

## ***Spondias pinnata* stem bark extract lessens iron overloaded liver toxicity due to hemosiderosis in Swiss albino mice**

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### ABSTRACT

The present study was designed to evaluate the ameliorating effect of 70% methanol extract of *Spondias pinnata* (SPME) on iron overload induced liver injury. Iron overload was induced by intraperitoneal administration of iron-dextran into mice and resulting liver damage was manifested by significant rise in serum enzyme markers (ALT, AST, ALP and bilirubin) and reduction in liver antioxidants (SOD, CAT, GST and GSH). Hepatic iron, serum ferritin, lipid peroxidation, protein carbonyl and hydroxyproline contents were measured in response to the oral administration of SPME of different doses (50, 100 and 200 mg/kg body weight). In order to determine the efficiency as iron chelating drug, the release of iron from ferritin by SPME was further studied. Enhanced levels of antioxidant enzymes were detected in SPME treated mice. SPME produced a dose dependent inhibition of lipid peroxidation, protein oxidation, liver fibrosis; and levels of serum enzyme markers and ferritin were also reduced dose dependently. The liver iron content was also found to be less in SPME treated group compared to control group. The reductive release of ferritin iron was augmented significantly after dose dependent addition of SPME. The ameliorating effect of SPME on damaged liver was furthermore supported by the histopathological studies that showed improved histological appearances. In conclusion, the present results demonstrate the hepatoprotective efficiency of SPME in iron intoxicated mice, and hence possibly useful as iron chelating drug for iron overload diseases.

**Key words.** Hepatotoxicity. Antioxidant enzymes. Ferritin. Lipid peroxidation. Hydroxyproline.

### INTRODUCTION

Liver, involved with numerous biochemical pathways related to nutrition and detoxification,<sup>1</sup> is often subjected to injuries induced by various hepatotoxins. Iron, a vital constituent of umpteen proteins<sup>2</sup> becomes a well-known hepatotoxin, when in excess. Hereditary hemochromatosis due to alteration of human hemochromatosis (HFE) gene<sup>3,4</sup> and hemosiderosis (secondary hemochromatosis) due to repeated blood transfusion along with dietary iron excess<sup>5</sup> leads to iron overloaded

toxicity in various organs of the body, most notably liver since it is the primary storage site of iron.<sup>6</sup> Free radical generation and lipid peroxidation are the proposed mechanisms of iron induced liver toxicity<sup>7</sup> and it was characterized by leakage of cellular enzymes into blood, decreased activities of antioxidant enzymes along with formation of protein carbonyl and collagen. Removal of excess iron by phlebotomy has been the most effective treatment for hereditary hemochromatosis,<sup>8</sup> whereas iron chelation therapy using deferasirox<sup>9</sup> and deferriprone<sup>10</sup> being better for hemosiderosis to effectively promote iron excretion. However, such compounds show several side effects and limitations<sup>11</sup> that direct towards the finding of a more effective and safe drug<sup>12</sup> which may rise to the therapeutic benefits for patients.

Phenolics and flavonoids from natural sources act effectively as potent antioxidants, through their respective iron chelating activity, for treatment of liver toxicity.<sup>12-16</sup> *Spondias pinnata* (Linn. f.) Kurz (Fam. *Anacardiaceae*) is previously studied for its

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antioxidant and iron chelating potential, which showed the presence of significant amounts of phenolic and flavonoid compounds.<sup>17</sup> The present study was performed to evaluate the ameliorating effect of 70% methanol extract of *Spondias pinnata* (SPME) on iron overload induced hepatotoxicity.

## MATERIAL AND METHODS

### 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 [(NH<sub>4</sub>)<sub>2</sub>Fe(SO<sub>4</sub>)<sub>2</sub>·6H<sub>2</sub>O], 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, was obtained from Cipla Ltd., Kolkata, India.

### Plant material

The bark of *Spondias pinnata* (SP) was collected from Bankura district of West Bengal, India. It was identified and authenticated through the Central Research Institute (Ayurveda), Kolkata, India and a voucher specimen (CRHS 111/08) was submitted there.

### Plant extract preparation

The stem bark of SP was dried at room temperature for 7 days, finely powdered and used for extraction. The powder (100 g) was mixed with 500 mL methanol:water (7:3) using a magnetic stirrer for 15 h, then the mixture was centrifuged at 2,850 x g and the supernatant was decanted. The process was repeated again with the precipitated pellet. The supernatants were collected, concentrated in a rotary evaporator and lyophilized. The dried extract was stored at -20 °C until use.

### Animals

Male Swiss albino mice (20 ± 2 g) were purchased from Chittaranjan National Cancer Institute (CNCI), Kolkata, India and were maintained under a constant 12 h dark/light cycle at an environmental temperature of 22 ± 2 °C. The animals were provided with normal laboratory pellet diet and water *ad libitum*. All experiments were performed after obtaining approval from the institutional animal ethics committee (IAEC) and care of the animals was taken as per the guidelines of the Committee for the purpose of control and supervision of experiments on animals (CPCSEA), Ministry of Environment and Forest, Government of India (Registration. No. 95/1999/CPCSEA).

### Experimental design

Thirty six mice were divided into six groups containing six mice in each group. One group served as blank (B) and received normal saline only. The other five groups were given five doses (one dose every two days) of 100 mg/kg b.w. each, of iron-dextran saline (i.p). One iron-dextran group (C) received normal saline and other four groups were orally administered with 50 mg/kg b.w. (S50), 100 mg/kg b.w. (S100), 200 mg/kg b.w. (S200) plant extract and 20 mg/kg b.w. desirox (D), respectively, for three consecutive 7 day periods, started from the day after the first iron-dextran injection.

### Sample collection and tissue preparation

The experiment ended on the 21st day. Mice were fasted overnight. They were anesthetized with ethyl ether and blood was collected by cardiac puncture. The blood samples were left to clot and sera were separated using cooling centrifuge and store at -80 °C until analysis. The liver was rapidly removed, washed with ice-cold saline to eliminate the blood cells and divided into three portions. One portion of the samples were weighed and homogenized in 10 volume of 0.1 M phosphate buffer (pH 7.4) containing 5 mM EDTA and 0.15 M NaCl, and centrifuged at 8,000 g for 30 min at 4 °C. The supernatant was collected and used for the assays of enzyme activities, protein oxidation, levels of hydroxyproline content and lipid peroxidation products. Protein concentration in the homogenate was estimated according to Lowry method<sup>18</sup> using BSA as standard. Another portion of the liver samples were weighed and digested

with equivolume (1:1) mixture of sulphuric acid and nitric acid and their iron content were analysed. The remaining portion of the liver was used for histopathological studies.

### Biochemical markers

Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and bilirubin in serum samples were measured using the commercial kits of Merck, Mumbai, India. Serum alkaline phosphatase (ALP) was estimated using the kit supplied by Sentinel diagnostics, Italy. Liver homogenate was used to measure antioxidant enzymes. Superoxide dismutase (SOD) was assayed by measuring the inhibition of the formation of blue colored formazan at 560 nm.<sup>19</sup> Catalase (CAT) activity was measured by following the decomposition of H<sub>2</sub>O<sub>2</sub> over time at 240 nm.<sup>20</sup> Glutathione-S-transferase (GST) was determined based on the formation of GSH-CDNB conjugate and increase in the absorbance at 340 nm.<sup>21</sup> Reduced glutathione (GSH) level was measured spectrophotometrically at 412 nm.<sup>22</sup>

### Assessment of liver damage

The lipid peroxide levels in liver homogenates were measured as thiobarbituric acid reactive substances (TBARS).<sup>23</sup> Protein oxidation was determined by spectrophotometric estimation of protein carbonyl contents.<sup>24</sup> Measurement of hydroxyproline content in the liver allows the actual quantitation of collagen content which is an important marker of liver fibrosis. Respective homogenates were hydrolyzed in 6 M HCl and hydroxyproline was measured by Ehrlich's solution.<sup>25</sup> The absorbances were read at 558 nm and results were calculated from 4-hydroxy-L-proline standard curve ( $R^2 = 0.9907$ ). The collagen content was determined by multiplying amount of total hydroxyproline content in each sample by a factor of 7.69.<sup>26</sup>

### Histopathological analysis

The excised portion of the liver samples was washed with normal saline prior to preparation for histological observations. Initially, the material was fixed in 10% buffered neutral formalin for 48 h. A paraffin embedding technique was carried out and sections were taken at 5  $\mu$ m thickness, stained with hematoxylin and eosin and examined microscopically for histopathological changes.

### Serum ferritin and liver iron

Serum ferritin levels were measured using enzyme-linked immunosorbent assay kit (from Monobind Inc., USA) according to the manufacturer's instructions. Liver iron was measured according to a formerly reported colorimetric method.<sup>27</sup> Briefly, samples were incubated with bathophenanthroline sulfonate for 30 min at 37 °C and absorbances were read at 535 nm.

### Iron release from ferritin

Iron reduction and release was determined spectrophotometrically, using ferrozine as metal chelator as previously described.<sup>28</sup> The reaction mixture (3 mL final volume) contained 200  $\mu$ g ferritin, 500  $\mu$ M ferrozine, in 50 mM pH 7.0 phosphate buffer. Reaction was started by the addition of 500  $\mu$ L plant extracts of different concentrations and the change in absorbance was measured continuously at 560 nm for 20 min. A cuvette containing ferritin, ferrozine and phosphate buffer but lacking plant extract was used as the reference solution.

### Statistical analysis

All data were reported as the mean  $\pm$  SD of six measurements. Statistical analysis was performed using KyPlot version 2.0 beta 15 (32 bit). Comparisons among groups were made according to pair t-test. In all analyses, a p value of < 0.05 was considered significant.

## RESULTS AND DISCUSSION

Iron, majorly through lipid peroxidation of biological membranes induces tissue damage, especially in liver.<sup>29</sup> Intraperitoneal iron-dextran injection resembles hemosiderosis secondary to ineffective erythropoiesis and high iron oral intake.<sup>30</sup> Moreover, intraperitoneal injection would avoid the direct interruption of plant extract on intestinal iron absorption leading to hepatic iron overload. The ability of a hepatoprotective agent to reduce injurious effect or preserve the normal hepatic physiological function is the index of its hepatoprotective effect, which was substantially achieved by treatment with SPME.

### Serum marker enzymes

Hepatic injury by iron results in the leakage of cellular enzymes into the bloodstream, resulting in

**Table 1.** The effect of stem bark extract of *S. pinnata* on serum parameters, liver antioxidants, liver damage parameters and liver iron content in iron overloaded mice.

Treatment	Serum parameters					Liver antioxidants					Liver damage parameters				
	ALT (unit/L)	AST (unit/L)	ALP (unit/L)	Bilirubin (unit/L)	Ferritin (µg/g protein)	SOD (unit/mg protein)	Catalase (unit/mg protein)	GST (unit/mg protein)	GSH (µg/mg protein)	TBARS (µM/mg protein)	Carbonyl content (µM/mg protein)	Collagen content (mg/liver)	Liver iron (µg/g)		
B	17.83 ± 0.99	30.61 ± 1.65	105.63 ± 25.96	1.16 ± 0.05	42.67 ± 3.31	0.52 ± 0.03	23.35 ± 3.02	4.41 ± 0.63	0.51 ± 0.04	1.86 ± 0.12	2.50 ± 0.45	7.71 ± 0.24	58.30 ± 3.20		
C	30.88 ± 2.33 <sup>x2</sup>	66.09 ± 7.44 <sup>x1</sup>	406.69 ± 59.09 <sup>x1</sup>	3.02 ± 0.34 <sup>x1</sup>	94.99 ± 3.85 <sup>x3</sup>	0.19 ± 0.02 <sup>x3</sup>	5.73 ± 2.22 <sup>x3</sup>	1.03 ± 0.58 <sup>x3</sup>	0.32 ± 0.02 <sup>x3</sup>	3.02 ± 0.07 <sup>x3</sup>	9.61 ± 0.25 <sup>x3</sup>	13.80 ± 0.19 <sup>x3</sup>	133.10 ± 2.54 <sup>x3</sup>		
S50	28.28 ± 1.10 <sup>x1</sup>	40.68 ± 1.19 <sup>x1</sup>	281.42 ± 19.32 <sup>x1</sup>	1.98 ± 0.15 <sup>x1</sup>	52.83 ± 2.13 <sup>x2</sup>	0.21 ± 0.02 <sup>x2</sup>	14.21 ± 2.71 <sup>x2</sup>	1.72 ± 0.54 <sup>x2</sup>	0.36 ± 0.05 <sup>x2</sup>	2.81 ± 0.08 <sup>x2</sup>	9.01 ± 0.18 <sup>x2</sup>	13.18 ± 0.17 <sup>x2</sup>	124.73 ± 2.85 <sup>x2</sup>		
S100	26.44 ± 2.11 <sup>x1</sup>	40.08 ± 0.91 <sup>x1</sup>	196.95 ± 18.03 <sup>x1</sup>	1.56 ± 0.07 <sup>x1</sup>	49.32 ± 1.67 <sup>x3</sup>	0.3 ± 0.03 <sup>x3</sup>	16.61 ± 2.41 <sup>x3</sup>	2.47 ± 0.64 <sup>x3</sup>	0.43 ± 0.05 <sup>x3</sup>	2.39 ± 0.09 <sup>x3</sup>	6.89 ± 0.37 <sup>x3</sup>	12.65 ± 0.23 <sup>x3</sup>	113.59 ± 3.53 <sup>x3</sup>		
S200	21.72 ± 1.22 <sup>x1</sup>	42.85 ± 3.75 <sup>x1</sup>	152.91 ± 16.97 <sup>x2</sup>	1.36 ± 0.08 <sup>x2</sup>	47.90 ± 0.82 <sup>x3</sup>	0.35 ± 0.03 <sup>x3</sup>	17.71 ± 2.05 <sup>x3</sup>	3.21 ± 0.56 <sup>x1</sup>	0.44 ± 0.03 <sup>x3</sup>	2.17 ± 0.07 <sup>x3</sup>	6.94 ± 0.43 <sup>x3</sup>	10.97 ± 0.28 <sup>x3</sup>	110.67 ± 3.38 <sup>x3</sup>		
D	23.55 ± 1.39 <sup>x2</sup>	44.67 ± 5.52 <sup>x1</sup>	151.58 ± 9.92 <sup>x1</sup>	1.59 ± 0.12 <sup>x2</sup>	54.48 ± 1.80 <sup>x3</sup>	0.40 ± 0.07 <sup>x2</sup>	18.97 ± 0.81 <sup>x3</sup>	3.13 ± 0.53 <sup>x1</sup>	0.48 ± 0.03 <sup>x3</sup>	2.24 ± 0.06 <sup>x3</sup>	7.30 ± 0.47 <sup>x3</sup>	12.33 ± 0.29 <sup>x3</sup>	90.46 ± 4.78 <sup>x3</sup>		

Values are mean ± SD of six observations. Treatment groups are B: normal mice; C: iron-dextran treated mice receiving normal saline; S50: 50 mg/kg b.w. SPME treated group; S100: 100 mg/kg b.w. SPME treated group; S200: 200 mg/kg b.w. SPME treated group; D: 20 mg/kg b.w. desirox treated group. X: significant difference from normal mice (B) group (<sup>x1</sup>: p ≤ 0.05, <sup>x2</sup>: p ≤ 0.01 and <sup>x3</sup>: p ≤ 0.001). Y: significant difference from iron overloaded (C) group (<sup>y1</sup>: p ≤ 0.05, <sup>y2</sup>: p ≤ 0.01 and <sup>y3</sup>: p ≤ 0.001).

augmented levels of serum ALT, AST, ALP and bilirubin.<sup>31</sup> The enhanced levels of serum ALT, AST, ALP and bilirubin are used as sensitive indicators of liver damage. As shown in table 1, iron induced liver injury resulted in the significant increase in ALT (73.23%), AST (117.63%), ALP (285.01%) and bilirubin (159.11%). Oral administration of plant extract markedly reduced the elevated levels of serum enzymes and bilirubin of iron overloaded mice to approach the normal control values.

### Effect on antioxidant enzymes

Cells are equipped with a repertoire of endogenous antioxidant defence machinery including enzymes such as SOD, CAT, GST or compounds such as GSH.<sup>32</sup> The activities of antioxidant enzymes SOD, CAT, GST and the levels of non-enzymatic antioxidant GSH were significantly decreased in iron overloaded control mice compared to normal mice. In the liver, 63% decrease in SOD activity observed in the test control compared with normal group, was successively reduced to 4%, 21% and 31% following the feeding of S50, S100 and S200 respectively (Table 1). The activity of CAT was shown in table 1. The overload of iron in liver resulted in 75% decrease in CAT activity compared to non-iron treated normal mice. But only 24% reduction was observed, when the iron intoxicated mice were fed S200 compared with normal control mice. GST activity was reduced 76% in iron treated control mice. Data from table 1 shows the dose dependant reversal of GST activity after administration with SPME. The activity of GSH was significantly reduced (37%) in iron intoxicated control mice. Administration with SPME (S50, S100 and S200) dose dependently increased the GSH levels about 8%, 22% and 25%, respectively (Table 1).

### Lipid peroxidation

The enhanced lipid peroxidation has been proposed as an initial step by which iron causes cellular injury.<sup>33</sup> Intraperitoneal injection of iron-dextran significantly enhanced (62%) lipid peroxidation in liver homogenates compared to normal control mice. However, the levels of TBARS were reduced by 12%, 32% and 46% in mice fed S50, S100 and S200 respectively (Table 1).

### Protein carbonyl and hydroxyproline content

Protein oxidation, another significant factor in the pathogenesis of iron overload induced hepatic

damage, disintegrates the structural integrity of various important proteins leading to formation of various diseases including cystic fibrosis and ulcerative colitis.<sup>34</sup> The results demonstrated in table 1 show a significant elevation of protein carbonyl content in iron overloaded mice compared to normal mice. When treated with SPME, the protein oxidation was found to be arrested dose dependently. The collagen content in liver is high during fibrosis, the extent of which could be assessed by the hydroxyproline content. From the values in table 1, it can be conferred that 78% upsurge of collagen content in iron overloaded mice compared to normal mice was reduced to 8%, 14% and 36% in SPME treated mice (S50, S100 and S200 respectively). Thus, treatment with SPME significantly reduced hydroxyproline content in iron intoxicated mice, indicating hepatic fibrosis inhibitory potency of the plant extract.

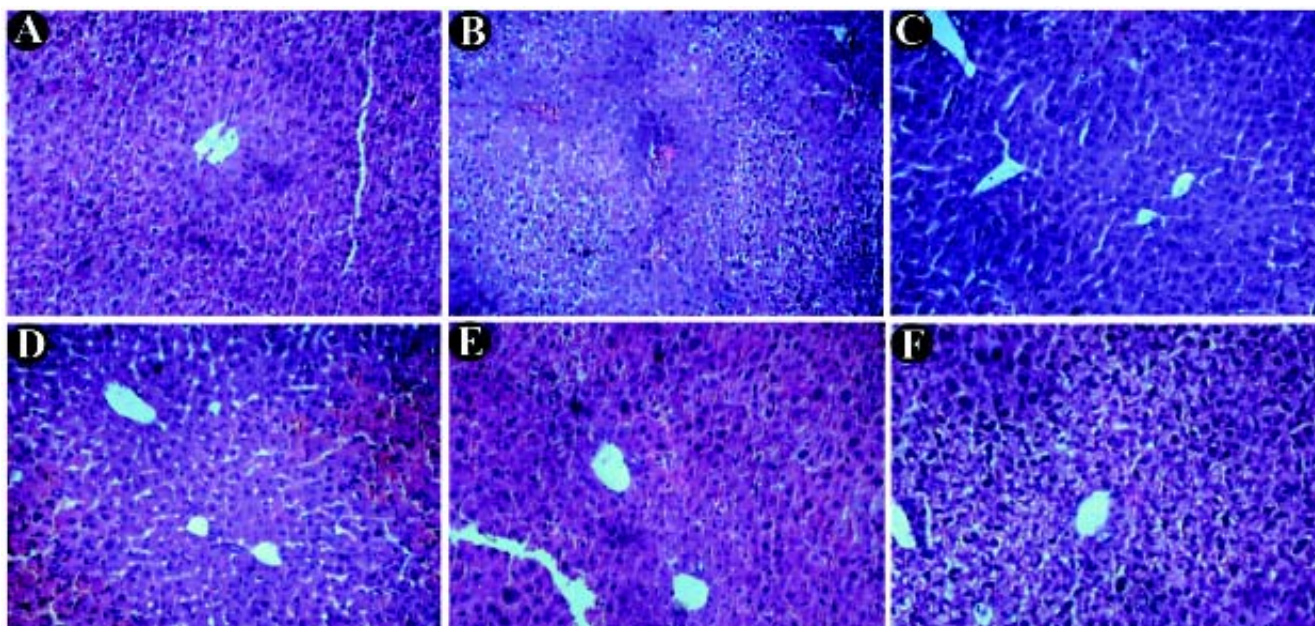
#### Histopathological study

The extent of hepatic damage is assessed by histological evaluation along with the level of various biochemical parameters in circulation. Histology of the liver sections of normal mice showed normal cell

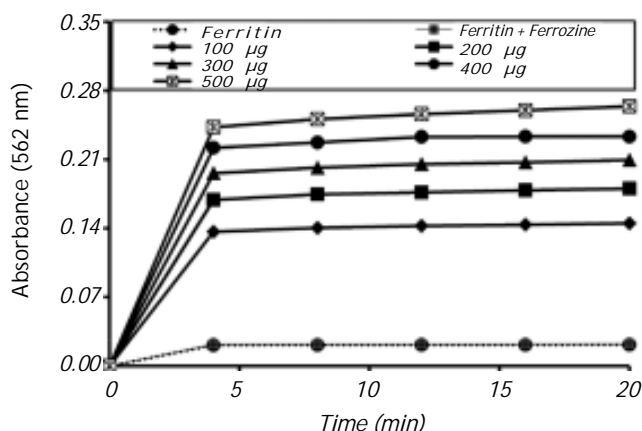
morphology with well-preserved cytoplasm, prominent nucleus and well brought out central vein (Figure 1A), whereas that of iron overloaded group showed severe hepatotoxicity evidenced by degeneration of fatty cells, necrosis, broad infiltration of lymphocytes and Kupffer cells around the central vein and loss of cellular boundaries (Figure 1B). SPME treated mice revealed marked reduction in hepatic lesions. The liver sections taken from SPME treated group showed attenuation of the pathological changes and gradual reversal to normal cytoarchitecture with higher dosage thus presenting protection against iron overload induced hepatic damage (Figures 1C-1E). Figure 1F presented the improved histology of liver sections taken from desirox treated group. Overall, SPME treated group produced a dose dependant improvement in histopathological alterations which signifies the *in situ* evidence of hepatoprotective effect of the plant extract.

#### Serum ferritin level and liver iron

The markedly elevated liver iron concentration, due to iron dextran intraperitoneal administration, is lowered dose dependently with SPME treatment



**Figure 1.** Photomicrograph of mice liver sections (staining with haematoxylin and eosin) x 20. **A.** Normal mice liver. **B.** Iron intoxicated (iron-dextran, 100 mg/kg b.w.) liver section showing necrosis, periportal inflammation and loss of cellular boundaries. **C.** Liver section treated with iron-dextran + 50 mg/kg b.w. SPME. **D.** Liver section treated with iron-dextran + 100 mg/kg b.w. SPME. **E.** Liver section treated with iron-dextran + 200 mg/kg b.w. SPME. All three groups (**C**), (**D**) and (**E**) shows reduced hepatocellular necrosis and inflammation dose dependently. (**F**) Liver section treated with iron-dextran + 20 mg/kg b.w. desirox also shows reduced necrotic area and increased number of hepatocytes.



**Figure 2.** Dose dependent formation of the  $[Fe(ferrozine)_3]^{2+}$  complex following release of  $Fe^{2+}$  from ferritin by SPME with time. The reductive release of ferritin iron was quantified by measuring the formation of the ferrous complex of ferrozine,  $[Fe(ferrozine)_3]^{2+}$  at 562 nm using a Shimadzu UV-VIS spectrophotometer.

(Table 1). Previously, it has been shown that SPME can bind iron<sup>17</sup> and the decrease in liver iron deposition induced by SPME treatment support its iron chelating potency. Body's iron level is positively correlated with ferritin, a ubiquitous intracellular storage protein that prevents iron from mediating oxidative damage to cell constituents.<sup>35</sup> The increased level of ferritin is usually noticed in iron overload induced liver toxicity. When SPME was administered, significant reduction in serum ferritin concentrations were observed dose dependently (Table 1).

#### Reductive release of ferritin iron

The ability of SPME to reductively release iron from ferritin was quantified by measuring the formation of the ferrous complex of ferrozine,  $[Fe(ferrozine)_3]^{2+}$ . Time course reaction (Figure 2) shows that after dose dependant addition of SPME the  $[Fe(ferrozine)_3]^{2+}$  complex formation was increased significantly with time. The reductive ability of SPME to release ferritin iron is significant as an iron chelating drug to treat iron overload.

#### CONCLUSION

From the present study, it can be inferred that SPME has protective effect against iron overload induced liver toxicity as evidenced by biochemical and histopathological studies. The hepatoprotective

action is probably related to its potent antioxidant and iron chelating property. The findings suggest its benefit in pathological sequence of iron overload linked liver disease. Further research is required to illustrate the active hepatoprotective principle.

#### ABBREVIATIONS

- **SPME:** *Spondias pinnata* methanol extract.
- **ALT:** alanine aminotransferase.
- **AST:** aspartate aminotransferase.
- **ALP:** alkaline phosphatase.
- **SOD:** superoxide dismutase.
- **CAT:** catalase.
- **GST:** glutathione-S-transferase.
- **GSH:** reduced glutathione.
- **g:** gram.
- **mg:** milligram.
- **kg:** kilogram.
- **mL:** milliliter.
- **M:** molar.
- **mM:** millimolar.
- **b.w.:** body weight.
- **i.p.:** intraperitoneal.
- **TBARS:** thiobarbituric reactive substances.
- **SD:** standard deviation.

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