Research Article

Hepatoprotective Potential of *Caesalpinia crista* against Iron-Overload-Induced Liver Toxicity in Mice

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The present study was carried out to evaluate the ameliorating effect of *Caesalpinia crista* Linn. (CCME) extract on iron-overload-induced liver injury. Iron overload was induced by intraperitoneal administration of iron dextran into mice. CCME attenuated the percentage increase in liver iron and serum ferritin levels when compared to control group. CCME also showed a dose-dependent inhibition of lipid peroxidation, protein oxidation, and liver fibrosis. The serum enzyme markers were found to be less, whereas enhanced levels of liver antioxidant enzymes were detected in CCME-treated group. In presence of CCME, the reductive release of ferritin iron was increased significantly. Furthermore, CCME exhibited DPPH radical scavenging and protection against Fe²⁺-mediated oxidative DNA damage. The current study confirmed the hepatoprotective effect of CCME against the model hepatotoxicant iron overload and the activity is likely related to its potent antioxidant and iron-chelating property.

1. Introduction

Liver is one of the largest organs in the human body and the main site for intense metabolism and excretion. It is involved with almost all the biochemical pathways to growth, fight against disease, nutrient supply, energy provision, and reproduction [1]. Hepatic damage is associated with distortion of these metabolic functions [2] and, sometimes, resulting in serious health problems. Hepatotoxicity is the most common finding in patients with iron overloading as liver is mainly the active storage site of iron in our body [3]. Iron, the most important transition element of the body, is found in functional forms in haemoglobin, myoglobin, the cytochromes, enzymes with iron sulphur complexes, and other iron-dependent enzymes [4]. Although an optimum level of iron is always maintained by the cells to balance between essentiality and toxicity, in some situations it is disrupted, resulting in iron overload which is associated to the oxidative stress induced disorders including anemia, heart failure, liver cirrhosis, fibrosis, diabetes, arthritis, depression, impotence, infertility, and cancer [5]. In all iron-overload-induced diseases, iron removal by iron chelation therapy is an effective life-saving strategy. The currently available iron-chelating agents used clinically are deferoxamine, 1,2-dimethyl-3-hydroxyppyrid-4-one (deferiprone, L1), and deferasirox. However, such compounds show several side effects and limitations [6,7] that direct towards the finding of a more effective and safe drug [8,9] which may rise the therapeutic benefits for patients.

Phytoconstituents including phenolics and flavonoids are most important representatives to offer alleviation of hepatic ailments. It has been found that most of them are effective antioxidants [10,11] and iron chelation is very important part of their antioxidant activity [12]. Thus, search for crude drugs of the plant origin with antioxidant activity has become a central focus of study of hepatoprotection. *Caesalpinia crista* Linn. (syn. *C. bonducella*(L.)Roxb.) (family-Fabaceae) is a large scandent prickly shrub widely distributed throughout the tropical and subtropical regions of Southeast Asia. In India, it is commonly known as *Katikaranja* or *Natakaranja* and used in different system of traditional medication for the treatment of diseases and ailments of human beings. Traditionally, in Ayurveda, various plant parts such as leaves, stem, root, seed, and oil are used as febrifugal, periodic, tonic, and vesicant for the treatment of gynaecological disorders, skin diseases, constipation, piles, and
sulfate hexahydrate \((\text{NH}_4)_2 \text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}\), 1-chloro-2,4- were obtained from Sisco Research Laboratories Pvt. Ltd.

### 2. Material and Methods

#### 2.1. Chemicals.
Iron dextran and guanidine hydrochloride \((\text{TBA})\), and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from Sigma-Aldrich, USA. Trichloroacetic acid \((\text{TCA})\), and 5,5′-dinitro phenylhydrazine (DNPH) were obtained from HiMedia Laboratories Pvt. Ltd., Mumbai, India. Phenazineme-thione (TMA), and 5,5′-dinitrobenzene (CDNB), chloramine-T, hydroxylamine were used and reported to have adaptogenic [19], antihelmintic [20], anti-inflammatory [21], antipyretic and analgesic [22, 23], antimalarial [24], antiamyloidogenic [25], antibacterial [26], antifilarial [27], antitumor [28], anticonvulsant [29], nootropic [30], immunomodulatory [31], hepatoprotective [32], anxiolytic [33], diuretic, and hypoglycemic activity [34]. Previously, 70% methanol extract of *Caesalpinia crista* (CCME) leaf has shown in vitro antioxidant and iron-chelating property and was found to be a rich source of phenolic and flavonoid compound [35]. Therefore, the present study was undertaken to assess whether the damage caused to liver by iron overload can be normalised by administration of CCME in mice.

#### 2.2. Plant Material.
The leaves of *Caesalpinia crista* (CC) were collected from the Bankura district of West Bengal, India. It was identified and authenticated by the Central Research Institute (Ayurveda), Kolkata, India, and a voucher specimen (CRHS 121/08) was deposited there.

#### 2.3. Preparation of Plant Extract.
The leaves of CC were dried at room temperature for 7 days, finely powdered and used for extraction. The powder \((100\, \text{g})\) was mixed with 500 ml methanol:water \((7:3)\) using a magnetic stirrer for 15 hours, then the mixture was centrifuged at 2850 \(\times\) g and the supernatant was decanted. The extraction was repeated again with the precipitated pellet. The supernatants were collected, concentrated in a rotary evaporator and lyophilized. The dried extract, denoted as CCME was stored at \(-20^\circ\text{C}\) until use.

#### 2.4. Animals.
Male Swiss albino mice \((20 \pm 2\, \text{g})\) 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 \(22 \pm 2^\circ\text{C}\). The animals were provided with normal laboratory pellet diet and water ad libitum. Institutional animal ethics committee (IAEC) approved all experiments performed 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.

#### 2.5. In Vitro Study

##### 2.5.1. DPPH Radical Scavenging
The free radical scavenging activity of CCME was evaluated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) using a standard method [36]. Briefly, the reaction mixture contains 0.05 ml of 1 mM DPPH solution, 0.5 ml of 99% ethanol, and 0.45 ml of sample and standard ascorbic acid at different concentrations. The solution was rapidly mixed and the reduction of DPPH was measured by reading the decrease in absorbance at 517 nm. All tests performed six times. Ascorbic acid was used as a reference compound.

##### 2.5.2. Oxidative DNA Damage
pUC18 plasmid DNA was used for DNA protection study by CCME, according to a previously described method [37] with minor modifications. In Hepes buffer \((\text{pH} 7.2, 100\, \text{mM})\), FeSO\(_4\) solution \((750\, \mu\text{M})\), CCME of varying doses \((0–30\, \mu\text{g/ml})\), DNA \((0.5\, \text{mg/ml})\), and water were added to make an initial reaction mixture. Finally, \(\text{H}_2\text{O}_2\) solution \((7.5\, \mu\text{M})\) was added to start the reaction. Desferal was used to stop the reaction after 10 min. 25 \(\mu\text{l}\) of each reaction mixture was loaded in 1% agarose gel. After migration, the gel was stained with ethidium bromide and visualized in a UV transilluminator. The DNA bands were quantified through densitometry and the following formulae were used to calculate the percentage of protection:

\[
\% \text{SC} = \left[ \frac{1.4 \times \text{SC}}{\text{OC} + (1.4 \times \text{SC})} \right] \times 100, \quad (1)
\]

where, SC is the supercoiled; OC is the open circular; 1.4 is the correction factor.

\[
\% \text{protection} = 100 \times \left[ \frac{\text{control SC} - \text{chelator SC}}{\text{control SC} - \text{no chelator SC}} - 1 \right]. \quad (2)
\]

The ability of the plant extract to protect the DNA supercoil can be expressed by the concentration of sample required for 50% protection, designated as the \([P]_{50}\) value.

#### 2.6. In Vivo Study

##### 2.6.1. 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.

2.6.2. Sample Collection and Tissue Preparation. Mice were fasted overnight after the experiment ended on the 21st day. They were anesthetized with ethyl ether and blood was collected by cardiac puncture. After the clotting of blood samples, sera were separated using cooling centrifuge and store at −80°C until analysis. The liver was dissected out and rinsed with ice-cold saline to eliminate the blood cells; half of them were cut, 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 8000 g for 30 min at 4°C. The supernatant was collected and used for the assay of enzyme activities, protein oxidation, levels of hydroxyproline content, and lipid peroxidation products. A standard graph of BSA was prepared to estimate the protein concentration in the homogenate by Lowry method [38]. The other half 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.

2.6.3. Serum Markers. Alanine amino transferase (ALAT), aspartate amino transferase (ASAT), 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.

2.6.4. Antioxidant Enzymes. Superoxide dismutase (SOD) was assayed by measuring the inhibition of the formation of blue colored formazan at 560 nm [39]. Catalase (CAT) activity was measured by following the decomposition of H2O2 over time at 240 nm [40]. Glutathione-S-transferase (GST) was determined based on the formation of GSH-CDNB conjugate and increase in the absorbance at 340 nm [41]. A spectrophotometric method was used to measure reduced glutathione (GSH) level at 412 nm [42].

2.6.5. Biochemical Parameters. The lipid peroxide levels in liver homogenates were measured in terms of thiobarbituric acid reactive substances (TBARS) as an index of malondialdehyde accumulation [43]. As a marker of protein oxidation, protein carbonyl contents were estimated spectrophotometrically by DNPH method [44]. Briefly, 50 µl streptomycin sulphate (10% w/v) was added to 450 µl homogenate samples and then centrifuged at 2800 g for 15 minutes. The supernatant (200 µl) was incubated with the same volume of 10 mM DNPH in 2M HCl at room temperature for 20 mins. 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, \( \varepsilon = 2.2 \times 10^{-4} \text{M}^{-1} \text{cm}^{-1} \). The measurement of hydroxyproline content in the liver allows the actual quantitation of collagen which is an important marker of liver fibrosis. Liver samples were hydrolyzed in 6 M HCl and hydroxyproline was measured by Ehrlich’s solution [45]. A standard curve \( R^2 = 0.9907 \) of 4-hydroxy-L-proline was prepared and results were calculated after taking absorbances at 558 nm. Total hydroxyproline content in each sample was multiplied by a factor of 7.69 to determine the collagen content [46]. Results are expressed as milligrams of collagen per liver (wet weight).

2.6.6. Histopathological Analysis. The liver samples were excised, washed with normal saline, and processed separately for histological study. 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 μm thickness, stained with hematoxylin and eosin, and examined microscopically for histopathological changes.

2.6.7. Liver Iron and Serum Ferritin. Liver iron was measured according to a formerly reported colorimetric method [47]. Samples were incubated with bathophenanthrolinesulphonate for 30 min at 37°C and absorbances were read at 535 nm. Serum ferritin levels were measured using enzyme-linked immunosorbent assay kit (from Monobind Inc., USA) according to the manufacturer’s instructions.

2.6.8. Iron Release from Ferritin. As previously described, iron reduction and release was determined spectrophotometrically [48]. The ferrous chelator, ferrozine, was used as a chromophore for this assay. The reaction mixture (3 ml final volume) contained 200 μg ferritin, 500 μM ferrozine, in 50 mM pH 7.0 phosphate buffer. Reaction was induced by the addition of 500 μl plant extracts of different concentrations and alteration 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.

2.7. 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 Origin professional 6.0. Comparisons among groups were made according to pair t-test. In all analyses, a P-value of <0.05 was considered significant.

3. Results and Discussion

3.1. In Vitro Study

3.1.1. DPPH Scavenging. The bleaching of DPPH indicates the free radical scavenging capacity of CCME. Figure 1 demonstrated the DPPH radical scavenging activity of the extract in comparison to the standard. The IC50 values of the sample and standard were 14.2 ± 0.63 μg/ml and 5.27 ± 0.27 μg/ml, respectively. The results clarify the antioxidant activity of CCME as it is an effective DPPH free radical scavenger.
3.2.1. Serum Markers. The serum enzymes are very important adjuncts to clinical diagnosis of diseases and tissue injury. Hepatic injury by iron results in the leakage of cellular enzymes into the bloodstream, resulting in augmented levels of serum ALAT, ASAT, ALP, and bilirubin [4]. Increase in the levels of serum enzymes, namely, ALAT (136.24%), ASAT (145.69%), ALP (149.21%), and bilirubin (265.48%) as shown in Table 1 clearly signifies iron-induced liver damage. Oral administration of CCME markedly reduced the elevated levels of serum enzymes and bilirubin of iron-overloaded mice to approach the normal control values.

3.2.2. Antioxidant Enzymes. Cells are armed with a stock of endogenous antioxidant defence machinery to protect them. These include enzymes such as SOD, CAT, and GST or compounds such as GSH [49]. In excess, iron is a major cause of oxidative stress and lowers the levels of SOD (77.04%), CAT (52.25%), GST (70.02%), and GSH (32.36%), whereas treatment with CCME arrested the iron-induced depletion of these enzymes dose-dependently (Table 2). Thus, CCME significantly mended the levels of antioxidant enzymes and helping revival from hepatic damage.

3.2.3. Biochemical Parameters. The enhanced lipid peroxidation has been proposed as an initial step by which iron causes structural and functional alterations in cell integrity [50]. The present result showed that 84% increment of lipid peroxidation in liver homogenates of iron-injected mice than blank was significantly reduced by 29%, 32%, and 41% in mice fed with S50, S100, and S200, respectively, (Figure 3). Protein oxidation is another outcome in iron-overload-induced hepatic damage. The iron-mediated oxidative modification of protein leads to their degradation and carbonyl formation [51], which was confirmed by elevated level of protein carbonyl (147%) from liver samples in iron-overloaded mice compared to normal mice. The results of current study clearly establish that CCME efficiently reduced the carbonyl content 11%, 20%, and 55% with gradual increase of concentration (S50, S100, and S200) (Figure 4). The increase in collagen content (197%) of iron-intoxicated mouse was a significant indicator of liver fibrosis. The excessive iron deposition in liver resulted in iron-catalysed oxidative stress contributing to the pathogenesis and progression of liver fibrosis [52]. The collagen content in iron-overloaded mice was found 15.56 ± 1.09 mg/liver compared to 5.23 ± 1.37 mg/liver of normal mice. The upsurge of collagen content was gradually reduced to 15.38 ± 1.2 mg/liver, 12.17 ± 1.29 mg/liver, and 8.95 ± 1.45 mg/liver in CCME treated mice (S50, S100, and S200, resp.), signifying the hepatic fibrosis inhibitory potency of the plant extract (Figure 5).

3.2.4. Histopathological Study. Histological observations are performed along with the level of various biochemical parameters in circulation to mark the extent of hepatic damage. The liver sections of normal mice showed normal cell morphology with well-preserved cytoplasm, prominent nucleus, and well-brought-out central vein (Figure 6(a)). Iron dextran control mice showed various degrees of pathological changes including hepatocellular necrosis, ballooning degeneration, and loss of cellular boundaries (Figure 6(b)). In contrast, the liver sections taken from CCME-treated mice showed lessenning of the pathogenesis and revealed marked reduction in hepatic injuries (Figures 6(c), 6(d), and 6(e)). Figure 6(f) exhibited the improved histology of liver sections taken from desirox-treated group. However, these observations indicate the in situ hepatoprotective evidence of the extract.

3.2.5. Liver Iron and Serum Ferritin. The elevated (142%) liver iron content was found in iron-overloaded mouse compared to normal mouse. Administration of CCME reduced the iron level 17%, 26%, and 45% with the effect of increased dose (S50, S100, and S200, resp.) (Figure 7). The decrease in liver iron deposition induced by CCME treatment supports its iron-chelating potency which was established previously [35]. Body’s iron level is positively correlated with ferritin, a ubiquitous intracellular protein that stores iron in a nontoxic form and also helps prevent iron from mediating oxidative damage to cell constituents [53]. The increased level of ferritin is generally noticed...
Liver section treated with iron dextran + 20 mg/kg b.w. desirox also shows reduced necrotic area and increased number of hepatocytes.

+200 mg/kg b.w. CCME. The two groups (d) and (e) show reduced hepatocellular necrosis, ballooning degeneration, and inflammation. (f) Portal inflammation (PI). (d) Liver section treated with iron dextran + 100 mg/kg b.w. CCME. (e) Liver section treated with iron dextran (I), and loss of cellular boundaries. (c) Liver section treated with iron dextran + 50 mg/kg b.w. CCME showing improved histology with

Figure 6: Histopathology. Photomicrograph of mice liver sections (staining with haematoxylin and eosin) x40; (a) normal mice liver. (b) Iron-intoxicated (iron dextran, 100 mg/kg b.w.) liver section showing necrosis (N), fatty ballooning degeneration (FD), inflammation (I), and loss of cellular boundaries. (c) Liver section treated with iron dextran + 50 mg/kg b.w. CCME showing improved histology with portal inflammation (PI). (d) Liver section treated with iron dextran + 100 mg/kg b.w. CCME. (e) Liver section treated with iron dextran + 200 mg/kg b.w. CCME. The two groups (d) and (e) show reduced hepatocellular necrosis, ballooning degeneration, and inflammation. (f) Liver section treated with iron dextran + 20 mg/kg b.w. desirox also shows reduced necrotic area and increased number of hepatocytes.
3.2.6. Reductive Release of Ferritin Iron and Its Correlation with Reducing Power. Within cell, ferritin served as the storage protein for excess iron in ferric state. In case of iron overload, various iron chelators are administered to attenuate the situation, but, most of these chelators have limited binding activity for ferric iron as well, as iron in ferritin is not properly accessed to them. So, iron chelation therapy is dependent on the reductive release of ferritin iron, which is achieved by supplemented addition of a reducing agent such as ascorbate to increase the availability of storage iron to chelators [54]. Figure 9 showed the reductive release of ferritin iron by CCME, that was measured with a ferrous complex of ferrozine [Fe(ferrozine)\(_3\)]\(^{2+}\). Control experiments without CCME produced negligible amounts of [Fe(ferrozine)\(_3\)]\(^{2+}\), whereas, after dose dependant addition of CCME the [Fe(ferrozine)\(_3\)]\(^{2+}\) complex formation was increased significantly with time. However, reducing property of an iron chelator should definitely increase the efficiency of iron chelation therapy to treat iron overload. Previous results had shown the reductive ability of CCME [35] as well as in the present study, a significant \(P < 0.001\) positive correlation \((R = 0.9571)\) between reducing power and \(\%\) iron released from ferritin has been well established (Figure 10).
4. Conclusions

From the present study, it might be concluded that CCME has protective effect against iron-overload-induced liver toxicity as evidenced by biochemical and histopathological studies. CCME may exert its hepatoprotective activity by upregulating antioxidant enzymes and chelating iron to excrete form the body. The findings suggest its benefit in pathological sequence of iron-overload-linked liver disease and can be used as promising hepatoprotective agent.

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