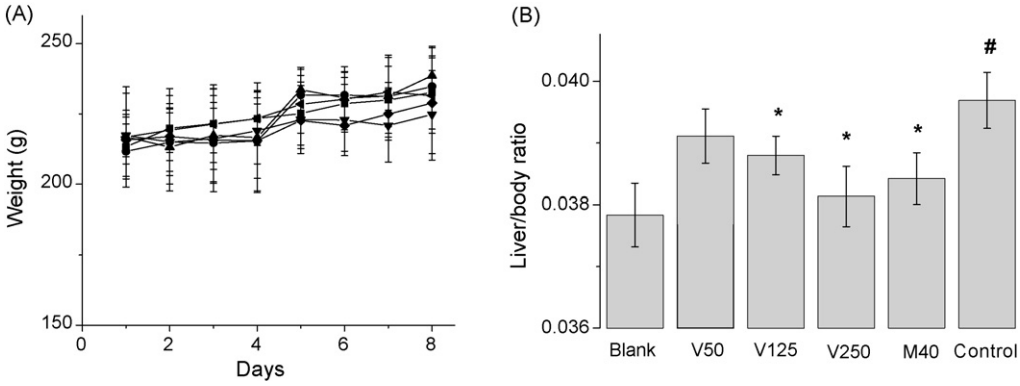

Fig. 1. Growth curves (A) and liver/body mass ratios (B) of rats following different treatments according to the protocol described under methods described in Section 2.4. Animals ($n = 10$/group), received orally, 50, 125 and 250 mg kg^{-1} body weight of Vimang (V50_▼, V125_▲ and V250_◄, respectively) or of mangiferin, 40 mg kg^{-1} (M40_♦) daily, during 14 days. Following the first 7 days of this treatment, they received in addition, by the intraperitoneal route, for 7 days, a daily dose of 100 mg kg^{-1} each, of iron–dextran in saline; during this period Vimang or mangiferin administrations were continued. Iron-dextran controls ($n = 10$), (■) received orally, 2 ml of saline instead of Vimang or mangiferin daily, for 14 days. After the seventh day, they received i.p. for another 7 days, a daily dose of 100 mg kg^{-1} each, of iron-dextran in saline. Blanks ($n = 10$), (●) received orally 2 ml of saline for 14 days. #$P < 0.05$ compared with blanks; *$P < 0.05$ compared with controls.

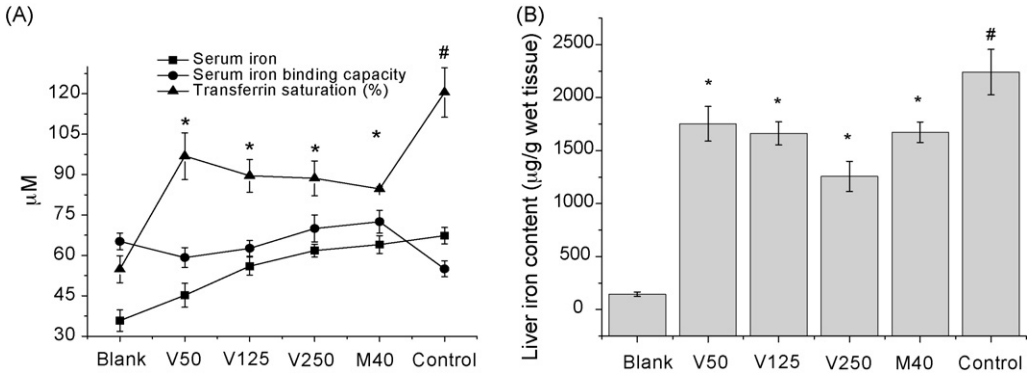

Fig. 3. Effect of Vimang or mangiferin on serum iron concentration (SIC_■), serum iron binding capacity (SIBC_●) and % transferrin saturation (TS_▲) (Panel A), and total iron liver content (Panel B) in iron loaded rats. Iron contents were assayed as described in Section 2.7. Values are means ± S.D. ($n = 10$). #$P < 0.05$ compared with blank; *$P < 0.05$ compared with control.

liver iron content of treated rats, were approximately doubled (SIC), or ten-fold increased (TS), following iron-dextran administration (Fig. 3B), while SIBC was significantly decreased (Fig. 3A). Treatment with Vimang decreased SIC but at higher doses showed a tendency to increase it, decreased TS as well as liver iron content, and increased SIBC. Mangiferin again closely reproduced these effects.

3.4. Effects of Vimang or mangiferin on serum SOD and GPx activities

Fig. 4 shows the 21% decrease in SOD activity in the serum of rats treated with iron-dextran. Treatment with Vimang or mangiferin significantly reversed this effect, estimated by the decreased slope of pyrogallol autoxidation curve at 420 nm. Increased GPx activity (by almost 47%) was hindered by Vimang or mangiferin in a dose-dependent manner (Fig. 5).

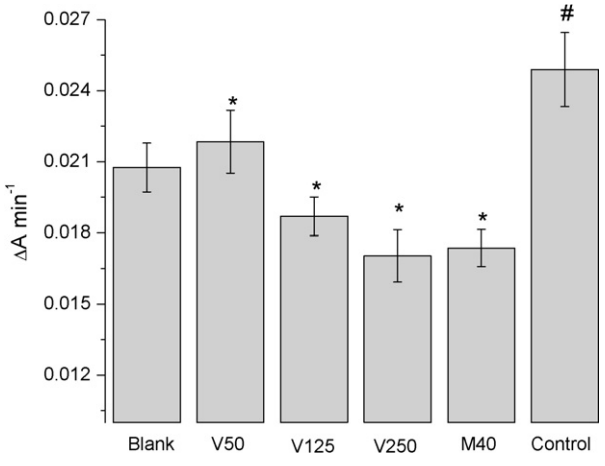

Fig. 4. Effect of Vimang or mangiferin on pyrogallol-generated superoxide radical scavenging activity of serum from iron loaded rats. Experimental conditions are the same as in Fig. 1. The reactions were initiated by the addition of pyrogallol (100 μM final concentration), and followed by absorbance monitored spectrophotometrically at 420 nm for 1 min, and calculation of the linear slope (with $r^2 > 0.99$). Bars represent means ± S.D. ($n = 10$). #$P < 0.05$ compared with blank; *$P < 0.05$ compared with control.

3.5. Effects of Vimang and mangiferin on thiobarbituric acid-reactive substances, GSH levels, and total antioxidant status of serum and liver

Thiobarbituric acid-reactive substances, glutathione levels, and total antioxidant status characterize the redox condition of the blood. In iron-loaded rats, serum GSH and total antioxidant status were significantly decreased, by nearly 60% and 70% respectively (Fig. 6A and B). Thiobarbituric acid-reactive substances were increased by approximately 60% (Fig. 6C), thus characterizing a condition of oxidative stress. Vimang or mangiferin significantly increased GSH levels and total antioxidant status (Fig. 6A and B), and decreased the levels of thiobarbituric acid reactive substances (Fig. 6C).

Iron-dextran administration contributed to the establishment of an oxidized environment in liver tissue, as evidenced by a 34% decrease of its GSH content (Fig. 7A) and a two-fold increase of thiobarbituric acid reactive substances (Fig. 7B). These effects were prevented by Vimang or mangiferin, clearly demonstrating these substances antioxidant abilities at the hepatic level.

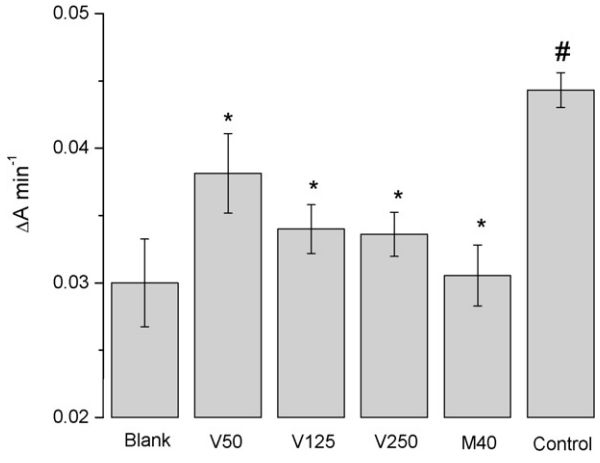

Fig. 5. Effect of Vimang or mangiferin on serum GPx activity of iron loaded rats. Experimental conditions were the same as shown in Fig. 1. GPx activity was proportional to the slope of the NADPH oxidation curve at 340 nm when cumene hydroperoxide was the substrate. Bars represent means ± S.D. ($n = 10$). #$P < 0.05$ compared with blank; *$P < 0.05$ compared with control.

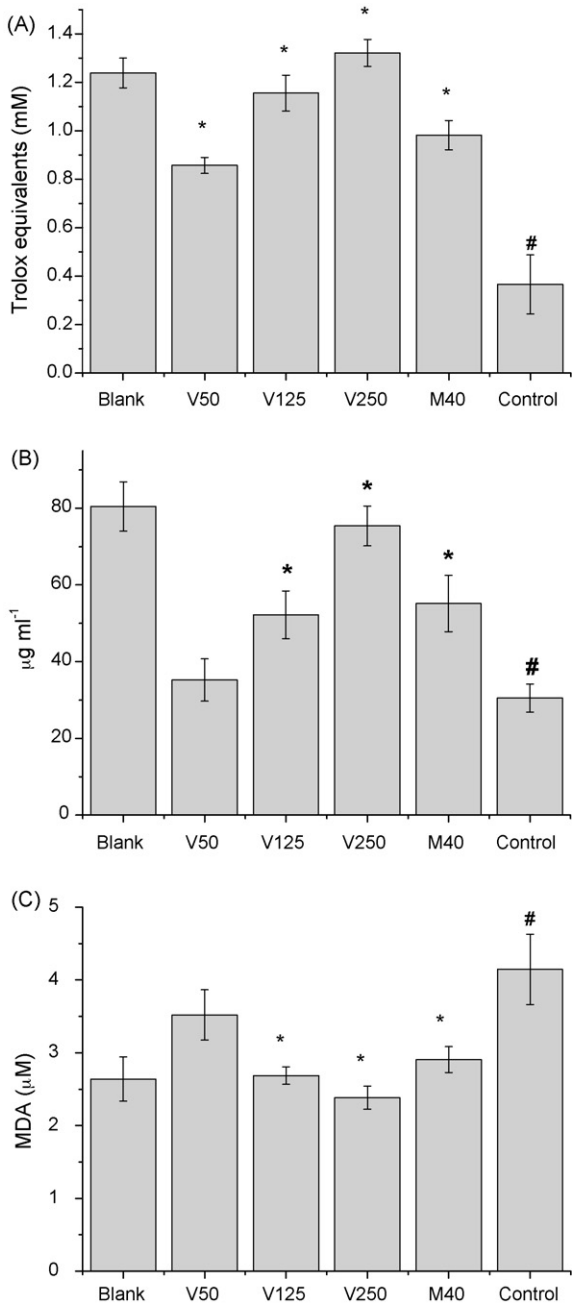

Fig. 6. Effect of Vimang or mangiferin administration on total antioxidant status (A) glutathione (B) and thiobarbituric acid-reactive substance (C) levels in the serum of iron loaded rats. Experimental details were as described in Section 2.3. Thiobarbituric acid-reactive substances, glutathione and total antioxidant status levels were assayed by the methods described in Section 2.4. Values are means ± S.D. ($n = 10$). #$P < 0.05$ compared with blank; *$P < 0.05$ compared with control.

3.6. Effects of Vimang or mangiferin on liver-resident macrophages

Iron-dextran administration approximately triplicated the number of resident macrophages in rat liver. Fig. 8 shows that Vimang or mangiferin decreased the number of phagocytic Kupffer cells in a dose-dependent manner.

3.7. Histopathological analyses

Hepatocytes of iron-dextran treated rats did not present ferric granule depots (Fig. 9B), showing a normal morphological appearance similar to that of non-iron loaded rats (Fig. 9A). Iron (ferric) granules were observed in the cytoplasm of Kupffer cells, and in free form, in the sinusoidal lumen (Fig. 9B). Moderate enlargement of these cells with hypertrophic nuclei having a vesicular appearance consistent with their activation, was also observed.

No qualitative signs of differences between the aspect of livers from rats treated with either one of three doses (50, 125 and 250 mg per kg) of Vimang or with mangiferin (40 mg/kg), were observed. Administration of 250 mg kg^{-1} Vimang or of 40 mg kg^{-1} mangiferin to iron dextran-treated rats resulted in total disappearance of signs of damage to the Kupffer cells. In these animals furthermore, ferric granules were totally phagocyted by these cells that showed a normal morphological appearance (Fig. 9C and D).

4. Discussion

Iron is the major catalyst of free-radical cell reactions and membrane lipid peroxidation, two major mechanisms of iron overload-induced tissue injury [1–3]. Deleterious effects of excessive iron deposition in liver, heart, pancreas and other endocrine organs, are believed to occur during genetic hemochromatosis, thalassemia major and transfusional siderosis. In such conditions, reactive molecular iron may initiate, propagate, and amplify (auto) oxidative phenomena in cells. The sequestering of low-molecular-weight, catalytically active iron present in the cytosol or released by chelation from other cell iron stores [30] could therefore be an effective therapeutic approach by simultaneously protecting membranes from the effects of cell-generated free radicals (i.e., antioxidant activity).

No compounds showing this broad spectrum of antioxidant activity are presently available for clinical use. We have therefore tested an aqueous extract of *Mangifera indica* L (Vimang) and its main polyphenol component mangiferin in this context.

In our experimental protocol, we ruled out the possibility of iron chelation prior to absorption or a direct interference of Vimang/mangiferin on intestinal iron absorption leading to serum and hepatic iron overloads. To avoid this, we have resorted to the i.p. injection of iron-dextran, rather than the commonly used oral administration of iron-carbonyl compounds. Fig. 3 shows that after the seventh dose of serial injections of 700 mg kg^{-1} iron, within one week, serum iron concentration had risen from 36 ± 4 to 67 ± 3 μmol l^{-1}, and total liver iron from 144 ± 20 to 2240 ± 215 μg g^{-1} wet weight. Liver/body weight ratios, as well as ASAT and ALAT activities were also significantly elevated (Fig. 2). This increased iron concentration led to an oxidative stress condition in serum and in liver, indicating that our experimental scheme was appropriate for their study.

Since all biochemical alterations originating from iron overload had been hindered by Vimang or by mangiferin, we were

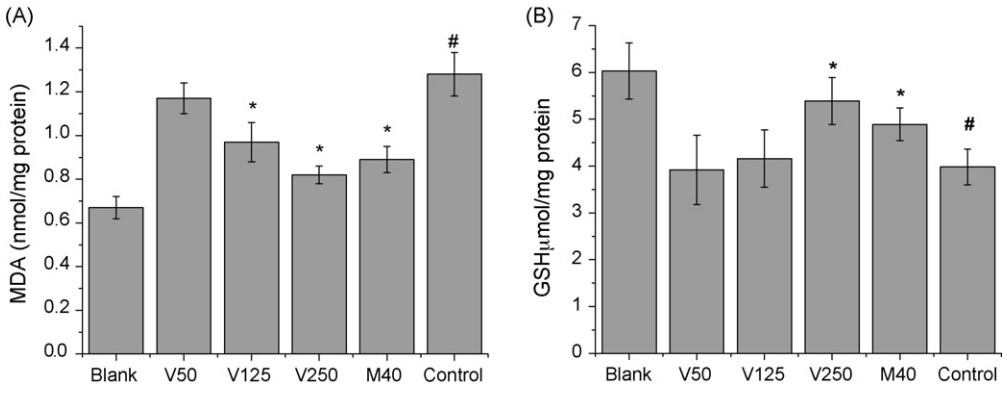

Fig. 7. Effect of Vimang or mangiferin on lipid peroxidation (A) and GSH (B) levels in liver homogenates of iron loaded rats. Thiobarbituric acid-reactive substances and GSH levels were assayed according to methods described in Section 2.4. Values are means ± S.D. ($n = 10$). #$P < 0.05$ compared with blanks; *$P < 0.05$ compared with controls.

led to suggest that the protective effects of Vimang on iron-mediated oxidative damage could at least in part, be attributed to mangiferin, acting not only by its free radical scavenging capacity, but also by its iron-complexing ability.

One characteristic of iron overloading is to increase non-heme iron serum levels. Vimang or mangiferin decreased hepatic total iron concomitantly with a dose-dependent tendency to increase of serum non-heme iron, suggesting that mangiferin and/or related compounds could gradually combine with hepatic non-heme iron, and take it into the blood stream for final excretion. Although, this hypothesis needs further experimental support, it is conceivable that Vimang/mangiferin release iron from liver ferritin, increasing serum iron concentration. Recent reports have documented the ability of baicalin and quercetin, both catechol polyphenols, to release iron from ferritin *in vitro* [20,21], accounting for their capacity to decrease liver iron by increasing serum iron levels in experimental iron overloading.

Serum iron concentration increased by Vimang was also associated with increased serum iron binding capacity and decreased percentage of transferrin saturation, supporting our assumption

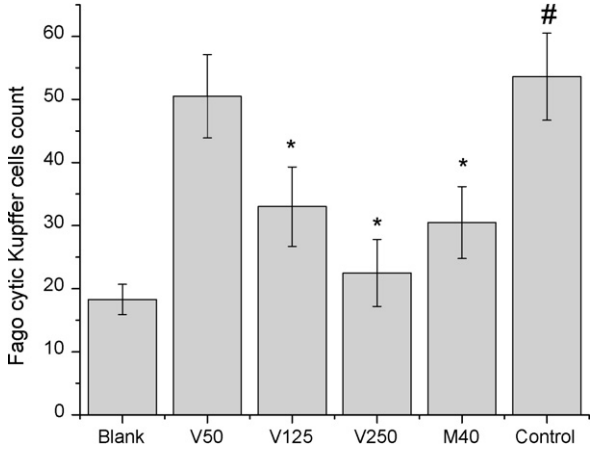

Fig. 8. Effect of Vimang or mangiferin administration on the number of liver-Kupffer cells loaded with ferric granules, counted at random at a magnification of 400×, in 10 microscope fields per liver section in differently treated rats. Bars represent means ± S.D. ($n = 10$). #$P < 0.05$ compared with blank; *$P < 0.05$ compared with control.

that iron chelation by mangiferin or possibly other polyphenolic Vimang components, act as a protective mechanism in iron-loaded rats.

We have recently shown that a Vimang-Fe(III) mixture prepared by using a 2:1 mangiferin content of the extract to define the ligand proportion in the mixture, was more effective than Vimang alone in scavenging DPPH and superoxide radicals, as well as in protecting hepatocytes from *tert*-butyl hydroperoxide-induced mitochondrial lipid peroxidation and hypoxia/reoxygenation [15]. This result suggests that the Vimang extract may not only decrease "free" or "loosely bound iron ions" by its coordination capacity rendering them inactive or poorly active in the Fenton reaction, but also by acquiring extra antioxidant capacity due to metal complex formation [18].

Vimang/mangiferin interaction with the plasma iron labile pool (LPIP) is another conceivable way involved in our results. LPIP is the pathologically relevant fraction of non-trasferrin bound iron that encompasses cell-penetrating redox active forms of iron [31,32] susceptible to chelation. It is therefore possible to postulate that the complexing capacity of Viman's polyphenol components, in special mangiferin, reduces iron available for translocation across cell membranes, preventing its accumulation in liver, heart, pancreas and further endocrine organs.

In vitro results [9] on the iron-chelating capability of Vimang have led us recently to demonstrate that Vimang prevents Fe(II)/citrate-induced lipid peroxidation in isolated rat liver mitochondria. This action may be due to its ability to stimulate oxygen consumption by Fe(II) autoxidation forming complexes with Fe(III) that do not participate in Fenton-type reactions and/or lipid peroxidation [13]. In this regard, it was also shown that Vimang protects 2-deoxyribose from oxidative damage by hypoxanthine, xanthine oxidase and Fe(III)/EDTA [14].

In the Vimang extract, mangiferin, a glucosylxanthone, is probably the major polyphenol component responsible for its antioxidant and antiinflammatory [10,33–35], as well iron-complexing properties. Confirming this hypothesis, our laboratory has accumulated several lines of evidence confirming that mangiferin's ability to interact with iron is its major antioxidant mechanism of action [16–19].

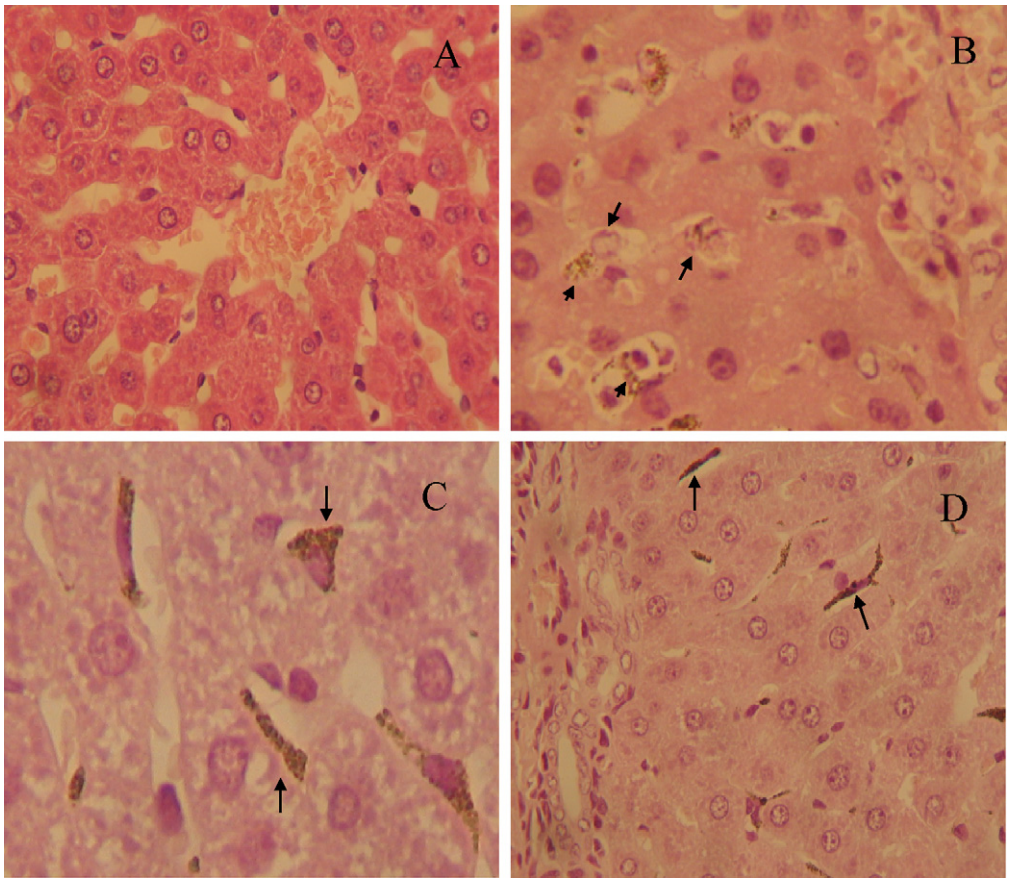

Fig. 9. Histological characterization of iron loaded rats. Rats were randomly divided into six groups of 10 animals each (blank group; control group; Vimang treated groups V50, V125 and V250; and mangiferin treated group, M40) and treated as described in Section 2.3. Pictures represent typical examples of the groups. Panel A, non-iron loaded rats (Blank); Panel B, iron-dextran-treated rats (arrows_degenerated Kupffer cells, arrowheads_ferric pigment); Panel C, 250 mg Vimang/kg rat body weight-treated rats (arrows_phagocyted ferric granules); Panel D, 40 mg/kg mangiferin-treated rats (arrows_phagocyted ferric granules).

Several observations have suggested that hepatic iron-loading may affect T-cell responses in liver [36,37]. It has also been proposed that activated Kupffer cells and leukocytes may release pro-inflammatory and pro-fibrogenic cytokines in the absence of overt necro-inflammation [37,38]. It is possible that the anti-inflammatory and immunomodulatory activities of Vimang and mangiferin, coupled to their ability to promote T cell survival [39], could explain their protective effects in iron-loaded rats.

Intraperitoneal administration of iron-dextran to rats resulting in iron uptake by Kupffer cells could be acting in two ways: (1) by production of oxidative species both by directly and by the Fenton reaction, and (2) via the activation of nuclear factor κB, producing fibrogenic mediators, pro-inflammatory cytokines, chemokines and adhesion molecules [40]; these may account for damage to Kupffer cells, as well as for the increased number of resident macrophages found in iron-loaded rats; both effects would increase hepatocyte damage. Vimang or mangiferin, by decreasing iron concentration in the liver and by inhibiting NF-κB activation, could decrease macrophage excess [33–35].

Further studies on *in vivo* formation and excretion of iron-mangiferin and Vimang-iron complexes will be needed, in special in the form of long term experiments with models reproducing significant end points like fibrogenesis and neoplasia. Nevertheless, our present findings, viewed together with the evidenced lack of toxicity and clinical safety of Vimang [35], constitute a rationale for the use of Vimang/mangiferin in the therapy of the consequences of iron-overload.

Acknowledgments

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