Chapter IV

Study of *in vitro* and *in vivo* iron chelating property of various isolated compounds using mouse model
4.1. Introduction

Iron is indispensable for virtually all aspects of life, while playing a role in immune function, cardiovascular health and cognitive development. Iron has the unique ability to alter its oxidation and redox states in response to liganding, which makes it essential for various cellular processes (Kruszewski, 2003). The cells maintain the free iron concentration to a minimum required level to avoid toxic effects of excess iron. But, in some situations the iron balance is disrupted and resulting iron overload in the body. Overloaded iron is potentially toxic as it generates Reactive Oxygen Species (ROS) such as hydroxyl and superoxide radicals and iron mediated oxidative stress, which is thought to be a significant factor in the disease formation such as anemias, heart failure, liver cirrhosis, fibrosis, gallbladder disorders, diabetes, arthritis, depression, impotence, infertility, and cancer (Goodman & Gilman, 2006). ROS initiate lipid peroxidation of cell membranes and oxidative damage of proteins, which in turn hampers membrane fluidity, disruption of microsomes and lysosomes, and accumulation of peptide fragments and cross-linked protein aggregates (Pietrangelo, 2003). As human beings have no active means of iron excretion, excess iron, regardless of the route of entry, accumulates in parenchymal organs and impend cell viability (Yajun et al., 2005). In our body, liver is the main active storage site of iron and, therefore, liver damage is the most common outcome in patients with iron overload (Papanastasiou et al., 2000). The only way to stop the detrimental effect of free or partially liganded ‘iron’ is to make sure that all of its six possible ligands are satisfied, whether by endogenous chelators or those added from the diet or as pharmaceuticals (Kell, 2010). The currently available iron-chelating agents used clinically are deferoxamine, 1,2-dimethyl-3-hydroxypyrid-4-one (deferiprone, L1), and deferasirox. However, such compounds show several side effects and limitations (Al-Refaie et al., 1992; Richardson, 1999) which in turn grow the interest to develop more effective and safer drug from natural resources. (Zhang et al., 2006; Pardo-Andreu et al., 2008) which may rise the therapeutic benefits for patients (Al-Refaie et al., 1992; Kontoghiorghes, 2003).

Among different bioactive phytoconstituents, phenolics and flavonoids are the most important representatives that offer alleviation of hepatic ailments. It has been found most of them are effective antioxidants and iron chelation is a very important part of their antioxidant activity (Cook & Samman, 1996). These iron-chelating agents consist of a range of bidentate, tridentate, and hexadentate ligands which are able to coordinate with iron, forming octahedral complexes and excrete from the body (Liu & Hider, 2002; Tam et al., 2003). Thus, the use of drugs from plant origin with antioxidant activity could be an alternative strategy to treat iron overload induced hepatic damage. Therefore, the present study was performed to assess whether the isolated phenolic antioxidant compounds, can be used to alleviate the damage caused to liver by iron overload in Swiss albino mice.

4.2. Materials and Methods

4.2.1. Chemicals

Iron-dextran and guanidine hydrochloride was purchased from Sigma-Aldrich, USA. Trichloroacetic acid (TCA), nitro blue tetrazolium (NBT), reduced nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), ferrozine, glutathione reduced, bathophenanthroline sulfonate disodium salt, thiobarbituric acid (TBA), and 5,5’-dithiobis-2-nitrobenzoic acid (DTNB) were obtained from Sisco Research Laboratories Pvt. Ltd, Mumbai, India. Hydrogen peroxide, ammonium iron (II) sulfate hexahydrate [(NH₄)₂Fe(SO₄)₂·6H₂O], 1-chloro-2,4-dinitrobenzene (CDNB), chloramine-T, hydroxylamine hydrochloride, dimethyl-4-
aminobenzaldehyde and 2,4-dinitro phenylhydrazin (DNPH) were obtained from Merck, Mumbai, India. Ferritin was purchased from MP Biomedicals, USA. Streptomycin sulphate was obtained from HiMedia Laboratories Pvt. Ltd, Mumbai, India. The standard oral iron chelating drug, desirox, with the parent group Deferasirox, was obtained from Cipla Ltd., Kolkata, India.

4.2.2. Animals

Swiss albino mice (Male, weighing 20 ± 2 g) were acquired from Chittaranjan National Cancer Institute (CNCI), Kolkata, India. The animals were kept under a continuous 12 h light / dark cycle (temperature-22 ± 2°C). The animals were fed with laboratory diet and water ad libitum. Experimental animals were taken care every 6 h during the treatment period and it was observed that there was no unwanted animal death.

4.2.3. Ethics

In vivo experiments were performed abiding by the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Govt. of India with due approval from the Institutional Animal Ethics Committee, Bose Institute (Registration. No. 95/1999/CPCSEA). All surgeries were done using ethyl ether as anesthetic (inside an appropriate fume hood), taking utmost care to reduce suffering.

4.2.4. In Vitro Study

4.2.4.1. Iron Chelation

The chelating activity of SPE2, Gallic acid (GA), Methyl gallate (MG) and SPW1 for ferrous ion was evaluated by a previously described method (Haro-Vicente et al., 2006). In a Hepes buffer (20 mM, pH 7.2) medium, test compounds (0-120 μg/ml) was added to ferrous sulfate solution (12.5 μM) and the reaction was started by the addition of ferrozine (75 μM). The mixture was shaken vigorously and left standing for 20 min at room temperature. The absorbance was then taken at 562 nm. All tests were performed six times. EDTA was used as a positive control.

4.2.4.2. Measurement of Reducing Power

The Fe$^{3+}$ reducing power of the test compounds was determined by the standard method (Oyaizu, 1986) with a slight modification. Different concentrations (0-1 mg/ml) of compounds (0.5 ml) were mixed with 0.5 ml phosphate buffer (pH 6.6) and 0.5 ml 0.1% potassium hexacyanoferrate, followed by incubation at 50°C in water bath for 20 min. After incubation, 0.5 ml 10% TCA was added to terminate the reaction. The upper portion of the solution (1 ml) was mixed with 1ml of distilled water and 0.1 ml 0.01% FeCl$_3$ solution was added. The reaction mixture was left for 10 min at room temperature and the absorbance was measured at 700 nm against appropriate blank solution. All tests were performed six times. Ascorbic acid was used as a positive control.

4.2.5. In Vivo Study

4.2.5.1. Experimental Design

Seventy two mice were distributed into twelve groups comprising six mice in each group. One group received normal saline only and served as blank (B). The other groups were given five doses (one dose every two days) of 100 mg/kg b.w. each, of iron-dextran saline (i.p).
Normal saline was administered to one iron-dextran group (C) and other groups were orally treated with 2 mg/kg b.w. GA (G2), 2 mg/kg b.w. MG (M2), 50 mg /kg b.w. SPW1 (W50), 4 mg/kg b.w. GA (G4), 4 mg/kg b.w. MG (M4), 100 mg /kg b.w. SPW1 (W100), 8 mg/kg b.w. GA (G8), 8 mg/kg b.w. MG (M8), 200 mg /kg b.w. SPW1 (W200) and 20 mg/kg b.w. desirox (D), respectively, for 21 days (Figure 4.1).

**Figure 4.1 Schematic Diagram of Experimental Design**

Thirty-six mice were divided into six groups comprising six mice in each group. Group B: received normal saline only and other five groups received iron-dextran. Out of these five groups, group C received normal saline and other four groups received different doses of test samples and standard drug desirox respectively.

4.2.5.2. Sample Collection and Tissue Preparation

Mice were fasted overnight after the experiment ended on the 21st day. All experimental animals were sacrificed on 22nd day under mild anesthesia (ethyl ether) and cardiac puncture was performed to collect blood and serum was separated and stored at -80°C. After collecting the blood, the liver was quickly excised, cleaned thoroughly with cold phosphate buffer saline (PBS) to remove the remaining blood and cut into three sections. The major liver portion was dissected and homogenized using 10 volumes of 0.1M phosphate buffer (pH7.4) supplemented with 0.15M NaCl and 5mM EDTA and centrifuged for 30min at 8000g in cold. The clear homogenate (supernatant) was collected and the protein concentration was quantified by Folin Lowry method (Lowry et al., 1951), where BSA was used as a standard; the remaining supernatant was then stored at -80°C. Second liver fragment was treated with a mixture of nitric acid and sulfuric acid (1:1) to analyse the iron content. The remaining portion was processed for histopathological examinations.

4.2.5.3. Liver Iron & Serum Ferritin Levels

Liver iron was measured according to a formerly reported colorimetric method (Barry & Sherlock, 1971). Samples were digested with HCl-TCA mixture, incubated with bathophenanthroline sulfonate and later with ammonium acetate for 30 min at 37°C and absorbances were measured at 535 nm.

Serum ferritin levels were measured using enzyme-linked immunosorbent assay kit (AccuBind Ferritin Kit from Monobind Inc., USA) according to the manufacturer’s instructions.
4.2.5.4. Measurement of Serum Markers Levels

In the serum samples, Alanine aminotransferase (ALAT) was measured using Ecoline® ALAT (Tris-GPT) kit; aspartate aminotransferase (ASAT) using Ecoline® ASAT (Tris-GPT) kit and bilirubin using Merckotest® Bilirubin kit. All kits were commercially procured from Merck, Mumbai, India. Alkaline phosphatase (ALP) was estimated using the ALP Liquid kit supplied by Sentinel Diagnostics, Italy.

4.2.5.5. Levels of Antioxidant Enzymes

Superoxide dismutase (SOD) was assayed by measuring the inhibition of the formation of blue colored formazan at 560 nm according to the technique reported previously (Kakkar et al., 1984). The procedure was followed likewise that for superoxide in the free radical scavenging assays as following: In a KOH-KH$_2$PO$_4$ buffer containing NBT (50 µM) and NADH (75 µM), samples are incubated with 15 µM PMS to get the formazan.

Catalase (CAT) activity was measured by following the decomposition of H$_2$O$_2$ over time at 240 nm according to a previously described method (Bonaventura et al., 1972), where the samples are incubated with 50 mM H$_2$O$_2$ in a phosphate buffer solution prior to spectrophotometric determination.

Glutathione-S-transferase (GST) was determined by a formerly reported method (Habig et al., 1974) based on the formation of GSH-CDNB conjugate in a phosphate buffer solution and increase in the absorbance at 340 nm.

Reduced glutathione (GSH) level was measured spectrophotometrically at 412 nm by a standard method (Ellman, 1959). Briefly, the sample was incubated in a phosphate buffered solution of Ellman’s Reagent (50 nM DTNB in 0.1% NaNO$_3$) and the gradually increasing O.D. was measured.

4.2.5.6. Evaluation of Liver Damage and Fibrosis

Lipid peroxidation levels in liver homogenates were determined in terms of Thiobarbituric Acid Reactive Substances (TBARS), as an index of malondialdehyde accumulation, in accordance to a formerly reported method (Buege & Aust, 1978).

Protein carbonyl contents were estimated spectrophotometrically by a previously described method (Reznick & Packer, 1994). Briefly, 450 µl sample homogenate was mixed with 50 µl streptomycin sulphate (10% w/v) and then centrifuged at 2800 g for 15 min. Then 200 µl of the supernatant was incubated with the same volume of 10 mM DNPH in 2 M HCl at room temperature for 20 min. After the reaction was completed, 10% cold TCA was added to precipitate the proteins and the precipitates were washed with ethyl acetate-ethanol mixture (1:1) for three times to remove unreacted DNPH. The final protein pellet was dissolved in 1 ml of 6 M guanidine hydrochloride solution and the absorbance was measured at 370 nm, using the molar extinction coefficient of DNPH, ε = 2.2x10$^{-4}$ M$^{-1}$ cm$^{-1}$.

Hydroxyproline content represents the content of collagen, which is closely related with liver fibrosis. Liver samples were hydrolized in 6 M HCl and hydroxyproline was measured by Ehrlich's solution according to the method described previously (Bergman & Loxley, 1963). A standard curve ($R^2 = 0.9907$) of 4-hydroxy-L-proline was prepared and results were calculated after taking absorbances at 558 nm. The collagen content was determined by multiplying amount of total hydroxyproline content in each sample by a factor of 7.69 (Kivirikko et al., 1967). Results are expressed as milligrams of collagen per liver (wet weight).
4.2.5.7. Iron Release from Ferritin

Iron release assay was performed according to a previously described method (Hynes & Cointeannainn, 2002). The release of ferritin iron was measured using the ferrous chelator ferrozine as a chromophore. The reaction mixture (3 ml final volume) contained 200 μg ferritin, 500 μM ferrozine, in 50 mM pH 7.0 phosphate buffer. Reaction was started by the addition of 500 μl plant samples of different concentrations (100-500 μg) and the change in absorbance was measured continuously at 560 nm for 20 min.

4.2.5.8. Histopathological Analysis

Excised liver samples were washed in normal saline and processed separately for histopathological evaluation. After fixing the sample in 10% buffered neutral formalin for 48 h, paraffin embedding technique was carried out and the sections were taken at 5 mm thickness using a microtome.

Liver sections were stained with hematoxylin to stain the nuclei blue and eosin for cytoplasm and the extracellular connective tissue matrix to stain pink and examined independently under microscope for morphological changes.

Prussian blue, a common stain used by pathologists to detect the presence of iron in biopsy specimens. Ferric iron deposits in tissue react with the soluble ferrocyanide in the stain, to form insoluble Prussian blue dye (a complex hydrated ferric ferrocyanide substance). They are then detected microscopically as blue or purple deposits, within cells.

Liver sections were also stained with Masson's trichrome stain to study the degree of liver fibrosis. It is a three-colour staining protocol used in histology. Red to stain keratin and muscle fibers, blue to collagen, light red or pink cytoplasm, and dark brown to black cell nuclei.

4.2.6. Statistical Analysis

All data are reported as the mean ± SD of six measurements. Statistical analysis was performed using KyPlot version 2.0 beta 15 (32 bit) and OriginPro 8.5.1 (OriginLab Corporation, USA). Comparisons among groups were made according to ‘pair t-test’ for in vitro studies and ‘One way ANOVA’ for in vivo studies. The IC₅₀ values were calculated by the formula, \( Y = 100 \times \frac{A_1}{X + A_1} \) where \( A_1 = IC_{50}, Y = response \) (\( Y= 100\% \) when \( X= 0 \)), \( X= \) inhibitory concentration. \( p< 0.05 \) was considered significant.

4.3. Results

4.3.1. In Vitro Study

4.3.1.1. Iron Chelation

\( \text{Fe}^{2+} \) ion reacts with ferrozine to form a violet colour complex. The formation of violet colored \( \text{Fe}^{2+}- \)ferrozine complex is disrupted by the presence of an iron chelator and as a result the violet colour is decreased. The results (Figure 4.2) demonstrated that the formation of \( \text{Fe}^{2+}- \)ferrozine complex is inhibited dose dependently in the presence of SPW1, MG, GA and reference compound EDTA, whereas SPE2 failed to possess any activity. The IC₅₀ values of the SPW1, MG, GA and standard EDTA were 59.98 ± 0.67 μg/ml, 129.85 ± 6.90 μg/ml and 1007.35 ± 141.31 μg/ml and 1.27±0.05 μg/ml, respectively.
Figure 4.2 Iron Chelating Study

Effects of (A) SPE2, GA, MG, SPW1 and (B) EDTA on ferrozine-Fe$^{2+}$ complex formation. The data expressed as percentage inhibition of chromogen formation. All data are expressed as mean ± S.D. (n = 6). *$p$< 0.05 and ***$p$< 0.001 vs. 0 μg/ml.

4.3.1.2. Fe$^{3+}$ Reducing Property

The reducing capacity of a compound may serve as a significant indicator of its potential iron reducing (Fe$^{3+}$ to Fe$^{2+}$) activity. The antioxidant power to reduce ferric ions by these active compounds was found to increase in a concentration-dependent manner (Figure 4.3) with MG possessing the highest activity. But in this assay also SPE2 failed to show any significant activity.

Figure 4.3 Total Reduction Capability

The reductive abilities of SPE2, GA, MG, SPW1 and the ascorbic acid. Each value represents mean±S.D. (n = 6). ***$p$< 0.001 vs 0 mg/ml.

4.3.2. In Vivo Study

4.3.2.1. Liver Iron & Serum Ferritin Levels

The iron overload condition was measured directly by the amount of iron in the liver and indirectly by ferritin content in serum. Compared to the normal mice the iron content (686%) and serum ferritin (143%) was elevated due to iron overload but on treatment with GA, MG and SPW1 reverted the iron content as well as ferritin content almost to the levels of normal
mice (Figure 4.4A, 4.4B respectively). The highest dose of MG demonstrated better activity than the standard drug desirox and significantly eliminated the excess iron, thus making the condition equal to the normal mice group.

**Figure 4.4** Iron Removal Potential of GA, MG and SPW1

(A) Hepatic iron content, (B) Serum ferritin content. Values are mean ± SD (n=6). *p < 0.05, **p ≤ 0.01, ***p ≤ 0.001 compared with the blank and *p < 0.01, **p ≤ 0.001 compared with the control.

### 4.3.2.2. Measurement of Serum Markers Levels

Levels of serum enzyme are checked in the clinical diagnosis to determine the condition of various diseases and tissue injury especially due to excess iron load. The elevated levels of serum enzymes and bilirubin markedly dose-dependently down regulated after oral administration of GA, MG and SPW1. From Table 4.1, it is clear that higher doses of MG displayed better activity than GA, SPW1 and the standard desirox in all cases except for bilirubin.

**Table 4.1: Effect of the Compounds on Serum Marker Levels of Hepatocellular Injury**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ALAT (Unit/L)</th>
<th>ASAT (Unit/L)</th>
<th>ALP (Unit/L)</th>
<th>Bilirubin (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GA</td>
<td>MG</td>
<td>SPW1</td>
<td>GA</td>
</tr>
<tr>
<td>B</td>
<td>14.42±1.91</td>
<td>14.42±1.91</td>
<td>14.42±1.91</td>
<td>62.46±6.32</td>
</tr>
<tr>
<td>C</td>
<td>64.60±3.32</td>
<td>64.60±3.32</td>
<td>64.60±3.32</td>
<td>234.91±8.30</td>
</tr>
<tr>
<td>M2, G2, W50</td>
<td>42.96±1.16</td>
<td>42.96±1.16</td>
<td>42.96±1.16</td>
<td>145.45±5.89</td>
</tr>
<tr>
<td>M4, G4, W100</td>
<td>32.17±2.16</td>
<td>32.17±2.16</td>
<td>32.17±2.16</td>
<td>114.78±8.36</td>
</tr>
<tr>
<td>M8, G8, W200</td>
<td>23.22±0.95</td>
<td>23.22±0.95</td>
<td>23.22±0.95</td>
<td>83.56±4.36</td>
</tr>
</tbody>
</table>

Values are mean ± SD from 6 animals in each group. X: significant difference from normal mice (B) group (X1: *P < 0.05, X3: **P < 0.001); Y: significant difference from iron overloaded (C) group (Y3: *P < 0.001)

### 4.3.2.3. Levels of Antioxidant Enzymes

The iron-overloaded condition stimulated an oxidative stress condition that significantly reduces levels of antioxidant enzymes and non-enzymatic antioxidant GSH compared with normal mice.
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Figure 4.5  Effect of GA, MG and SPW1 Treatment on Liver Antioxidant Enzyme Levels.

(A) SOD, (B) Catalase, (C) GST, (D) GSH. Values are expressed as the mean ± SD (n=6). *p < 0.05, **p ≤ 0.01, ***p ≤ 0.001 compared with the blank and #p ≤ 0.05, ##p ≤ 0.01, ###p ≤ 0.001 compared with the control.

Oral administration of GA, MG and SPW1 indicated significantly restore the dropped levels of SOD (89%), Catalase (73%), GST (72%), GSH (37%) almost to the normal state. In the case of SOD (Figure 4.5A), catalase (Figure 4.5B), GST (Figure 4.5C) and GSH (Figure 4.5D) SPW1 demonstrated better activity than standard desirox treatment. Among the compounds the activity is as follows-SPW1>MG>GA.

4.3.2.4 Evaluation of Liver Damage and Fibrosis

Iron overload increases lipid peroxidation product which is a sensitive indicator of hepatocellular oxidative stress. The intraperitoneal injection of iron-dextran significantly enhanced (~70-100%) lipid peroxidation in liver homogenates compared to normal control mice. The increased levels of lipid peroxidation were reduced after oral administration of GA, MG and SPW1 (Figure 4.6A). Another consequence of iron overload induced toxicity is oxidative modification of proteins and carbonyl formation which may be used an early marker for protein oxidation. A significant elevation (149%) of protein carbonyl content in iron overloaded mice was found to be arrested by MG and SPW1 better than standard desirox (Figure 4.6B). The hydroxyproline content was determined as it signifies the enhanced level of collagen content in liver fibrosis. Hydroxyproline content was observed to rise by 122% in disease group but treatment with the test compounds reduced the collagen content dose dependently (Figure 4.6C). Overall treatments with the compounds reduce the liver damage as well as fibrosis due iron overload.
4.3.2.5. Iron Release from Ferritin

A dose dependent increase in the formation of the ferrous-ferrozine complex \([\text{Fe(ferrozine)}^2+]\) was measured to quantify the efficiency of GA, MG and SPW1 in releasing the reduced iron from ferritin. Experiments without test samples formed insignificant quantities of the reduced iron ferrozine complex, whereas increasing concentrations significantly released iron from ferrozine with time (Figure 4.7A). SPW1 demonstrated better activity followed by MG and GA. But when the percentage of iron released from ferritin and the reducing power of the test samples were correlated (Figure 4.7B) GA was found to be most significant than the other two compounds.

Figure 4.7  Iron Release from Ferritin.

(A) Iron release from ferritin, (B) Correlation between iron released from ferritin with reducing power. Iron released in response to increasing amounts (100-500 μg) of GA, MG and SPW1 was plotted against reducing power displayed at the same doses.
4.3.2.6. Histopathological Analysis

Liver sections were stained with hematoxylin and eosin for morphologic evaluation, Perls' Prussian Blue stain for assessment of iron load and Masson’s trichrome stain for assessment of fibrosis. Liver sections from normal mice demonstrated normal cell morphology with prominent nuclei in well-preserved cytoplasm and prominent central vein without cellular infiltration (Figure 4.8A), whereas iron dextran overloaded mice demonstrated various degrees of pathological changes including ballooning degeneration, inflammation, loss of cellular boundaries, and hepatocellular necrosis (Figure 4.8B).

Figure 4.8 Microscopic Observation of Mouse Liver Sections that had been Stained with Hematoxylin and Eosin at ×400

(A) Liver sections from control mice with normal cytoarchitecture. (B) Iron-overloaded (iron dextran, 100 mg/kg b.w.) liver section demonstrates degeneration of cellular boundaries, fatty ballooning deterioration, inflammation "I", and necrosis "N". (C) Desirox-treated liver sections demonstrate reduced necrotic area. (D) Liver section from the G2 mouse group improved histology with portal inflammation "PI" (E) Liver section from the G4 mouse group (F) Liver section from the G8 mouse group (G) Liver section from the M2 mouse group (H) Liver section from the M4 mouse group (I) Liver section from the M8 mouse group (J) Liver section from the W50 mouse group (K). Liver section from the W100 mouse group (L) Liver section from the W200 mouse group demonstrates improved histology with minimal necrotic area.
In contrast, the liver sections taken from GA (Figure 4.8D-F), MG (Figure 4.8G-I) and SPW1 (Figure 4.8J-L) treated mice groups displayed attenuation of pathogenicity and gradual reversal to normal cyto-architecture with the increasing dosage, thus restoring the normal condition. Figure 4.8C represents liver sections of the desirox-treated group with improved histology, which is similar to the highest doses of GA, MG and SPW1. Another detrimental effect of excess iron in liver is deposition of iron in the form crystalline ferritine and amorphous hemosiderin. The hemosiderin in tissue sections stained with Perls’ Prussian blue as blue patches. The liver sections from untreated iron overloaded mice demonstrated increased hemosiderin deposition (Figure 4.9B) compared with normal mice (Figure 4.9A). However, sections from the treated mice groups demonstrated a gradual decrease in hemosiderin deposition patches (Figure 4.9D-F for GA, Figure 4.9G-I for MG and Figure 4.9J-L). The highest dose of MG and SPW1 exhibited a parallel effect to the standard desirox-treated group (Figure 4.9C).

Figure 4.9  Microscopic Observation of Mouse Liver Sections that had been Stained with Perls’ Prussian Blue at x400
(A) Liver section from control mice demonstrates normal hemosiderin deposition patches (very low). (B) Liver sections from iron-overloaded mice display excess blue patches. (C) Desirox-treated liver section (D) Liver section from the G2 mouse group with lesser blue patches. (E) Liver section
from the G4 mouse group (F) Liver section from the G8 mouse group (G) Liver section from the M2 mouse group (H) Liver section from the M4 mouse group (I) Liver section from the M8 mouse group. (J) Liver section from the W50 mouse group (K) Liver section from the W100 mouse group (L) Liver section from the W200 mouse group. MG and SPW1 demonstrates improved histology, and a gradual reduction of blue patches indicates effective iron removal from the liver.

Accumulated collagen in liver was also stained blue using Masson’s trichrome. The microscopic observation suggested that the liver section of control mice revealed normal lobular architecture and distribution of collagen (Figure 4.10A). From the liver section of iron-overloaded mice it is evident that the normal architecture of the liver is destroyed and the nodules surrounded by accumulated collagen indicating fibrous cirrhotic (Figure 4.10B). However, after treatment with the test samples, a gradual decrease in the degree of collagen deposition was observed (Figure 4.10D-F for GA, 10G-I for MG and Figure 4.10J-L for SPW1). Here, the highest MG dose also demonstrated a similar scenario compared with the standard desirox-treated group (Figure 4.10C).

![Figure 4.10 Microscopic Observation of Mouse Liver Sections that had been Stained with Masson’s Trichrome Stain ×100](image-url)

(A) Liver sections from control mice displayed normal cellular integrity with no fibrosis. (B) Liver sections from iron-overloaded mice displayed elongated fibrous septa and collagen accumulation.
(blue). (C) Desirox-treated liver section (D) Liver section from the G2 mouse group with lesser blue patches. (E) Liver section from the G4 mouse group (F) Liver section from the G8 mouse group (G) Liver section from the M2 mouse group (H) Liver section from the M4 mouse group (I) Liver section from the M8 mouse group. (J) Liver section from the W50 mouse group (K) Liver section from the W100 mouse group (L) Liver section from the W200 mouse group. M8 demonstrates a nearly negligible collagen accumulation and healthy liver. Higher doses of GA, MG and SPW1 demonstrate reduced collagen deposition, fibrous septum and necrotic cells in periportal veins, indicating a trend of restoration of normal cellular integrity.

4.4. Discussion

The liver, which is involved in numerous biochemical pathways related to nutrition and detoxification (Anusha et al., 2011), is often subjected to injuries induced by various hepatotoxins. Iron, a vital constituent of countless proteins especially within the oxygen handling biological machinery (Bothwell et al., 1979), becomes a well-known hepatotoxin when in excess. In iron-overloaded liver, the metal initiates and propagates several ROS, leading to the oxidative damage of many vital biomolecules, resulting in the cellular lipid peroxidation, mitochondrial damages, DNA fragmentation and, finally, cell death (Sayre et al., 2005). However, the preventive system of our body is inadequate to handle excess free radicals; therefore, external antioxidant iron chelator supplements are essential to maintain healthy physiological conditions. An effective remedial strategy should act in a dual manner by decreasing the oxidation rate: one manner sequestering and chelating the stored iron in cells (Rothman et al., 1992) and other as a radical scavenger (i.e., antioxidant activity).

Because all the compounds depicted excellent antioxidant and free radical scavenging activities, the present study primarily incorporates the in vitro iron chelation potency of them and Fe$^{3+}$ reducing power capacity. The results obtained clearly show the SPW1 and MG significantly chelate iron and GA, MG and SPW1 possessed excellent reducing power potential. But SPE2 failed to show any activity in both the assays. Although the basic structure of MG and GA is alike, MG exhibited better iron chelation than GA. So, this iron chelation result further supported the findings of Yang et al., (2014) who suggested that Fe(III) in Hepes buffer, pH 7.4 interacted with three molecules of MG and produced a Fe(III)–MG3 complex with a stable octahedral geometry and chelate-free iron better than GA.

So, the in vivo ameliorating potency of GA, MG and SPW1 on accumulation of iron and oxidative damage by iron overload in the mouse liver was studied. The hemochromatosis condition was created by the intraperitoneal injection of iron-dextran. This process will not hamper the intestinal iron absorption by the test compounds, which ultimately leads to iron overloaded toxicity in liver and serum as well as create liver fibrosis in mice (Farinati et al., 1995).

As 90% of excess iron is deposited in the hepatic cells as ferritin or hemosiderin, most techniques focus on measuring liver iron levels, and it is widely accepted that liver iron content provides an accurate measure of whole-body iron concentration (Zuyderhoudt et al., 1983). The decrease in hepatic iron deposition after the treatment justified the in vitro iron-chelating effectiveness of the compounds. Ferritin, a ubiquitous intracellular iron-binding protein, generally stores iron in a non-toxic ferric form and releases it in a controlled fashion whenever needed (Harrison, 1977). In general, the amount of ferritin in blood reflects the extent of iron storage in the liver, making it one of the key parameters in the management of hemochromatosis and other iron overload diseases. The increased level of ferritin is generally noticed in iron-overload induced liver toxicity as serum ferritin is one of the markers developed
as a consequence of iron overload. In this study, the elevated ferritin level was found to be significantly decreased after the treatment with GA, MG and SPE2. The iron removal potentials of the compounds also further visual confirmed by the liver tissue biopsy stained with Perls’ Prussian blue. It revealed that with the increase treatment doses the blue patches of stored hemosiderin disappeared gradually.

In hepatocytes, ferritin stores excess iron in the ferric state. An ideal chelator designed for the decorporation of a particular metal from the body should be able to bind, carry, and remove the metal out of the body without causing any toxicity (Kontogiorghes et al., 1987). At the molecular level, almost all the chelating agents form complexes with Fe$^{2+}$ ion in solution and remove iron from ferritin in vitro (Sheftel et al., 2012). However various iron chelators administered to attenuate the situation in vivo, but most struggle with a narrow binding capacity for ferric iron (Fe$^{3+}$) as well as iron in ferritin is not properly accessed to them. Thus, reducing agents such as ascorbic acid need to be administered as supplements to upsurge the accessibility of stored iron to chelators along with the iron chelation drugs. (O’Brien, 1974). However, it is obvious that intrinsic reducing property of an iron chelator should definitely increase the efficiency of iron chelation therapy to treat iron overload. The result (Figure 4.6) signified that increasing concentrations of the compounds specially SPW1 significantly released iron (Fe$^{3+}$) from ferritin with time and the correlation between the percentage of iron released from ferritin and the reducing power was quite significant ($R^2 \geq 0.78$). This result also validates that the compounds alone can effectively chelate the excess iron by reducing it from its ferric form.

Levels of serum enzyme and bilirubin are checked in the clinical diagnosis to determine the condition of various diseases and tissue injury (Khatri et al., 2009; Liu et al., 2012). These enzymes are predominantly found in the hepatic cell and liver damage due to excess iron leads to the release of these intracellular enzymes into the blood, resulting in amplified levels of serum ALAT, ASAT, ALP and bilirubin (Pulla Reddy & Lokesh, 1996) as evident in Table 4.1. The administration of all three compounds substantially reduced the increased level of the serum marker enzymes almost similar to the standard desirox. This result indicated towards their healing capabilities of hepatic parenchyma and regeneration of hepatocyte as well as its functional efficiency (Thabrew & Joice, 1987). Among the compounds, MG possessed better hepato-amelioration activity compared with SPW1 and GA.

Living cells are equipped with an array of endogenous antioxidant enzymes such as GST, SOD, CAT and the small compound GSH, which are first line of defenses against excessive free radicals. Evaluating the levels of these antioxidant enzymes is a proper indirect way to assess pro-oxidant-antioxidant combat in tissues (Sabir et al., 2012). SOD destroys superoxide free radicals by converting them into H$_2$O$_2$, whereas catalase further decomposes excess H$_2$O$_2$ to H$_2$O and O$_2$. Catalase efficiency is so commendable that it cannot be saturated by any concentration of H$_2$O$_2$ (Lledias et al., 1998). The cellular GSH system is probably the most important cellular defense mechanism, which not only acts as a ROS scavenger but also regulates the intracellular redox state (Eruslanov & Kusmartsev, 2010). The results suggested that SPW1 restore the antioxidant enzymes much better than MG and GA, even better than standard drug Desirox.

Cell membrane lipid peroxidation is the most detrimental result of iron-induced oxidative stress mainly via hydroxyl radicals (Ryan & Aust SD, 1992). Lipid peroxidation (LPO) and the resulting end products such as malondialdehyde (MDA) act as an important factors of liver fibrosis by activating hepatic stellate cells, resulting in increased pro-collagen α$_1$ (I) gene.
expression (Lee et al., 1995; Farinati et al., 1995; Reeves et al., 1996). The most important hypotheses for the mechanism of hepatocellular injury in chronic iron overload is the peroxidative damage to the lipid membranes of cellular organelles resulting in structural and functional alterations in cell integrity through loss of lysosomal membrane (Bonkowsky et al., 1981). Iron-induced liver pathogenicity also leads to the oxidation of various important structural and functional proteins and forms protein carbonyls. Thus, it serves as a marker of oxidative stress and leads to the onset/development of various diseases including cystic fibrosis and ulcerative colitis (Zuyderhoudt et al., 1983). The results presented in this study (Figure 4.6A, B) clearly establish that all the compounds efficiently reduced the lipid peroxidation and protein oxidation. The results also supported by the histological study as liver biopsy is considered to be the gold-standard method for assessment of the degree of inflammation and fibrosis alongside other biochemical tests. The live sections stained with hematoxylin and eosin exhibited various degrees of inflammation, necrosis as well as cell wall degeneration in iron overloaded condition, but dose dependent treatment reduced the tissue damage.

The relationship between lipid peroxidation and hepatic fibrosis is well established in a variety of liver diseases including iron overloaded hepatotoxicity, hemochromatosis, alcohol-induced liver injury and chronic hepatitis C (Plebani & Burlina, 1991). Subsequent to hepatic cellular damage, collagen production predominates over hepatocellular regeneration as an immediate healing response, thereby occupying the injured areas instead of destroyed hepatocytes. Collagen content is therefore considered to be a major marker of liver fibrosis and hepatotoxicity (Dalle-Donne et al., 2003). Reducing the level of collagen content (Figure 4.6C) by removing the excess iron by MG, GA and SPW1 further strengthen the position of the compounds in significantly overcoming hepatic damage/fibrosis in iron intoxicated mice. On the other hand, Masson’s trichrome staining of the liver tissue sections disclosed the reduction of liver fibrosis as the collagen content gradually decreased with the increasing doses of the test compounds, which also confirmed the anti-fibrotic effect of the compounds along with the test for collagen content. Overall results signify the in situ evidence of ameliorating the effect of the iron overload-induced liver toxicity.

Excess iron plays a critical role in oxidative stress-related hepatotoxicity and liver fibrosis, which is profoundly supported by the results of the present study. Among the isolated compounds from S. pinnata bark, MG and SPW1 exhibited exceptional amelioration potential for hepatotoxicity mainly due to its iron chelation capacity. In contrast, GA failed to demonstrate brilliant iron chelation activity in vitro, but it displayed a pleasing amendment of oxidative stress and liver damage as it neutralized excess generated free radicals due to iron overload before they could attack any bio-entities. Thus, these compounds act in different pathways to achieve the same goal. These findings suggest their beneficiary role in the pathological sequence of iron-overload-linked liver disease and fibrosis. Conclusively, all the compounds may be used for the development of potential orally administrable iron chelating drug in the treatment of iron overload-induced liver toxicity.