Protective effect of *Clerodendrum colebrookianum* leaves against iron-induced oxidative stress and hepatotoxicity in Swiss albino mice

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Liver toxicity due to iron overload leads to oxidative damage of proteins, lipids and nucleic acids which in turn manifests several human diseases. Here, we evaluated the improving effect of *Clerodendrum colebrookianum* leaf on iron overload induced liver injury along with *in vitro* iron chelation and the protection of Fenton reaction induced DNA damage was conducted. Iron overload was induced by intraperitoneal administration of iron-dextran into mice. Post oral administration of different doses of the extract (50, 100 and 200 mg/kg body weight) showed significant decrease in different biochemical markers such as liver iron, serum ferritin and serum enzyme levels, along with decreased lipid peroxidation, protein oxidation and collagen content. In addition, the extract effectively enhanced the antioxidant enzyme levels and also exhibited the potential activity of the reductive release of ferritin iron. The protective effect of *C. colebrookianum* extract on injured liver was furthermore supported by the histopathological studies that showed improvement histologically. In conclusion, the present results demonstrated the hepatoprotective efficiency of *C. colebrookianum* leaf in iron overloaded mice, and hence, a potential iron chelating drug for iron overload diseases.

**Keywords:** Antioxidant enzymes, East Indian Glory Bower, Folk medicine, Ferritin, Hypertension, Iron overload, Lipid peroxidation, Protein carbonyl, Traditional medicine

Being an imperative trace element of the body for its growth and survival, iron exists in hemoglobin, myoglobin, cytochromes, iron-dependent metalloenzymes and enzymes with iron sulfur complexes¹. Although an optimum level of iron is always maintained by the cells to balance between essentiality and toxicity, in some situations the balance is disrupted, resulting in iron overloaded toxicity. Generation of highly reactive HO’ by virtue of Fenton’s reaction causes immense injury to various biomolecules leading to oxidative stress², resulting in inflammatory responses in hepatic cells. The first line of defense against oxidative injury, the intrinsic antioxidant system involving superoxide dismutase (SOD), catalase (CAT) and glutathione-s-transferase (GST) enzymes and reduced glutathione (GSH), starts behaving abnormally as their levels hugely deviate from their respective normal conditions. The major mechanism of iron-induced hepatotoxicity appears due to increased hepatic lipid peroxidation (LPO)³ and single and double-strand breaks in DNA⁴, which have been implicated in the pathophysiology of iron overload diseases including hemochromatosis, L-thalassemia, ischemic heart disease and cancer⁵,⁶. Fatty ballooning degeneration is one of the major detriments resulting from oxidative damage to lipids of hepatic parenchyma. Damage to hepatocellular proteins causes factors like collagen and protein carbonyl contents to strongly deviate from their normal course. This leads to serious pathological conditions like loss of overall tissue integrity, perportal vein inflammation and hepatocellular necrosis which is followed by the migration of fibrosis inside the deformed perportal vein; the same being a hallmark of hepatic damage. Hepatic damage also induces leakage of cellular enzymes into the bloodstream, resulting in elevated levels of serum ALAT, ASAT, ALP and bilirubin¹. Since total iron content in liver increases, ferritin, a storage protein, is incapable of hoarding iron and thus spills it in its Fe³⁺form in the blood. Analyzing the reductive release of ferritin becomes another hallmark for estimating the extent of damage due to excess iron. In order to bridge the gap between calculated assumptions and

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certain inferences upon the efficacy of any drug which is believed to be capable of alleviating iron induced hepatic injury, considering the above factors as experimental parameters in an animal model becomes vital.

At present, the only chelator in widespread clinical use is desferrioxamine (DFO) which however suffers from a number of serious disadvantages like its high cost, limited membrane permeability and unavailability as oral drug. The contemporary pharmaceutical research is thus directed towards the development of more economical, effective, orally absorbed and safe drugs thereby raising the therapeutic benefits for patients. Among different bioactive phytoconstituents, phenolics and flavonoids are the most important representatives that offer alleviation of hepatic ailments. It has been found that most of them are elective antioxidants and iron chelation is a very important part of their antioxidant activity. These iron-chelating agents consist of a range of bidentate, tridentate, and hexadentate ligands which are able to coordinate with iron, forming octahedral complexes.

*Clerodendrum colebrookianum* Walp. (Fam. Lamiaceae) was previously studied for its phytochemical constituents and antioxidant potential, which showed the presence of significant amounts of phenolic and flavonoid compounds. Apart from that, traditionally *C. colebrookianum* has shared an impressive inclusion in the folklore medicinal practices of different parts of North-East India for the treatment of various ailments and disorders such as colic pain, diabetes, helminthic infections, cough, dysentery, skin diseases, high blood pressure, malaria and other liver dysfunctions. Considering these traditional uses and high antioxidant efficacy, the present study was performed to evaluate the ameliorating effect of aqueous and 70% methanolic extract of *C. colebrookianum* leaf on oxidative stress induced hepatic damage in iron overloaded mice and insight the possible mechanism behind this efficiency.

**Materials 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), ferrozone, glutathione reduced, bathophenanthroline sulfonate disodium salt, Thiobarbituric acid (TBA) and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) were obtained from Cipla Ltd., Kolkata, India. Hydrogen peroxide, ammonium iron (II) sulfate hexahydrate [(NH₄)₂Fe(SO₄)₆H₂O], 1-chloro-2,4-dinitrobenzene (CDNB), chloramine-T, hydroxyamine 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 and preparation of extract*—Fresh leaves of *C. colebrookianum* were collected from East Khasi Hills district of Meghalaya in the winter season and specimens of the same were submitted and authenticated by the herbarium curator, Department of Botany, North-Eastern Hill University, Shillong, Meghalaya, India and a voucher no. 6786 was obtained. The leaves were cleaned and dried at room temperature, finely powdered and used for extraction. The leaf powder was mixed with the respective solvents (water and 70% methanol) in a 1:10 ratio w/v, i.e. 100 g in 1000 ml, using a magnetic stirrer for 15 h and then centrifuged at 5000 rpm for 15 min to obtain the supernatant. The process was repeated by mixing the precipitated pellet with 1000 ml fresh solvent. The supernatants from both the phases were mixed and concentrated under reduced pressure in a rotary evaporator at 40 °C, followed by lyophilisation. The aqueous and 70% methanol extracts of *C. colebrookianum* leaves were marked as CCLA and CCLM, respectively.

*Animals*—Male Swiss albino mice (20±2 g) were purchased from the 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.
In vitro study

Iron chelation activity—The Fe$^{2+}$ chelating activity of CCLA and CCLM was evaluated according to a standard method$^{21}$. Briefly, in a HEPES buffer (20 mM, pH 7.2) medium, CCLA and CCLM (0-120 μg/ml) and positive control EDTA (0-20 μg/ml) were separately added to a 12.5 μM ferrous sulfate solution and 75 μM ferrozine was added to start the reaction. After rapid vortexing, the mixture was left at room temperature for 20 min and the absorbance was taken at 562 nm. All tests were performed six times.

DNA protection assay—The protection of the pUC18 plasmid DNA damaged by Fenton reaction generated OH$^-$ radicals was studied by quantifying the decrease of supercoiled DNA after oxidative attack, using a previously described method with minor modifications$^{22}$. FeSO$_4$ solution (15 μM), plant extracts of varying doses, DNA (1 μg) and water were added in Heps buffer (pH 7.2, 13 mM) to make an initial reaction mixture. H$_2$O$_2$ solution (0.0125 mM) was then added to start the reaction. After 10 min, the reaction was stopped by adding Desferal (0.2 mM) as stopping reagent followed by loading buffer. 20 μl 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.

\[
\% \text{ protection} = \frac{100 \times [(\text{control SC} - \text{chelator SC})/(\text{control SC} - \text{no chelator SC}) - 1]}{1.4 \times \text{SC}/(\text{OC}+(1.4 \times \text{SC}))} \times 100
\]

where, SC = supercoiled; OC = open circular; 1.4 = correction factor

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.

In vivo study

Experimental Design—Thirty six mice were divided into 6 groups containing 6 mice in each group. One group served as blank (B) and received normal saline only. The other 5 groups were given 5 doses (one dose every two days) of 100 mg/kg body wt. each, of iron-dextran saline (i.p.). One iron-dextran group (C) received normal saline and other 4 groups were orally administered with 50 mg/kg body wt. (S50), 100 mg/kg body wt. (S100), 200 mg/kg body wt. (S200) plant extract and 20 mg/kg body wt. desirox (D), respectively, for 3 consecutive 7 day period, started from the day after the first iron-dextran injection.

Sample collection and tissue preparation—At the end of the experiment on the 21$^{st}$ day, mice were fasted for overnight before sacrifice. 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 stored at $-80 \degree$C until analysis. The liver was rapidly removed and washed with ice-cold saline to eliminate the blood cells and divided into three portions. One major portion of the liver samples was weighed and homogenized in 10 volumes 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 \degreeC. The supernatant was collected and used for the assay of enzyme activities, protein oxidation, levels of hydroxyproline content and lipid peroxidation products. Protein concentration in the homogenate was estimated according to the Lowry method$^{23}$ using BSA as standard. Another portion of the liver samples was weighed and digested with equimol (1:1) mixture of sulfuric acid and nitric acid and their iron content were analyzed. The remaining portion of the liver was used for histopathological studies.

Serum enzymes and Ferritin level—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. Serum ferritin levels were measured using enzyme-linked immunosorbsent assay kit (from Monobind Inc., USA) according to the manufacturer’s instructions.

Antioxidant enzymes—Superoxide Dismutase (SOD) was assayed by measuring the inhibition of the formation of blue colored formazan at 560 nm.$^{24}$ Catalase (CAT) activity was measured through time course decomposition of H$_2$O$_2$ at 240 nm.$^{25}$ A formerly reported method was followed to determine Glutathione-S-Transferase (GST) based on the formation of GSH-CDNB conjugate.$^{26}$ Reduced Glutathione (GSH) level was measured spectrophotometrically at 412 nm by a standard procedure.$^{27}$

Assessment of liver damage parameters—The lipid peroxide levels in liver homogenates were measured
in terms of thiobarbituric acid reactive substances (TBARS) as an index of malondialdehyde accumulation. Protein carbonyl contents as a marker of protein oxidation was determined by a spectrophotometric method. Hydroxyproline content represents the content of collagen, closely related with liver fibrosis. Liver samples were hydrolyzed in 6 M HCl and hydroxyproline was measured by Ehrlich’s solution. 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. Results are expressed as milligrams of collagen per liver (wet weight).

**Liver Iron**—Liver samples were incubated with bathophenanthroline sulfonate for 30 min at 37 °C and absorbances were measured at 535 nm to determine the liver iron content.

**Iron release from Ferritin**—The release of ferritin iron was determined spectrophotometrically according to a previously described method. 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. The reaction was started by the addition of 500 μl CCLA of different concentrations (100-500 μg) 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.

**Histopathological analysis**—The liver samples were excised, washed with normal saline, and processed separately for histological study. Initially, the material was fixed at 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.

**Statistical analysis**—All data are reported as the mean ± SD of six measurements. Statistical analysis was performed using Ky Plot 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.

**Results**

**In vitro studies**

**Iron Chelation activity**—The formation of violet colored Fe$^{2+}$-ferrozine complex is disrupted by the presence of an iron chelator. The results (Fig. 1a and 1b) demonstrated that the formation of Fe$^{2+}$-ferrozine complex is inhibited dose dependently in the presence of CCLA, CCLM and reference compound EDTA. The $IC_{50}$ values of the CCLA, CCLM and EDTA were 121.10 ± 10.04 μg/ml, 250.59 ± 3.41 μg/ml and 1.27 ± 0.05 μg/ml, respectively. Among both the extracts CCLA exhibited better iron chelation activity which inhibited 50.44% of the complex formation at the highest dose, i.e., 120 μg/ml.

**DNA protection potential**—The protective effect of CCLA and CCLM against Fe$^{2+}$-H$_2$O$_2$ mediated DNA breakdown was demonstrated in Fig. 2a and 2b. pUC18 supercoiled DNA was used as a control (lane 1). Lane 2 comprised only of the open circular DNA.
form of DNA generated by Fenton reaction. Addition of gradually increasing concentrations of CCLA and CCLM resulted in the restoration of DNA in the supercoiled form (lane 3-8 and 3-7, respectively). The results in fig. 2c and 2d showed the dose dependent protection by CCLA and CCLM with a P$_{50}$ value of 11.65 ± 0.25 µg and 63.18 ± 1.79 µg, respectively. This observation concretes the antioxidative, specially hydroxyl radical scavenging as well as iron chelating ability of the extract.

**In vivo study**

Results from *in vitro* studies lead the present investigation primarily incorporates the *in vivo* ameliorating effect of CCLA on iron accumulation and oxidative damage in liver of iron overloaded mice.

**Serum enzymes**—Iron overloaded liver injury resulted in a significant rise in the levels of ALAT, ASAT, ALP and bilirubin. As evidenced in Table 1, iron induced liver injury resulted in the significant increase in ALT (347.88%), AST (276.13%), ALP

![DNA protection activity of CCLA. In vitro protection against oxidative damage to pUC 18 by (a) CCLM; and (b) CCLA. Picture of agarose gel of pUC18 DNA showing bands of supercoiled (SC) and open circular (OC) forms. Lanes on the gel represent: (Lane 1) control DNA (no H$_2$O$_2$ or Fe$^{2+}$), (Lane 2) reaction mixture without extract; reaction mixture with extract of increasing concentration and the graphs represents its % of protection, for (c) CCLM; and (d) CCLA, respectively. The results are mean ± S.D (n=6).***P < 0.001 vs. 0 µg/ml](image)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ALT (Unit/L)</th>
<th>AST (Unit/L)</th>
<th>ALP (Unit/L)</th>
<th>Bilirubin (mg/dl)</th>
</tr>
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<tr>
<td>B</td>
<td>14.42 ± 1.91</td>
<td>62.46 ± 6.31</td>
<td>84.62 ± 3.48</td>
<td>1.58 ± 0.10</td>
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<td></td>
<td>64.6 ± 4.32$^{X1}$</td>
<td>234.91 ± 8.30$^{X3}$</td>
<td>252.63 ± 4.92$^{X3}$</td>
<td>3.15 ± 0.18$^{X3}$</td>
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<td></td>
<td>(347.88)</td>
<td>(276.13)</td>
<td>(198.56)</td>
<td>(99.67)</td>
</tr>
<tr>
<td>S50</td>
<td>49.08 ± 3.89$^{XY2}$</td>
<td>186.52 ± 14.74$^{X3Y3}$</td>
<td>206.78 ± 9.40$^{X3Y3}$</td>
<td>2.98 ± 0.15$^{X3}$</td>
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<td></td>
<td>(240.28)</td>
<td>(198.65)</td>
<td>(144.37)</td>
<td>(88.90)</td>
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<tr>
<td>S100</td>
<td>38.38 ± 0.42$^{X3Y3}$</td>
<td>166.83 ± 13.05$^{X3Y3}$</td>
<td>174.36 ± 4.54$^{X3Y3}$</td>
<td>2.53 ± 0.19$^{X3}$</td>
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<tr>
<td></td>
<td>(166.11)</td>
<td>(167.12)</td>
<td>(106.06)</td>
<td>(60.17)</td>
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<tr>
<td>S200</td>
<td>31.76 ± 0.86$^{X3Y3}$</td>
<td>129.13 ± 13.45$^{X3Y3}$</td>
<td>144.81 ± 5.08$^{X3Y3}$</td>
<td>2.25 ± 0.13$^{X3}$</td>
</tr>
<tr>
<td></td>
<td>(120.17)</td>
<td>(106.76)</td>
<td>(71.13)</td>
<td>(42.87)</td>
</tr>
<tr>
<td>D</td>
<td>19.85 ± 0.79$^{X3Y3}$</td>
<td>88.06 ± 10.3$^{X3Y3}$</td>
<td>114.97 ± 6.12$^{X3Y3}$</td>
<td>1.72 ± 0.14$^{X3}$</td>
</tr>
<tr>
<td></td>
<td>(37.59)</td>
<td>(40.99)</td>
<td>(35.87)</td>
<td>(8.92)</td>
</tr>
</tbody>
</table>

Treatment groups are: B, Normal mice; C, Iron-dextran treated mice receiving normal saline; S50, S100 and S200: 50, 100 and 200 mg/kg body wt. CCLA treated groups, respectively; D, 20 mg/kg body wt. desirox treated group. X, significant difference from normal mice (B) group (X1: P ≤0.05, X2: P ≤0.01 and X3: P ≤0.001). Y, significant difference from iron overloaded (C) group (Y1: P ≤0.05, Y2: P ≤0.01 and Y3: P ≤0.001). Values given in parentheses are % change.

Table 1—The effect of CCLA on serum parameters in iron overloaded mice.

[Values are mean ± SD of six observations]
(198.56%) and bilirubin (99.67%). Oral administration of increased doses of plant extract prominently abridged the elevated levels of serum enzymes and bilirubin of iron overloaded mice to approach the normal (control) values. Although in all cases, the standard drug desirox, showed the best activity in reducing the respective enzyme levels to near normal.

**Antioxidant enzymes**—Iron overload liver toxicity reduces the level of antioxidant enzymes- SOD, CAT and GST along with compounds such as GSH, than the normal physiological condition which thereby give rise to major oxidative stress. The results showed (Table 2) that when treated with the highest dose of CCLA the iron-induced depletion of antioxidant enzymes SOD, CAT, GST and the levels of non-enzymic antioxidant GSH were arrested markedly viz. SOD by 34.15%, CAT by 33.49%, GST by 19.44% and GSH by 14.35%, whereas a reduction of 14.8%, 16.09%, 13.57% and 6.09% was found in the standard desirox treated group for SOD, CAT, GST and GSH, respectively.

**Lipid peroxidation products**—The intraperitoneal injection of iron-dextran significantly enhanced (101.64%) lipid peroxidation in liver homogenates compared to normal control mice. The level of elevated lipid peroxidation was considerably reduced by 63.09, 42.89 and 28.20% in mice receiving oral treatments of S50, S100 and S200, respectively whereas standard desirox generated 11.57% reduction (Fig. 3a).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SOD (Unit/mg protein)</th>
<th>CAT (Unit/mg protein)</th>
<th>GST (Unit/mg protein)</th>
<th>GSH (µg/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>0.37 ± 0.02</td>
<td>16.66 ± 8.99</td>
<td>5.71 ± 0.43</td>
<td>0.46 ± 0.02</td>
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<tr>
<td>C</td>
<td>0.04 ± 0.02X3</td>
<td>4.43 ± 5.12X3</td>
<td>1.6 ± 0.42X3</td>
<td>0.29 ± 0.01X3</td>
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<td></td>
<td>(88.62)</td>
<td>(73.38)</td>
<td>(71.98)</td>
<td>(37.39)</td>
</tr>
<tr>
<td>S50</td>
<td>0.05 ± 0.02X3</td>
<td>5.08 ± 5.35XY1</td>
<td>1.8 ± 0.34X3</td>
<td>0.33 ± 0.02XY1</td>
</tr>
<tr>
<td></td>
<td>(87.94)</td>
<td>(69.47)</td>
<td>(68.48)</td>
<td>(28.26)</td>
</tr>
<tr>
<td>S100</td>
<td>0.15 ± 0.05XY2</td>
<td>9.16 ± 4.24XY3</td>
<td>3.0 ± 0.42XY2</td>
<td>0.38 ± 0.01XY3</td>
</tr>
<tr>
<td></td>
<td>(59.35)</td>
<td>(44.99)</td>
<td>(47.46)</td>
<td>(18.26)</td>
</tr>
<tr>
<td>S200</td>
<td>0.24 ± 0.03XY3</td>
<td>11.08 ± 5.08XY3</td>
<td>4.6 ± 0.47Y3</td>
<td>0.39 ± 0.01XY3</td>
</tr>
<tr>
<td></td>
<td>(34.15)</td>
<td>(33.49)</td>
<td>(19.44)</td>
<td>(14.35)</td>
</tr>
<tr>
<td>D</td>
<td>0.31 ± 0.06Y3</td>
<td>13.98 ± 6.54XY3</td>
<td>4.94 ± 0.39Y3</td>
<td>0.43 ± 0.02Y3</td>
</tr>
<tr>
<td></td>
<td>(14.80)</td>
<td>(16.09)</td>
<td>(13.57)</td>
<td>(6.09)</td>
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</table>

Treatment groups are B, Normal mice; C, Iron-dextran treated mice receiving normal saline; S50, S100 and S200: 50, 100, and 200 mg/kg body wt. CCLA treated groups, respectively; D, 20 mg/kg body wt. desirox treated group. X, significant difference from normal mice (B) group (X1: P ≤ 0.05, X2: P ≤ 0.01 and X3: P ≤ 0.001). Y, significant difference from iron overloaded (C) group (Y1: p ≤ 0.05, Y2: p ≤ 0.01 and Y3: p ≤ 0.001). Values given in parentheses are % change.

**Protein carbonyl and hydroxyproline content**—A significant elevation (149.34%) of protein carbonyl content in iron overloaded mice was found compared to normal mice. This study clearly establishes that the highest dose of extract efficiently reduced the protein carbonyl content and approaches to the activity near about that of the standard oral iron chelator drug desirox (Fig. 3b). As depicted in fig. 3c, the upsurge level of hydroxyproline content (122.46%) was observed in the iron overloaded mice group than the normal group. This rise of collagen content was gradually reduced by 105, 68 and 56% in CCLA treated mice (S50, S100 and S200, respectively). Desirox although reduced it further to a level of 4.9±0.11 mg/liver (30.52%).

**Liver iron and Serum ferritin level**—A raised up liver iron content (685.79%) was found in the iron-overloaded mice compared to normal mice. Oral administration of CCLA reduced the iron level to 442.62, 286.89, and 101.09% with respect to doses S50, S100 and S200, respectively (Fig. 3d) which also confirm dose-dependent in vitro iron chelation property of CCLA as earlier defined in the text. Consequently, the serum ferritin levels increased up to 58.99±5.04 µg/g protein which is more than double what the normal mice displayed (24.25±1.72 µg/g protein). As per fig. 3e administration of CCLA, a notable tendency of dose dependent reduction in serum ferritin concentrations was observed; the S50, S100 and S200 dose groups showed the serum ferritin levels of 55.3 ± 1.83, 49.06 ± 2.06 and 42.83 ± 3.68 µg/g protein, respectively.
Fig. 3—Effect of CCLA on (a) Hepatic lipid peroxidation levels; (b) Protein oxidation levels; (c) Collagen content; (d) hepatic iron content in different treated mouse liver; (e) Serum ferritin levels in different treated mice. Mice were randomly divided into six groups (blank, B; control, C; 50 mg/kg body wt. CCLA, S50; 100 mg/kg body wt. CCLA, S100; 200 mg/kg body wt. CCLA, S200; desirox group, D) and treated as described in ‘experimental design’ section. Values are expressed as mean ± SD of six mice. ***P <0.001, **P <0.01 compared with blank and ###P <0.001 compared with control.

Fig. 4—Dose dependent formation of the [Fe(ferrozine)]$_3^{2+}$ complex following release of Fe$^{2+}$ from ferritin by different doses of CCLA and single dose of desirox. 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.

Iron release from ferritin and its correlation with reducing power—The ability of plant extracts to release iron from ferritin was tested using ferrous chelator ferrozine as a chromophore. Ferrozine formed a complex with ferrous ion, [Fe(ferrozine)$_3^{2+}$], which was quantified to measure the reductive release of ferritin iron. Control experiments devoid of CCLA treatment produced negligible amount of released iron, whereas, after dose dependant (100-500 μg) addition of CCLA; significant amount of iron was released within 20 min. Fig. 4 showed iron released with time in response to the dose dependent addition of CCLA.

Fig. 5—Correlation of released ferritin iron with reducing power. The iron released in response to the increasing concentrations (100-500 μg) of CCLA was plotted against reducing power displayed by the same doses.

On the basis of the previous in vitro results showing a dose-dependent moderate reductive ability of CCLA, as well as from the present study, a significant positive correlation coefficient ($R^2$=0.902) between reducing power and the amount of iron (%) released from ferritin has been well established (Fig. 5). This analysis interprets that CCLA may be used as an effective iron chelating drug for the treatment of iron overload.

Histopathological study—Histological observations are executed along with the level of various biochemical parameters in circulation to mark the extent of hepatic...
Fig. 6—Photomicrograph of mice liver sections (staining with haematoxylin and eosin) ×40. (a) Control mice liver shows normal cellular integrity; (b) Iron-intoxicated (iron dextran, 100 mg/kg body wt.) liver section showing necrosis (N), fatty ballooning degeneration, inflammation (I), and loss of cellular boundaries; (c) Liver section treated with iron dextran + 50 mg/kg body wt. CCLA shows improved histology with portal inflammation (PI). d. Liver section treated with iron dextran + 100 mg/kg body wt. CCLA; e. Liver section treated with iron dextran + 200 mg/kg body wt. CCLA; (f) Liver section treated with iron dextran + 20 mg/kg body wt. desirox shows reduced necrotic area and the increased number of hepatocytes. S100 and S200 show reduced hepatocellular necrosis, ballooning degeneration, and inflammation indicating a trend of restoration of normal cellular integrity.

damage. The liver sections of normal mice showed normal cell morphology with well-preserved cytoplasm, prominent nucleus, and well-brought-out central vein (Fig. 6a). Iron dextran control mice showed various degrees of pathological changes including hepatocellular necrosis, ballooning degeneration, inflammation and loss of cellular boundaries (Fig. 6b). In contrast, the liver sections taken from CCLA-treated mice groups displayed evidence of lessening of the pathogenesis and revealed a marked reduction in hepatic injuries (Fig. 6c-e). Fig. 6f exhibited the improved histology of liver sections taken from desirox-treated group that is almost similar to S200 group. However, these observations indicate the in situ hepatoprotective evidence of the extract.

Discussion

Iron is considered to be the most common cofactor within the oxygen handling biological machinery. Excess iron mediated uncontrolled overproduction of free-radicals give rise to ominous pathogenic conditions which is not always effectively antagonized by the intrinsic physiological antioxidants. On the other hand, existence of excess iron in body systems owing to threatening xenobiotics along with dietary overload gives rise to hepatic siderosis, ensuing free-radical generation, thus forming the vicious cycle. An effective therapeutic approach could include the confiscation of catalytically active iron present in the cytosol or its release by chelation from other cell iron stores and simultaneously defending the cellular membranes from free radicals generated in the cells (i.e., antioxidant activity).

The present investigation established the protective effect of CCLA against iron overload induced oxidative stress in Swiss albino mice. With the intention to investigate the underlying principle of traditional usage of the plant as medicine, biologically active total phytocomponents present in the plant are needed to be well extracted with different solvents. As is also evident from the phytochemical screening and HPLC standardization of the samples, the likely presence of bioactive compounds (such as tannic acid, quercetin, catechin, reserpine, ascorbic acid and gallic acid) also justifies the procedure for extraction of the plant material. From figures 1 and 2 it was revealed that CCLA has shown better in vitro iron chelation, alongside the ability to dose dependently protect
Iron-dextran injections given intraperitoneally to the mice resembled the hemochromatosises secondary to iron loaded anemias (anemias treated with repeated transfusions) and high iron oral intake, while avoiding direct interruption of extract on intestinal iron absorption resulting in hepatic and serum iron overload. A gradual increase in the oral administration of CCLA greatly reduced hepatic iron content dose dependently compared to the iron overload control group, which also support its in vitro iron chelation property. Chronic iron overload mediated lipid peroxidation is the initial step by which iron causes structural and functional alterations in cell integrity through loss of lysosomal membrane, leading to hepatocellular injury. In the present study CCLA has been found to be an effective lipid peroxidation inhibitor, which is also well demonstrated by corresponding decrement in diffused ballooning degeneration found from histopathological studies. Other consequence of iron overloaded liver toxicity is oxidative modification of proteins causing harmful alterations in their catalytic and structural integrity which successively develop liver fibrosis which in turn increase the formation of protein carbonyl as well as collagen content. Assessment of the extent of protein oxidation is considered as an early biomarker of oxidative stress in the diagnosis of iron-overload induced hepatic injury. Treatment with CCLA dose dependently reduced protein oxidation as well as hydroxyprolin content, a marker of hepatic fibrogenesis. Similarly, the liver sections of the extract treated mice from histological data showed restoration from severe hepatic lesions and neutrophilic cellular inflammations thus gradually impending towards normal cytoarchitecture with increasing CCLA concentration per kg body weight. Along with that, the present result proposed that CCLA halted the iron induced depletion of the antioxidant enzymes, (SOD, CAT and GST along with non enzymatic GSH) inevitable performers of the intrinsic defense mechanism of the cells against oxidative stresses and thus reduce oxidative damage comparable to that of standard desirox.

Serum enzymes and bilirubin level are among the important parameters in the clinical diagnosis of liver diseases. Iron overloaded hepatic injury leads to leakage of cellular enzymes into the bloodstream, resulting in augmented levels of serum ALAT, ASAT, ALP and bilirubin. Enhanced levels of serum markers are used as sensitive indicators of liver damage. CCLA reduced the serum enzymes and total bilirubin levels, indicating its protective effect over liver and improving in its functional efficiency.

On the basis of the fact that 5/6th part of the surfeited iron in our body is settled in the liver, most procedures seek the measurement of liver iron levels for diagnosis. Iron in the body is generally stored in a non-toxic ferric form by ferritin, a ubiquitous intracellular protein which also helps to prevent iron from mediating oxidative damage to cell constituents. Serum ferritin is one of the key markers developed as a consequence of iron overload induced hepatic toxicity as the amount of ferritin in blood indirectly reflects the amount of iron present in the liver. Live iron content and ferritin level in this study was found enhanced in iron overloaded mice, whereas, the level significantly reduced after the treatment with CCLA.

The therapeutic approach of administering iron chelators to attenuate the situation of iron-overload depends largely on the availability of Fe2+ as the chelators suffer a limited binding ability for Fe3+. Apart from this, efficient access to the iron held up by ferritin is not available to them. The need for a higher rate of reductive release of iron from ferritin in iron overload disorders thus becomes more favorable for iron chelation therapy, which is provided by a supplementary addition of reducing agents like ascorbic acid. However, it is obvious that an iron chelator pertaining an intrinsic reducing property should definitely increase the efficiency of iron chelation therapy which is successfully done in vitro by CCLA. Moreover reducing capability of CCLA has been found to be significantly correlated to the ferritin iron release capacity of the extract.

The present investigation concludes that the drugs which possess both reducing power and iron chelating activity can successfully reduce the toxic levels of iron in iron overload mice and hence protect liver from oxidative stress and fibrosis. Serum enzyme and serum ferritin levels, indicators of severe iron overload, are also effectively lowered owing to the administration of CCLA. The hepatoprotective activity of CCLA was further confirmed from improved histopathological conditions of the liver.
The findings confer the benefit of extract, derived from *Clerodendrum colebrokianum* leaves, on the pathological sequence of iron-overload-linked liver disease by the upregulation of antioxidant enzymes as well as excretion of iron from body by effective chelation and that the same can be used as a promising hepatoprotective agent.

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