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Mangifera indica Leaves Extracts Mitigate Experimentally Induced Oxidative Stress and Iron-Overload *via* Iron Chelation and Modulation of HO-1, Nrf2, and MMP-9

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ABSTRACT

Objective: The current study aimed to investigate and compare the efficiency of the total extract and ethyl acetate (EtOAc) extract of *Mangifera indica* leaves to attenuate iron overload-induced hepatic and splenic dysfunctions, using an *in-vivo* iron-overload rat model. **Methods:** Rats received water supplemented with iron for 2 months, followed by treatment with either total extract or EtOAc extract of *M. indica* leaves for an extra 2 months. **Results:** Rats treated with EtOAc fraction of *M. indica* leaves, and the total extract, had significantly diminished iron accumulation within hepatic and splenic tissues. Both extracts normalized the hepatic enzymatic and non-enzymatic biomarkers like alkaline phosphatase (ALP), alanine aminotransferase (AST), and total bilirubin. Moreover, they restored the oxidants/antioxidants balance within the liver and spleen tissues. Additionally, they suppressed HO-1 levels, increased Nrf2 expression, and downregulated MMP-9 expression. **Conclusion:** Briefly, *M. indica* extracts showed iron-chelation, antioxidant, and antifibrotic potentials. These findings pointed out the usefulness of the mango leaves as a natural, affordable by-product in managing iron overload complications. However, the results also demonstrated that EtOAc extract is more accountable for such activities.

Keywords: *HO-1; Iron overload; Mangifera indica; MMP-9; Nrf2.*

INTRODUCTION

Iron is an indispensable micronutrient that is vital for cellular integrity. It is an active part of the heme, responsible for transporting oxygen to different body tissues, as well as an integral part of electron-transport and several critical enzyme systems within the body [1]. The presence of iron in the interconvertible ferrous or ferric oxidation states is indispensable for normal biological functions of iron, yet it is critical, as free iron is a potent generator for reactive oxygen species (ROS) and lipid peroxidation products [2, 3]. Interestingly, either iron overload or deficiency states are deleterious and are accompanied by several health problems; hence it is crucial to maintain iron homeostasis within the body [4]. Disruption of iron homeostasis and the resultant iron

accumulation is known as hemochromatosis. The etiology of hemochromatosis is either hereditary, caused by mutations in proteins that regulate iron absorption, or acquired, caused by long-term iron supplementation as in chronic anemia and thalassemia patients [5].

In hemochromatosis, iron overload induces iron dumping within many organs such as liver, spleen, kidney, and heart. Being major storage sites of iron, liver as well as spleen are highly vulnerable to iron overload-induced damage [6]. The pathogenesis of such damage could be attributed to surplus free iron generated from the heme breakdown by hemeoxygenase (HO)-1, induced in liver tissues in such noxious condition [7]. This free iron initiates ROS generation, which, in turn, damages cellular constituents, including vital organelles, proteins, DNA, and membrane lipids besides antioxidants depletion [2, 8].

Moreover, Iron overload has been correlated with many other diseases such as hepatic fibrosis/cirrhosis, heart failure, diabetes, arthritis, and cancer [9, 10]. Unfortunately, the human body lacks a defined mechanism to excrete the excess iron [4]. Hence, iron chelators can remove this excessive iron by forming soluble complexes, therefore, facilitates its excretion in the urine and/or feces. Deferoxamine (Desferal®), deferasirox (Exjade®), and deferiprone (Ferriprox®) are commercially available ion chelators, yet their expensive price, and adverse drug reactions, as well as other limitations such as low oral bioavailability and/or limited plasma half-life, make them suboptimal [10, 11]. Accordingly, recently published researches interested in finding an effective, safe, and economic iron chelator to alleviate iron overload with fewer side effects and broader safety margin is a justifiable interest. Natural products continue to be a major platform for bioactive entities. Krill oil, curcumin, and *Terminalia chebula* extract have proven to be effective in reducing the toxic level of iron, hence protect against oxidative stress [8, 12, 13]. Previously, our research team reported, through *in-vitro* studies, that the *M. indica* leaves contain high polyphenolic compounds and exhibited the highest iron-chelating and antioxidant activities, among several selected plant extracts [14].

However, the action of *M. indica* leaves on hepatic and splenic disorder caused by iron overload is still unclearly investigated. The present research focused on a comparative study of the methanolic extract, and EtOAc fraction of *M. indica* leaves to pinpoint their potential to attenuate liver and spleen abnormalities caused by iron overload as well as to determine the possible underlying mechanisms.

MATERIAL AND METHODS

Plant material and extracts preparation

Leaves of *M. indica* were collected from *Mansoura* city in August 2017 and have been authenticated by *Professor Ibrahim Mashaly, Ph.D.*, professor of Ecology and Botany, Faculty of Science, Mansoura University. The leaves were carefully washed to remove dust and other foreign particles, using running tap water, then shade dried, crushed into powder (2 Kg), and, finally, exhausted by methanol (70% v/v) (5 ×10 L). The methanolic extract was evaporated under reduced pressure to a constant weight (150 g). The EtOAc extract was prepared using liquid-liquid partitioning of 150 gm of total methanolic extract (dissolved in 50% methanol) and evaporated under reduced pressure to a constant weight (69.77 gm). Both methanolic extract and EtOAc fraction of *M. indica* were suspended in 0.5% w/v CMC solution for oral use [14, 15].

Fractionation, phytochemical screening, and determination of in-vitro antioxidant and iron chelation activity of total methanolic extract and EtOAc fraction of M. Indica leaves

Fractionation, phytochemical screening, and determination of *in-vitro* antioxidant and iron chelation activity of total methanolic extract and EtOAc fraction of *M. Indica* leaves have been previously reported by our research team [14]. In brief, the total extract showed a comparable *in-vitro* antioxidant capacity (96.95±0.98%) and iron chelating activity (69.7±0.27%) in comparison with ascorbic acid (91.95±0.29%) and ethylenediamine-tetraacetic acid (EDTA) (70.30±0.08%), respectively. Phytochemical screening of the total extract revealed a total phenolic content of 405.5± 1.25 µg/ g (expressed as gallic acid equivalent) and total flavonoid content of 336.9±3.56 µg/g (expressed as quercetin equivalent). Total methanolic extract of *M. Indica* leaves was fractionated using EtOAc, n-butanol, petroleum ether, and methylene chloride. EtOAc fraction of *M. Indica* leaves showed the highest *in-vitro* iron-chelating activity (29.28±2.52%), antioxidant capacity (99.9±0.29%), total phenolic content (631.82±1.26 µg/g), and total flavonoid content(616.126±1.97 µg/g) compared with other tested fractions [14].

Furthermore, EtOAc fraction of *M. Indica* leaves was subjected to phytochemical investigation using thin-layer chromatography (TLC) (using precoated silica gel plate GF_{254}) and column chromatography (using silica gel glass column (68.7 cm × 4.5 cm, 400 gm) packed in petroleum ether and eluted with different proportions of EtOAc in petroleum ether (0-100%, gradient elution)) to identify and isolate the compounds responsible for the iron chelation activity. The separated fractions were purified, dried, and analyzed with spectroscopic analysis (IR-spectrum, H-NMR, APT, and HMBC) for identification and characterization. The spectroscopic analysis revealed the presence of mangiferin and Iriflophenone-3-C-β-D-glucoside as major components that showed *in-vitro* iron chelation activities of 97.28 ± 2.01% and 57.54 ± 2.74%, respectively.

Chemicals

Sodium carboxymethyl cellulose (CMC) was obtained from (*EL-Nasr* Company, *Egypt*), and Tris-HCl was purchased from *Prevest* (*Prevest Denpro* Limited - *India*).

Experimental animals

Twenty-eight male Sprague-Dawley rats, each weighing 200 ± 10 gm, were used in the current study. Rats have been purchased from *Theodor Bilharz* research institute (*Giza, Egypt*). Rats were kept under standard conditions of 12-h light/dark schedule, 25 ± 2°C, and humidity up to 50±5%, in opaque polypropylene well-ventilated cages. All rats were granted free access to standard laboratory diet and water *ad libitum*. Rats were allowed to acclimatize to the housing conditions for at least 1 week prior to iron-overload induction. All animal experiments described in the current study were carried out in compliance with Laboratory Animal Care guidelines approved by the Research Ethics Committee, Faculty of Pharmacy, Mansoura University.

Experimental design

Rats were divided randomly into four groups (n=5): control group, Iron-overload (Fe-overload) group, total methanolic extract-treated (Fe/TM) group, and EtOAc fraction-treated (Fe/EM) group. Control group rats received tap water for 4 months and received neither medicine nor vehicle. Rats in Fe-overload group received 2 months of iron supplementation in drinking water and then left untreated for another 2 months. Rats in Fe/TM or Fe/EM groups received 2 months of iron supplementation in drinking water and then treated orally with 600mg/kg/day of methanolic extract or EtOAc fraction of *M. indica* (suspended in 0.5% w/v CMC solution), respectively, for another 2 months.

All rats received drinking water containing 8.3 mg of $FeSO_4$/L of drinking water for the first 2 months, except rats in control group received only tap water [8]. According to the Egyptian Ministry of Health, the maximum permissible concentration (MPC) for Fe^{2+} is 3 mg Fe^{2+}/L of drinking water. Accordingly, the concentration of iron supplemented in drinking water has been determined to exceed MPC for this metal.

Sample collection and preparation of tissue homogenates

At the end of the experiment, rats have been fasted overnight. They were anesthetized with urethane (1.2gm/kg, intraperitoneal) and sacrificed by cervical dislocation. Blood samples were collected via cardiac puncture, allowed to clot, and centrifuged for 10 min at 12000 rpm to separate serum. The supernatant serum was collected and stored at −80°C for liver functions biomarkers analysis.

Liver and spleen tissues were excised, rinsed with 0.9% normal saline, divided into small portions, and immediately frozen at −80°C for tissue homogenates preparation. Liver or spleen homogenates were freshly homogenized in 10% w/v 20 mM Tris-HCl buffer; pH 7, centrifuged for 30 min at 30,000 x g at 4°C and the supernatant was used as tissue homogenate for biochemical analysis [8, 12].

Determination of liver and spleen tissues content of iron and copper

For iron and copper determination in tissue homogenates, liver and spleen tissues have been digested with HNO_3. The digestion residues were then dissolved in 0.1 mol/L HNO_3 and then analyzed by air acetylene flame atomic absorption spectroscopy (*Perkin-Elmer, Norwalk, CT, USA*) [8].

Estimation of serum alkaline phosphatase (ALP) and alanine aminotransferase (AST) activities and total bilirubin level

Serum was analyzed for total bilirubin level and ALP and AST activities. All the analyses have been performed using commercially available *Sigma-Aldrich Corp Diagnostic Kits* (*St. Louis, MO, USA*).

Estimation of oxidative stress biomarkers, superoxide dismutase (SOD) and catalase (CAT) activities, and malondialdehyde (MDA)content in liver and spleen tissues

SOD and CAT activities and MDA content in spleen and liver homogenates have been determined using the commercial *Bio-Diagnostic* assay kits (*Giza, Egypt*) as instructed by the kit instructions.

Determination of DNA protection in liver and spleen tissues

This parameter was determined by using DNA nicking assay with some modifications [16]. The assay depends on the ability of total extract or EtOAc fraction of *M. indica* to protect DNA against damage caused by hydroxyl radicals. Bromophenol blue dye (0.25% in 50% glycerol) was mixed with tissue homogenates. 20 μL of the reaction mixture was loaded on agarose gel (0.8% solution, prepared by dissolving 0.4 g of agarose in 50 mL of 1 × Tris-Borat-EDTA puffer. The electrophoresis was performed at 90V for 60 min. and finally, stained with ethidium bromide. LAS-4000 MINI Gel Documentation system was used for visualization and quantification of complexed (protected) form pf pUC19, as described before[16].

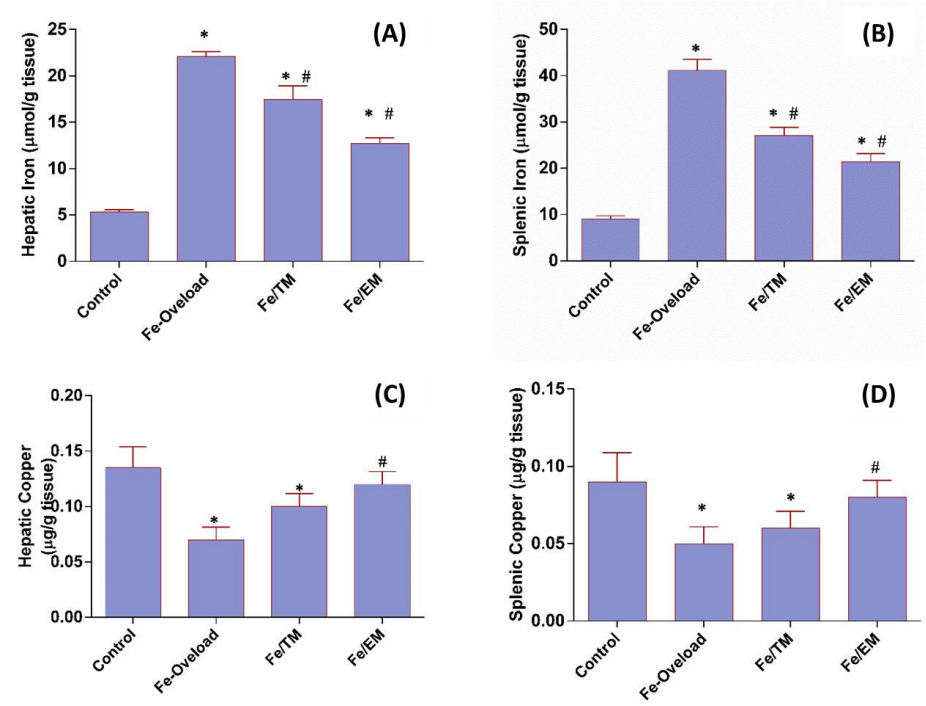

Figure 1. The effect of *Mangifera indica* leaves total extract and ethyl acetate fraction on the levels of (A) hepatic iron (Fe^{++}), (B)hepatic copper (Cu^{++}), (C) splenic iron (Fe^{++}), and (D)splenic copper (Cu^{++}). Data are expressed as mean ±SD (n=5). Values differ significantly at*p<0.05 vs. control & #p<0.05 vs. Fe-overload (ANOVA, followed by Tukey–Kramer multiple comparison test)

Determination of total protein in liver and spleen tissues

Total protein contents in homogenized liver and spleen tissues were quantified as previously described by *Adilakshmi and Laine.*, (2002) using commercial *Pierce™ Protein Reagent Assay BCA* assay kit (*Thermo Fisher Scientific., MA, USA)* as instructed by kit manual [17].

Quantification of matrix metalloproteinase (MMP)-9, nuclear factor erythroid 2-related factor 2 (Nrf2), and HO-1 expression in liver and spleen tissues

Freshly prepared liver and spleen homogenates were utilized for determination of MMP-9, Nrf2, and HO-1 tissue expression by using commercial ELISA kits; catalog No. SEA553Ra (*Cloud-Clone Corp., Katy, USA)*, ab207223 (*Abcam., MA, USA*), and MK124 (*Takara Bio Inc., Japan*), respectively, as specified by the manufacturers.

Data analysis and statistics

The results of the current study were expressed as Mean ± SD. Statistical analysis of the results has been accomplished using one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test at the levels of significance p<0.05. Statistical analysis was carried out using the *Graph Pad Instat* version 3.06 statistical package (*GraphPad Software, Inc., La Jolla, CA, USA*).

RESULTS

Determination of liver and spleen tissues contents of iron and copper:

The Fe-overload group exhibited a significant elevation in hepatic and splenic iron content by about 4-fold compared with control group. The hepatic iron content was significantly decreased in *Fe/TM and Fe/EM groups by about 21% and 43%, respectively. Meanwhile, the splenic iron content was reduced by about 34% and 48%, respectively*, all compared with Fe-overload group (**Figure 1A** and **1B**).

For hepatic and splenic copper level, Fe-overload group showed a significant reduction in copper levels in both tissues, by about 49% and 45%, respectively, compared with normal control. The hepatic and splenic copper contents were elevated, yet non-significant, in *Fe/TM group by about 43% and 20%, respectively, compared with* Fe-overload group. *Moreover, the* hepatic and splenic copper contents were

significantly elevated in *Fe/EM group by about 71% and 60%, respectively,* in comparison with Fe-overload group (**Figure 1C** and **1D**). Results indicated that the EtOAc fraction of *M. indica* was more potent than the total extract in reducing hepatic and splenic iron overload and elevation of the copper content in both tissues.

Estimation of serum ALP and AST activities and total bilirubin level

The Fe-overload rats showed a significant increase in serum total bilirubin content, and AST and ALP activities, by about 1.5, 2.2, and 1.6 folds, respectively, compared with normal rats. Upon treatment with total extract of *M. indica* leaves, rats in Fe/TM group showed a significant decrease in AST and ALP activities by about 30 and 31%, respectively, compared with Fe-overload group; meanwhile, the bilirubin level was not improved. The treatment with EtOAc fraction of *M. indica* leaves protected against iron-induced liver damage. This was evidenced by the significant decrease in bilirubin content and AST and ALP activities by about 32%, 48%, and 37%, respectively, compared with Fe-overload rats. Once again, EtOAc fraction of *M. indica* leaves showed higher potential than total extract in protection against iron-induced liver damage (**Figure 2**).

Estimation of oxidative stress biomarkers; SOD and CAT activities, and MDA content in liver and spleen tissues

Fe-overload rats revealed a significant depletion of the antioxidant activities of SOD and CAT enzymes in hepatic tissues, by about 48.8% and 72.5%, and in splenic tissues, by about 48% and 84%, respectively, as well as a significant elevation in MDA level up to 5.8 and 7 folds in both hepatic and splenic tissues, respectively, all in comparison with normal group. Total extract of *M. indica* leaves significantly increased the alleviated activities of SOD and CAT enzymes in hepatic tissues by up to 2.5 and 3.7 folds, respectively, and in the spleen by up to 2.8 and 3.5 folds, respectively, all compared to Fe-overload group. Moreover, EtOAc fraction of *M. indica* leaves significantly elevated the depleted activities of SOD and CAT enzymes in hepatic tissues, by up to 3 and 4.7 folds, respectively, and in splenic tissues by up to 3.5 and 4.4 folds, respectively, all compared to Fe-overload group. This was naturally associated with a significant decrease in MDA levels in Fe/TM and Fe/EM groups, by about 45% and 54%, respectively, in liver tissues and by about 53% and 56%, respectively, in splenic tissues, all in comparison with Fe-overload group (**Figure 3**).

Determination of DNA protection and *total protein* in liver and spleen tissues

In context, the generated state of oxidative stress in Fe-overload group was also accompanied with a significant decrease in hepatic and splenic DNA protection by about 61.8% and 68.8%, respectively, and a significant reduction of hepatic and splenic total protein levels, by about 21.7% and 28%, respectively, all compared to normal group, which denoted oxidative DNA damage. The total extract of *M. indica* leaves showed a significant increase in hepatic and splenic DNA protection by about 43.5% and 71.7%, respectively, as well as a significant elevation in hepatic and splenic total protein levels, by about 10.3 % and 14.9%, respectively, as compared with Fe-overload group (**Figure 4**). Furthermore, EtOAc fraction of *M. indica* leaves revealed significant boosting of hepatic and splenic DNA protection by about 98% and 76%, respectively, and significant augmentation of hepatic and splenic total protein levels, by about 14.2% and 20.6%, respectively, all compared to normal group, as compared with Fe-overload group (**Figure 4**).

Quantification of Nrf2 expression in liver and spleen tissues

Likewise, Fe-overload group denoted a significant retraction of Nrf2 expression in both hepatic and splenic tissues, approximately 32% and 43%, respectively, in comparison with normal control group. Rats in Fe/TM and Fe/EM groups significantly normalized the Nrf2 expression in hepatic tissues, as compared with Fe-overload group. Regarding Nrf2 expression in spleen tissues, Rats in Fe/TM and Fe/EM groups significantly increased the Nrf2 expression, by about 49% and 57%, respectively, compared with Fe-overload group (**Figure 5A & 5B**).

Quantification of HO-1 expression in liver and spleen tissues

Interestingly, rats in Fe-overload group revealed a significant increase in HO-1 expression in both hepatic and splenic tissues by about 5.5 and 1.5-fold, respectively, compared with normal control rats. Rats in Fe/TM group showed a significant decrease in HO-1 expression, by about 48%, in hepatic tissues, and a nonsignificant reduction in expression of HO-1, by about 17%, in splenic tissues, as compared with Fe-overload group. Moreover, rats in Fe/EM group showed a significant decrease in HO-1 expression, by about 57%, in hepatic tissues, and a nonsignificant reduction in HO-1 expression, by about 22%, in splenic tissues, as compared with Fe-overload group (**Figure 5C & 5D**).

Quantification of MMP-9 expression in liver and spleen tissues

In the same context, rats in Fe-overload group revealed a significant elevation of MMP-9 expression, approximately 2.6-fold, in both hepatic and splenic tissues compared with normal control rats. Upon treatment with total extract of *M. indica* leaves, rats in Fe/TM group showed a significant reduction in MMP-9 expression in hepatic and splenic tissues, by about 38%

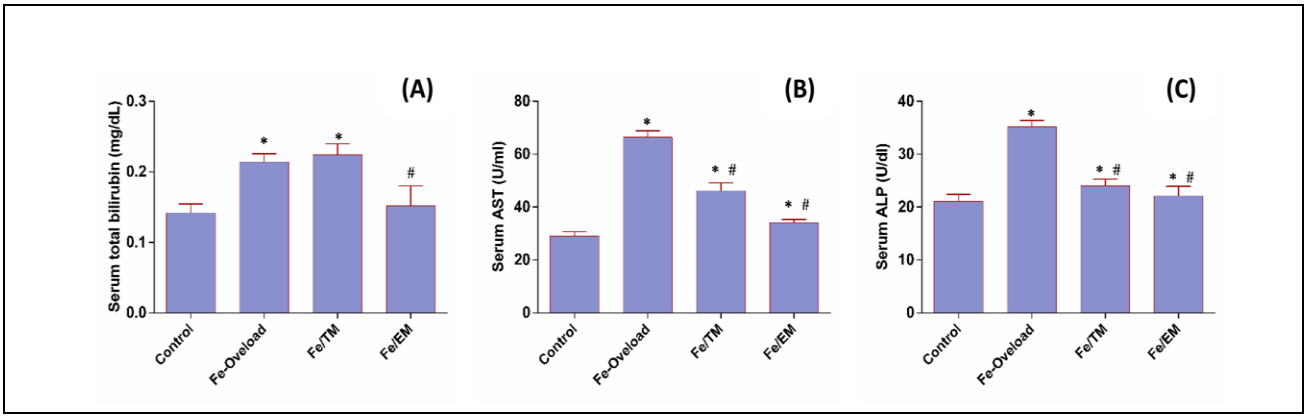

Figure 2. The effect of *Mangifera indica* leaves total extract and ethyl acetate fraction on (A) serum total bilirubin content, (B)serum AST activities, and (C) Serum ALP activities. Data are expressed as mean ±SD (n=5). Values differ significantly at*p<0.05 vs. control & #p<0.05 vs. Fe-overload (ANOVA, followed by Tukey–Kramer multiple comparison test).

and 29%, respectively, compared with Fe-overload group. Moreover, treatment with EtOAc fraction, rats in Fe/EM group exhibited a significant reduction in MMP-9 expression in hepatic and splenic tissues, by about 46% and 33%, respectively, as compared with Fe-overload group (**Figure 5E & 5F**).

DISCUSSION

Despite being a pivotal element in normal living cell physiology, dramatic iron accumulation in parenchymal cells of many vital organs as liver, spleen, kidney, and heart results in cellular and tissue injuries, which usually progress to organ fibrosis [1, 18]. Cellular injury is induced by iron-liberated ROS, reactive nitrogen species (RNS), and lipid membranes peroxidation. Lipid peroxidation triggers oxidative damage to cellular organelles, which can propagate to hepatocyte necrosis, apoptosis, and ultimately hepatic fibrogenesis [19].

The current study demonstrated that chronic iron supplementation substantially increased the hepatic and splenic iron content. These alterations have been followed by the disruption of oxidants/antioxidants homeostasis. Chronic deposition of excess iron in parenchymal cells of hepatic tissues has been reported to induce liver injury [2]. According to the hypothesis, iron can interfere with copper metabolism. Moreover, copper deficiency can result in iron overload [8, 20]. Consequently, there is an inverse relationship between iron and copper levels within the body. Administration of either total extract or EtOAc fraction of *M. indica* leaves to Fe-overload rats revealed significant alleviation of the increased iron levels within hepatic and splenic tissues, as well as restoration of iron overload-induced reduction of copper content within hepatic and splenic tissues. These results are in lined with a previous study, where curcumin successfully attenuated iron overload-increased hepatic and splenic levels of iron and normalized copper contents in these tissues [8].

Under iron overload-induced oxidative stress, free, redox-active iron catalyzes many reactions within erythrocytes, where these redox assets render iron one of the major ROS generators within biological systems [18]. Free iron produces ROS via the Fenton reaction, where ferric iron is reduced to ferrous iron by the superoxide radical. Ferrous iron, in turn, reacts with hydrogen peroxide to generate hydroxyl radicals, which are highly reactive and can destroy hepatocytes as well as splenic tissues [21, 22]. The iron-induced ROS can directly damage biomolecules, with subsequent boosting in protein oxidation, membrane lipids peroxidation, and DNA damage [20, 23]. Similarly, iron supplementation showed a significant increase in hepatic and splenic MDA content, the final biomarker of lipid peroxidation, and DNA damage as well as a significant reduction in total protein content in these tissues in comparison with the control group. Moreover, ROS mediate oxidative degradation of SOD with a subsequent reduction in SOD activity. SOD is pivotal for the neutralization of superoxide radical into hydrogen peroxide and oxygen. The unneutralized superoxide radicals promote the inactivation of CAT activity[24, 25].

Oral administration of total extract or EtOAc fraction of *M. indica* leaves significantly revoked iron-induced oxidative damage in both hepatic and splenic tissues, as revealed by diminished MDA content and DNA damage as well as increased total protein levels and significant boosting of antioxidant CAT and SOD enzymatic activities in the liver and spleen. These results are in harmony with earlier studies where Krill oil and curcumin have been reported to attenuate oxidative damage induced by iron overload in similar rat models[8,13].

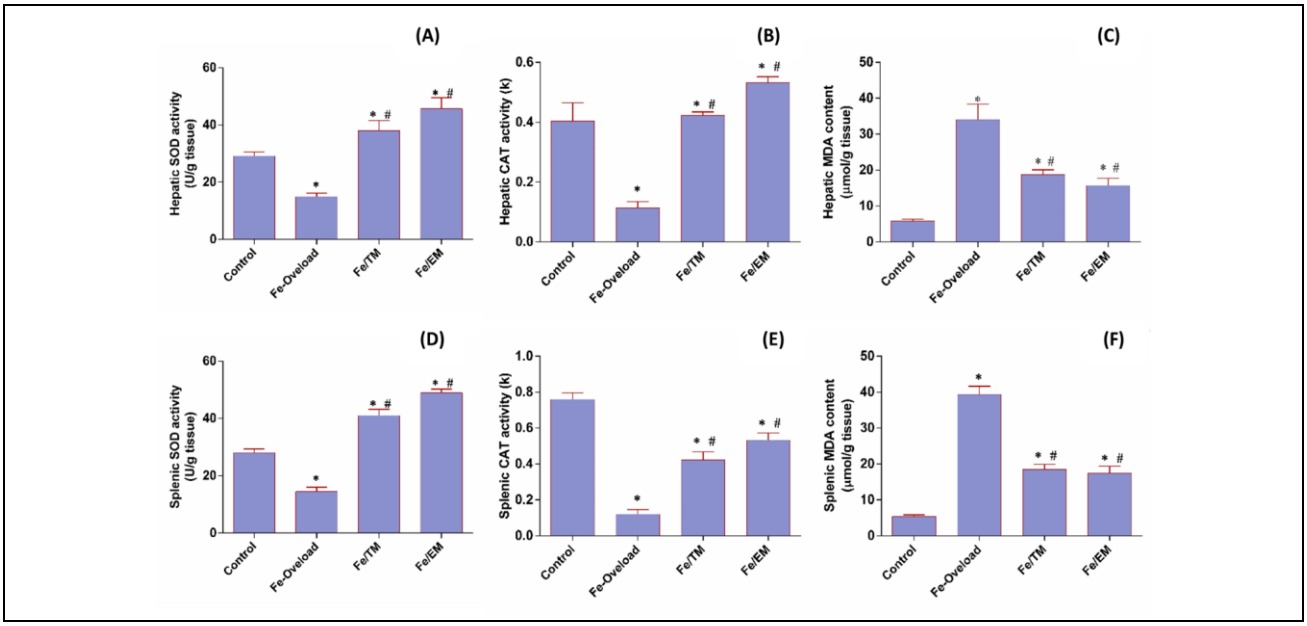

Figure 3. The effect of *Mangifera indica* leaves total extract and ethyl acetate fraction on (A) hepatic SOD activity, (B)hepatic CAT activity, (C) hepatic MDA level, (D) splenic SOD activity, (B)splenic CAT activity, and (C) splenic MDA levels. Data are expressed as mean ±SD (n=5). Values differ significantly at*$p<0.05$ vs. control & $^{\#}p<0.05$ vs. Fe-overload (ANOVA, followed by Tukey–Kramer multiple comparison test)

All the above data indicated that both total extract and EtOAc fraction of *M. indica* leaves re-established the impaired oxidants/antioxidants balance and attenuated the induced DNA damage. These protective effects might be credited to the antioxidant as well as iron chelation impact of *M. indica* as evidenced by increased copper levels and enzymatic activities of CAT and SOD as well as decreased iron and MDA levels in both hepatic and splenic tissues. Our previous *in-vitro* study also asserts these effects, where it reported significant iron-chelating and antioxidant activities of *M. indica* leaves extract, owing to its high content of polyphenolic compounds [14].

Moreover, iron overload-induced reduction in SOD activity might be attributed to copper deficiency. Consequently, improvement of SOD activity in liver and splenic tissues following treatment with total extract and EtOAc fraction of *M. indica* leaves could be credited to the modulation of copper content [8].

One issue that attracted considerable attention is the antioxidant Nrf2. Nrf2 controls cytoprotective genes transcription, which are mostly involved in the regulation of ferroptosis. Ferroptosis, a regulated cell death, has been reported to be initiated by disturbance of the glutathione system and exacerbated by iron overload. Ferroptosis ultimately results in mitochondrial dysfunction and lipid peroxidation [26]. Nrf2 is normally present in the cytoplasm chelated by Kelch-like ECH–associating protein 1 (Keap1) in an inactive form. Under stress, Nrf2 is released from Keap1 and translocated into the nucleus, where cytoprotective genes expression is initiated [27]. However, oxidative stress and ROS generation induced by iron overload effectuated a significant decline in the expression of Nrf2 in hepatic and splenic tissues relative to the control group. Oral treatment with total extract or EtOAc fraction of *M. indica* leaves significantly augmented hepatic and splenic Nrf2 expression compared to Fe-overload group. These results give more credence to our postulation that total extract and EtOAc fraction of *M. indica* leaves provide a potential antioxidant and iron chelating effects.

As a response to oxidative stress-induced hepatic injury, cellular enzymes may leak into the bloodstream. Augmented serum levels of liver enzymes such as ALP, AST, alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) can emphasize damage to hepatic tissue as functional integrity of cellular membranes have been lost [12, 28]. These findings are in harmony with our results, where iron supplementation provoked significant elevation in serum levels of AST and ALP enzymes in Fe-overload group. In contrast, supplementation with total extract or EtOAc fraction of *M. indica* leaves significantly abolished the raised AST and ALP serum levels. These results indicated that treatment with total extract or EtOAc fraction of *M. indica* leaves significantly attenuated iron overload-induced liver damage.

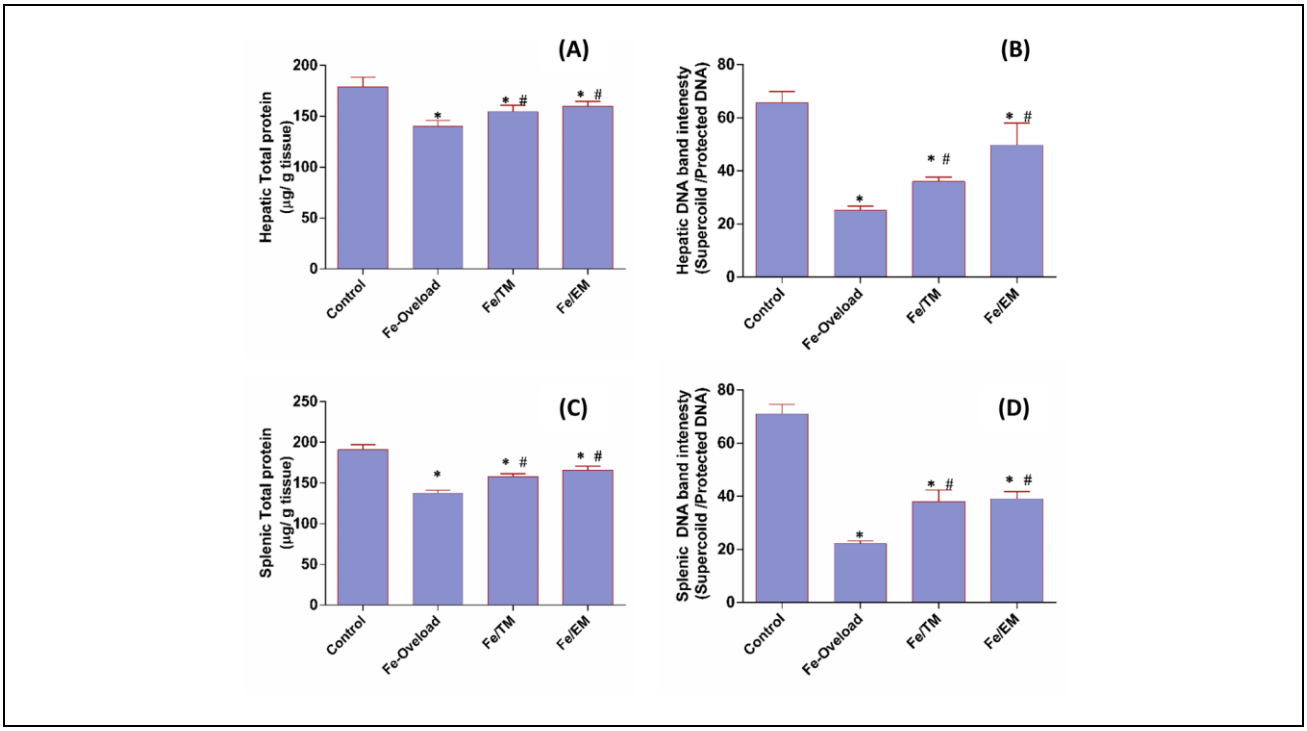

Figure 4. The effect of *Mangifera indica* leaves total extract and ethyl acetate fraction on (A) hepatic total protein content, (B) Hepatic DNA protection, (C) splenic total protein content, and (D) splenic DNA protection. Data are expressed as mean ± SD (n=5). Values differ significantly at*p<0.05 vs. control & $^{\#}p$<0.05 vs. Fe-overload (ANOVA, followed by Tukey–Kramer multiple comparison test)

Moreover, under iron-induced noxious cellular damage, hepatic and splenic macrophages, as well as neutrophils, release a surge of ROS and RNS, which are scavenged by circulating erythrocytes. Oxidative damage to erythrocytes with subsequent heme cleavage as well as transformation into senescent erythrocytes, induce expression of HO-1 enzyme [29]. HO-1, an inducible isoform of HO enzyme system, showed limited expression in normal liver [30]. In contrast, HO-1 exhibited a greater expression in splenic tissues, where it has a pivotal role in the recycling of iron from damaged heme [31]. HO-1 catalyzes cleavage of non-functional heme to biliverdin/ bilirubin, carbon monoxide, and free iron. Bilirubin exhibits an antioxidant activity via scavenging peroxy radicals and inhibition of lipid peroxidation. Ferritin, is also co-induced with HO-1, permitting safe chelation of free iron released from heme cleavage [32]. HO-1 induction is believed to be a defensive feedback mechanism for removal and neutralization of excessive free iron resulting from the breakdown of erythrocytes and heme as well as combating Fe-overload induced oxidative damage [13, 33]. Khan *et al*. (2004) have reported that pro-oxidative iron could accumulate in hepatic cells even with elevated activity of HO system, leading to augmented oxidative stress and hepatic dysfunction [30]. Similarly, our results revealed that iron supplementation triggered a marked rise in hepatic and splenic HO-1 and serum bilirubin levels compared with normal control group. Consequently, Supplementation with total extract or EtOAc fraction of *M. indica* leaves diminished the upregulated levels of HO-1 and bilirubin in comparison with Fe-overload rats. These results could account for total extract, and EtOAc fraction of *M. indica* leaves antioxidant potentials and its ability to counteract oxidative stress and hepatic and splenic damage.

Cellular injury, induced by iron-induced ROS and lipid peroxidation, and subsequent hepatocellular organelles damage is believed to contribute to necrosis and apoptosis of hepatocytes, and eventually progress to hepatic fibrogenesis [18]. Notably, one of the major phenomena of this hepatic fibrosis is the accumulation of extracellular matrix (ECM) in hepatic tissues. MMPs have been reported to be implicated in ECM degradation and hepatic fibrosis progression [34]. Among different MMPs, the upregulated expression of MMP-9 has been reported in the initial phases of hepatic fibrogenesis. MMP-9 is responsible for the turnover and degradation of several ECM proteins, including collagen of type IV and fibronectin. Moreover, it can release/activate the profibrotic cytokine, transforming growth factor-beta (TGF-β), from ECM reservoirs[35, 36, 37]. In addition to ECM breakdown and TGF-β upregulation, MMP-9 may

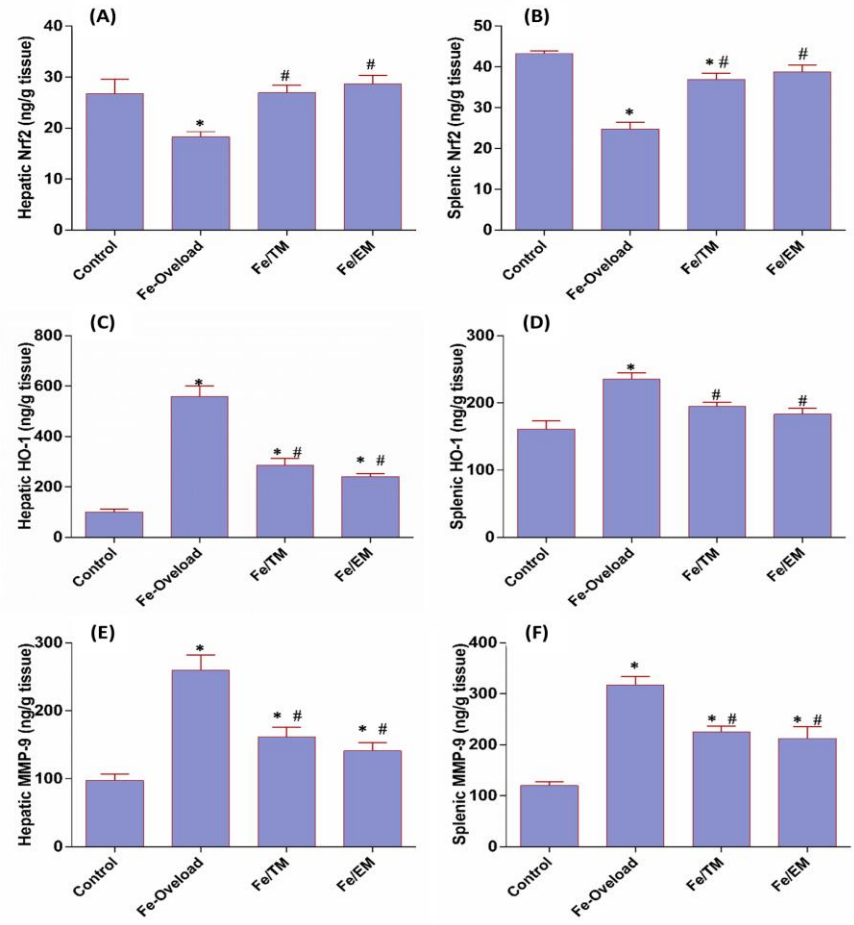

Figure 5. The effect of *Mangifera indica* leaves total extract and ethyl acetate fraction on (A) hepatic Nrf2 expression, (B) splenic Nrf2 expression, (C) hepatic HO-1 expression, (D) splenic HO-1 expression, (E) hepatic MMP-9 expression, and (F) splenic MMP-9 expression. Data are expressed as mean ±SD (n=5). Values differ significantly at*p<0.05 vs. control & $^{\#}p$<0.05 vs. Fe-overload (ANOVA, followed by Tukey–Kramer multiple comparison test)

enhance apoptosis of hepatic stellate cells in the presence of low levels of tissue inhibitor of matrix metalloproteinase (TIMP)-1 [19, 37]. Similarly, Abdalkader *et al*. (2018) has reported overexpression of MMP-9 as well as its tissue inhibitors, TIMP-1, in Fe-overload livers [26].

In the current study, iron markedly enhanced hepatic and splenic MMP-9 expression compared to normal rats. Rat supplemented with total extract or EtOAc fraction of *M. indica* leaves showed significant declination in the hepatic and splenic expression of MMP-9 in comparison with the Fe-overload rats. This observation suggested that total extract and EtOAc fraction of *M. indica* leaves might confer protection to hepatic and splenic tissues through antifibrotic impact.

Several previous studies have proved that plants rich in polyphenolic compounds could act as a potential source for compounds that possess an iron-chelating as well as antioxidant properties [38, 39]. In particular, polyphenolic compounds containing *ortho*-dihydroxy polyphenol groups can exhibit a strong ability to chelate iron metal [39, 40]. Several previously conducted studies on stem bark extract of *M. indica* known as (Vimang®) and seed kernel parts reported that they have potent hydroxyl radicals scavenging and a potential iron-chelating activities [41, 42]. Interestingly, our previously published study demonstrated that each of total extract and EtOAc fraction of *M. indica* leaves showed potential chelation and antioxidant activity using 2, 2`- bipyridyl assay, among the tested extracts. Also, EtOAc fraction of *M. indica* leaves exhibited higher phenolics and flavonoids compared to methanolic extract [14]. In the current study, EtOAc fraction of *M. indica* leaves showed preferable activity to restore all biological parameters associated with chronic Fe-overload disorder toward the normal values compared to total extract. The higher protective

potentials of EtOAc fraction of *M. indica* leaves might be credited to its high content of polyphenol compounds. Therefore, the current study supports the previous findings of our research team[14].

CONCLUSION

The current study demonstrated that the EtOAc extract is accountable for the activity of Mango leaves as an iron chelator. EtOAc fraction of *M. indica* leaves attenuated iron accumulation, exhibited antioxidant as well as antifibrotic potentials, and protected against iron overload-induced liver and spleen damage in chronic Fe-overload rats. Interestingly, total extract was less effective in the management of iron- overload complications. Future studies on EtOAc extract of *M. indica* leaves are needed to identify the bioactive compounds responsible for the observed activities, even if some literatures cited that mangiferin is the principal constituent responsible for such activities.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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