

Anti-oxidative protection against iron overload-induced liver damage in mice by *Cajanus cajan* (L.) Millsp. leaf extract

Rhitajit Sarkar, Bibhabasu Hazra & Nripendranath Mandal*

Division of Molecular Medicine, Bose Institute, P-1/12 CIT Scheme VII M, Kolkata 700054, India

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In view of the contribution of iron deposition in the oxidative pathologic process of liver disease, the potential of 70% methanolic extract of *C. cajan* leaf (CLME) towards antioxidative protection against iron-overload-induced liver damage in mice has been investigated. DPPH radical scavenging and protection of Fenton reaction induced DNA damage was conducted *in vitro*. Post oral administration of CLME to iron overloaded mice, the levels of antioxidant and serum enzymes, hepatic iron, serum ferritin, lipid peroxidation, and protein carbonyl and hydroxyproline contents were measured, in comparison to deferasirox treated mice. Oral treatment of the plant extract effectively lowered the elevated levels of liver iron, lipid peroxidation, protein carbonyl and hydroxyproline. There was notable increment in the dropped levels of hepatic antioxidants. The dosage of the plant extract not only made the levels of serum enzymes approach normal value, but also counteracted the overwhelmed serum ferritin level. The *in vitro* studies indicated potential antioxidant activity of CLME. The histopathological observations also substantiated the ameliorative function of the plant extract. Accordingly, it is suggested that *Cajanus cajan* leaf can be a useful herbal remedy to suppress oxidative damage caused by iron overload.

Keywords: *Cajanus cajan*, DNA protection, Ferritin, Hepatoprotection, Iron overload

Cajanus cajan (L.) Millsp. (syn. *Cajanus indicus*; en. Pigeon pea; family. Fabaceae), cultivated widely in the Indian subcontinent, Eastern Africa and Central America, rich in important amino acids, fatty acids and minerals¹, is nutritionally significant². Amongst its many medicinal uses, *C. cajan* is traditionally used in treating diabetes, sores, skin irritations, hepatitis, measles, jaundice, dysentery, expelling bladder stones and stabilizing menstrual cycles and various liver and kidney diseases^{3,4}. Being a forage crop, *C. cajan* is widely used for ethnomedical purposes worldwide, in diabetes and as stimulant⁵, in food poisoning, colic and constipation⁶, to cure gingivitis and stomatitis⁷, and in relief of pain in Chinese traditional medicine⁸. Chemical investigations revealed that *C. cajan* leaves are rich in polyphenolic compounds, especially flavonoids like luteonin and apigenin⁹, and also contain stilbenes, saponins, tannins, resins and terpenoids. Chemical studies reveal 2'-2' methyl cajanone, 2'-hydroxy genistein, isoflavones, cajanin, cahanones etc., which impart antioxidant properties¹⁰. Roots are also found to possess genistein and genistin.

It also contains hexadecanoic acid, α -amyrin, β -sitosterol, pinostrobin, longistylin A, longistylin C and the most important isoflavonone Cajanol, which impart anticancer activity^{11,12}. Presence of cajanuslactone, a coumarin imparts antibacterial activity³. Presence of cajaninstilbene acid, pinostrobin, vitexin and orientin is responsible for antiplasmodic activity¹³. Few preliminary studies regarding the antidiabetic, neuroactive, hypocholesterolemic, and anthelmintic potential have also been observed¹⁴. The antioxidant studies of *C. cajan* indicated presence of significant amount of phenolic and flavonoid compound¹⁵.

In presence of molecular oxygen, 'loosely-bound' iron is able to redox cycle between the two most stable oxidation states thereby generating oxygen-derived free radicals such as the infamous hydroxyl radical. Tissues that become subject to oxidative stress generated by the free radicals undergo damage through all biomacromolecules (polynucleotides, proteins, lipids and sugars) that can lead to a critical failure of biological functions and ultimately cell death. Moreover, excess iron deposition in the liver (hepatic siderosis) may result from increases in plasma iron due to hemolysis, blood transfusion, dietary iron overload, inflammatory syndrome, diabetes, or chronic liver diseases^{16,17}. Defence

*Correspondent author

Telephone: +91-33-2569-3265/85;

Fax: +91-33-2355-3886

E-mail: mandaln@rediffmail.com, nrופן@boseinst.ernet.in

mechanisms against iron-mediated-free radical-induced oxidative and/or nitrosative stress(es) involve: (i) preventive mechanism (ii) repair mechanism (iii) physical defence and (iv) antioxidant defence. Enzymatic antioxidant defences include superoxide dismutase, catalase and glutathione; but these are sometimes not enough to overcome the oxidative stress. Owing to the side effects of synthetic antioxidants (e.g. butylated hydroxytoluene and butylated hydroxyanisole) and oral iron chelators (e.g. deferasirox), their use has been restricted for therapeutic purposes.

Therefore, in view of the contribution of iron deposition in the oxidative pathologic process of liver disease and the need for a new drug to combat alongside intrinsic defences, effect of 70% methanolic extract of *Cajanus cajan* leaf has been studied on antioxidative iron releasing potential in iron-overload-induced liver damage in mice.

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), 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. HPLC grade acetonitrile, ammonium acetate, hydrogen peroxide, ammonium iron (II) sulfate hexahydrate $[(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}]$, 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. 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) and ferritin were purchased from MP Biomedicals, USA. Streptomycin sulphate was obtained from HiMedia Laboratories Pvt. Ltd, Mumbai, India. The standard oral iron chelating drug, Desirox (composing deferasirox), was obtained from Cipla Ltd., Kolkata, India.

Plant material—The leaves of *C. cajan* were collected from Bankura district of West Bengal, India. The plant was identified by the Central Research Institute (Ayurveda), Kolkata, India, where a specimen (CRHS 119/08) was deposited.

Animals—Male Swiss albino mice (20 ± 2 g) are purchased from Chittaranjan National Cancer Institute (CNCI), Kolkata, India and maintained under a

constant 12 h dark/light cycle at an environmental temperature of 22 ± 2 °C. The animals were fed with normal laboratory pellet diet and water *ad libitum*. The experiments were conducted according to the Institutional Animal Ethics Committee regulations approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Govt. of India (Registration. No. 95/1999/CPCSEA).

Plant extract preparation—Air dried and pestle grinded leaves of *C. cajan* (100 g) were mixed with 70% methanol (methanol:water::70:30) (500 mL) and kept in a shaking incubator overnight (12h, 37 °C, 160 rpm). The mixture was centrifuged at 2850 g for 20 min. The supernatant collected and pellet mixed with 500 mL of 70% methanol and extracted similarly. The supernatants collected from both the phases were mixed and filtered. The resultant filtrate was concentrated in a rotary evaporator under reduced pressure. The concentrated extract was lyophilized and stored at -20 °C until further use. An aqueous solution of the extract was used for all the experiments.

Standardization of the plant extract—Qualitative characterization: Phytochemical screening for the plant extract was done for carbohydrate, alkaloids, tannins, terpenoids, triterpenoids, anthraquinones, saponins and glycosides following standard methods^{18,19}.

HPLC standardization of the extract: Stock solutions (10 µg/mL) were prepared in mobile phase for the sample and catechin and tannic acid as standards. All the samples were filtered through 0.45 µm polytetrafluoroethylene (PTFE) filter (Millipore). Analysis was performed using a HPLC-Prominence System RF10AXL (Shimadzu Corp.) equipped with degasser (DGU-20A₅), quaternary pump (LC-20AT), auto-sampler (SIL-20A) and detectors of Reflective Index (RID-10A), Fluorescence (RF-10AXL) and Diode Array (SPD-M20A). Gradient elution of the samples was done with consecutive mobile phases of acetonitrile and 0.5 mM ammonium acetate in water, at a flow rate of 1 mL/min for 65 min through the column (Z1C-HILIC) that was maintained at 25 °C. The detection was carried out at 254 nm. The injection volume was 20 µL, and the sample and standards were analyzed in triplicates.

Estimation of primary and secondary metabolites: The extract was analyzed for carbohydrates²⁰, alkaloids²¹ and tannins²² following standard procedures.

In vitro assays—DPPH radical scavenging: Scavenging of the stable DPPH radical by different concentrations (0-100 µg/mL) of the extract and the standard ascorbic acid was done as per Sarkar *et al.*²³ at 517 nm. The percentage radical scavenging activity was calculated from the following formula:

$$\text{scavenging [DPPH] (\%)} = [(A_0 - A_1)/A_0] * 100$$

where A_0 is the absorbance of the control and A_1 is the absorbance in presence of the samples and standard.

Protection on Fe^{2+} mediated DNA breakdown: The assay was performed according to a previously standardized method²³. The degradation of pUC-18 plasmid DNA through Fenton reaction in Fe^{3+} -ascorbate-EDTA- H_2O_2 system, and subsequent rejuvenation of the nicked DNA to its supercoiled form by different CLME concentrations (3-30 µg/mL) was studied in agarose gel migration. The DNA bands were quantified through densitometry and the following formula used to calculate the percentage of protection:

$$\text{SC (\%)} = [1.4 \times \text{SC}/(\text{OC} + (1.4 \times \text{SC}))] \times 100$$

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

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

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

In vivo study of hepatoprotective activity—Experimental design and sample collection: Randomly divided Swiss albino mice comprised six groups of 6 mice each. Of them, normal saline treated group served as blank (B). Intraperitoneal iron-dextran injection was administered to each mouse (100 mg/kg body weight) of the other five groups in five doses (one dose every two days). Out of them, four groups were orally treated with 50, 100 and 200 mg/kg body weight of CCME and 20 mg/kg body weight Desirox (marked S50, S100, S200 and D respectively), for 21 days starting the day following first iron injection. Normal saline is administered to one iron-dextran injected group assigned as C. After overnight fasting on the 21st day, mice were anesthetized with ethyl ether and blood collected by cardiac puncture. Serum from blood was separated using cooling centrifuge

and stored at -80°C until analysis. Liver was dissected fast, washed, cut into half and weighed. One half was 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 assays. Protein concentration in the homogenate was estimated according to Lowry method using BSA as standard. The other halves were digested with equivolume (1:1) mixture of sulphuric acid and nitric acid and their iron content analysed.

Liver iron: Liver iron was measured according to colorimetric method²⁴. Samples were incubated with bathophenanthroline sulfonate for 30 min at 37°C and absorbances are read at 535 nm.

Lipid peroxidation product: The lipid peroxide levels in liver homogenates were measured as thiobarbituric acid reactive substances (TBARS) following Buege and Aust²⁵.

Protein carbonyl content: Protein carbonyl content was estimated spectrophotometrically by the method of Reznick and Packer²⁶. Briefly, 10% (w/v) streptomycin sulphate was mixed to homogenate samples and centrifuged. Equivolume supernatant was incubated with 10 mM DNPH in 2 M HCl. Post reaction, protein was precipitated with 10% TCA and subsequently washed with ethyl acetate-ethanol mixture (1:1) to remove unreacted DNPH. The protein pellet was dissolved in 6 M guanidine hydrochloride solution and the absorbance measured at 370 nm, using the molar extinction coefficient of DNPH, $\epsilon = 2.2 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

Hydroxyproline content: Hydroxyproline content of liver homogenates hydrolyzed in 6 M HCl was calculated from 4-hydroxy-L-proline standard curve ($R^2 = 0.9907$), using Ehrlich's solution method²⁷ of spectrophotometric measurement at 558 nm. The collagen content, expressed in mg/liver (wet weight), was determined as a product of hydroxyproline content in each sample with a factor of 7.69¹⁶.

Antioxidant enzymes: Superoxide dismutase (SOD)²⁸, catalase (CAT)²⁹, glutathione-S-transferase (GST)³⁰ and reduced glutathione (GSH)³¹ levels were measured.

Serum enzymes and ferritin levels: Alanine aminotransferase (ALAT), Aspartate aminotransferase (ASAT) and bilirubin in serum samples were measured using the commercial kits of Merck, Mumbai, India. Alkaline Phosphatase (ALP) was estimated using the kit supplied by Sentinel Diagnostics, Italy. Serum ferritin

level was measured using enzyme-linked immunosorbent assay kit (from Monobind Inc., USA) according to the manufacturer's instructions.

Histopathological analysis: Liver samples collected from each mouse were washed, and then fixed in 10% neutral formalin. The fixed tissue were embedded in paraffin wax and sectioned into 5 μm thick, stained with hematoxylin and eosin. Then the sections were examined on light microscope and photographed by using a microscopic camera.

In vitro ferritin iron release—Reductive iron release using known metal chelator ferrozine was determined spectrophotometrically³². Reaction was started by adding plant extracts of different concentrations into 50 mM pH 7.0 phosphate buffer containing 200 μg ferritin and 500 μM ferrozine. The change in absorbance was measured continuously at 560 nm for 20 min against reference solution containing buffer along with ferritin and ferrozine.

Statistical analysis—All data are reported as the mean \pm SD of 6 measurements. KyPlot version 2.0 beta 15 (32 bit) was used for plotting and statistical analyses of all data. The IC_{50} values were calculated by the formula $Y = 100 * A1 / (X + A1)$, where $A1 = \text{IC}_{50}$, $Y = \text{response}$ ($Y = 100\%$ when $X = 0$), $X = \text{inhibitory concentration}$. Comparisons among groups were made according to pair *t*-test. In all analyses, a *P* value of < 0.05 was considered significant.

Results

Qualitative and quantitative standardization of CLME—Phytochemical analysis of CLME showed qualitative and quantitative presence of primary and secondary metabolites (Table 1). HPLC analysis of the sample was performed to identify the presence of bioactive compounds in CLME by comparing with the respective retention time of the reference standards. The chromatogram obtained from HPLC analysis of the extract and two main peaks having retention times 3.29 and 19.06 min was determined to be corresponding to tannic acid and catechin, respectively (Fig. 1).

DPPH radical scavenging and protection of DNA damage—The effect of CLME to effectively scavenge DPPH radical indicated its potential antioxidant activity, nearly as good as the standard, as is indicated from the IC_{50} values of CLME ($12.23 \pm 0.31 \mu\text{g}/\text{mL}$) and ascorbic acid ($5.29 \pm 0.28 \mu\text{g}/\text{mL}$), and significant enough with $P < 0.001$ when compared to the standard.

Fenton reaction damaged pUC-18 DNA is protected from OC form to SC form gradually with increasing dose of CLME. As is observed from Fig. 2(a), Lane 1 contained unreacted DNA (no H_2O_2 or Fe^{2+}) and hence only, SC-form is present. Lane 2 reacted DNA with only the OC-form and lanes 3-9 contained CLME of increasing concentration (3-30 $\mu\text{g}/\text{mL}$) that is protecting DNA with increasing retention of the SC-form. Fig. 2(b) also corroborates the fact with about 87% retention of SC-form from the nicked DNA at the highest concentration, as is also evident from the $[\text{P}]_{50}$ value of the plant extract being $9.49 \pm 0.02 \mu\text{g}/\text{mL}$.

Effect of CLME on liver injury marker levels—The most direct result of iron overload, i.e., liver iron accumulation was dose dependently decreased by

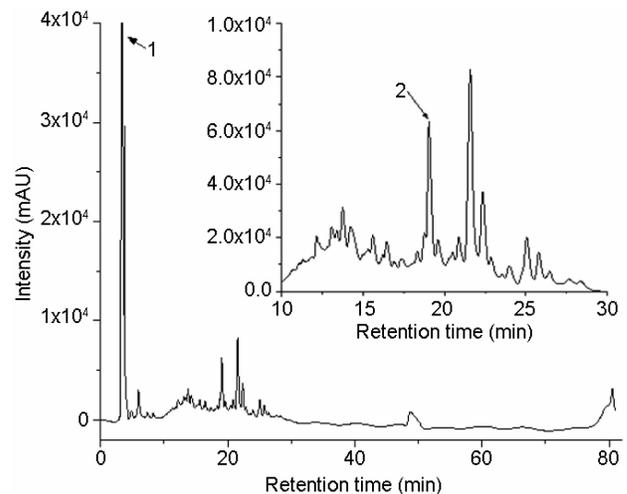


Fig. 1—HPLC chromatogram of CLME. Inset shows expanded region of the chromatogram with retention time of 10-30 min. Peaks marked 1 (3.29 min) and 2 (19.06 min) signify the retention peaks of tannic acid and catechin, respectively.

Table 1—Phytochemical screening of *C. cajan* leaf extract

Phytochemicals	Carbohydrates	Alkaloids	Tannins	Terpenoids & Triterpenoids	Anthraquinones	Saponins	Glycosides
Qualitative	+	+	+	+	-	-	-
Quantitative	8.62 ± 0.05 mg/100 mg extract glucose equivalent	55.21 ± 0.78 mg/100 mg extract reserpine equivalent	3.32 ± 0.15 mg/100 mg extract catechin equivalent	--	--	--	--

treatment with CLME in a fashion as good as the standard (Table 2). The increased level of TBARS, the end product of lipid peroxidation in liver as a result of oxidative stress, was significantly decreased by CLME (Table 2). The levels of protein carbonyl, a marker of protein oxidation, were diminished gradually with increasing dose of the extract from the augmented level as observed in C group, with the

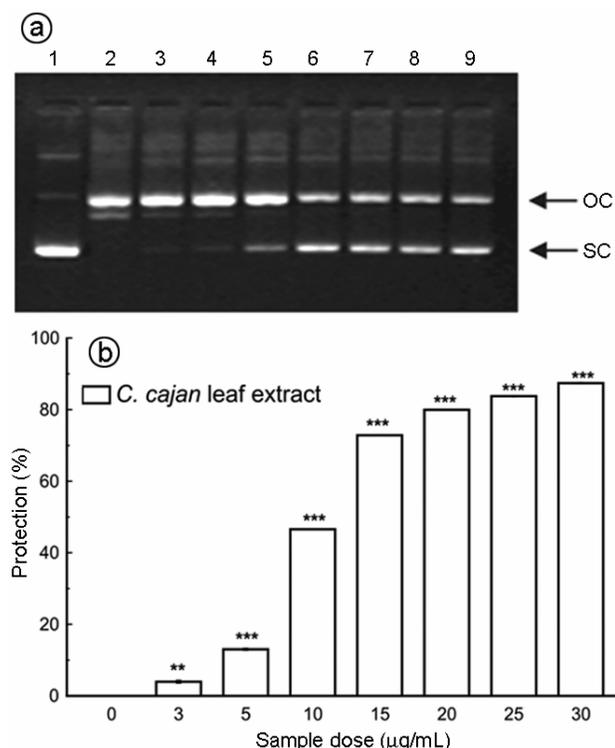


Fig. 2—Protection against oxidative damage to pUC18 by CLME. ***P* values: < 0.01, *** < 0.001 vs 0 µg/mL.

activities of the higher two doses being better than that of the standard (Table 2). Hydroxyproline content, representing collagen content as a result of liver fibrosis, was high in the iron-overloaded mice; but the contents lowered with oral treatment of gradually increasing concentration of CLME.

Effect on liver antioxidants—The activities of antioxidant enzymes SOD, CAT, GST and the levels of non-enzymic antioxidant GSH were significantly decreased in iron overloaded control mice compared to normal mice (Table 3). The levels of the antioxidants were significantly increased in the iron-overloaded mice orally treated with CLME in a dose-dependent manner. In all the studies, it was found that the highest dose of the extract approached the activity near about that of the standard oral iron chelator drug desirox, especially in the cases of GST and GSH.

Effect on serum marker levels of hepatocellular injury—The abnormal high levels of serum ALAT, ASAT, ALP and bilirubin denoted the damage to the hepatic cells. Iron induced liver injury resulted in the significant increase in ALAT, ASAT, ALP and bilirubin, by more than 100% and higher (Table 4). Oral administration of CLME markedly reduced the elevated levels of serum enzymes and bilirubin of iron overloaded mice to approach the normal control values. It is also apparent from Table 4 that significant increase of serum ferritin level in iron loaded mice is substantially reduced dose dependently with the same treatment.

Release of redox iron from ferritin—Reductively released iron from ferritin by CLME was quantitated by time dependent measurement of the formation of

Table 2—Effect of *C. cajan* leaf extract on iron accumulation, lipid peroxidation, oxidative stress for liver toxicity and liver fibrosis levels [Values are mean ± SD from 6 animals in each group. Figures in parentheses are % change.]

Treatment	Liver iron (µg/g)	TBARS (µM/mg protein)	Protein carbonyl (µM/mg protein)	Hydroxyproline (mg/liver)
B	43.43 ± 2.58	1.29 ± 0.15	3.77 ± 0.41	4.32 ± 1.00
C	117.21 ± 4.57 ^{X3} (169.90)	2.64 ± 0.07 ^{X3} (104.64)	8.89 ± 0.46 ^{X3} (135.82)	18.12 ± 0.93 ^{X3} (318.99)
S50	107.64 ± 3.04 ^{X3Y2} (147.87)	2.50 ± 0.24 ^{X3} (93.71)	8.26 ± 0.37 ^{X3Y1} (119.16)	15.87 ± 0.69 ^{X3Y2} (267.11)
S100	104.04 ± 4.70 ^{X3Y1} (139.59)	2.45 ± 0.06 ^{X3Y2} (90.19)	6.72 ± 0.26 ^{X3Y3} (78.34)	15.62 ± 0.56 ^{X3Y3} (261.16)
S200	90.20 ± 3.62 ^{X3Y3} (107.71)	2.27 ± 0.09 ^{X3Y3} (75.99)	6.60 ± 0.35 ^{X3Y3} (75.12)	14.34 ± 0.77 ^{X3Y3} (231.77)
D	90.46 ± 4.78 ^{X3Y3} (108.30)	2.24 ± 0.06 ^{X3Y3} (73.80)	7.30 ± 0.47 ^{X3Y2} (93.70)	12.33 ± 0.29 ^{X3Y3} (185.19)

X: significant difference from normal mice (B) group (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); TBARS—thiobarbituric acid reactive substances

ferrous complex of ferrozine, $[\text{Fe}(\text{ferrozine})_3]^{2+}$, (Fig. 3). The time course of the experiments with plant extract showed gradually increasing formation of $[\text{Fe}(\text{ferrozine})_3]^{2+}$, in comparison to the control experiments. The release of iron from ferritin by CLME furthermore compared to its reducing power produced a significantly ($P < 0.001$) positive correlation between the two activities (Fig. 4). The high value of the correlation coefficient (R) signified that higher reducing power of CLME reductively released higher amount of ferritin iron.

Histopathologic observations—The sections of livers obtained from control rats showed the characteristic hepatic architecture of the normal cell morphology with well-preserved cytoplasm (Fig. 5A),

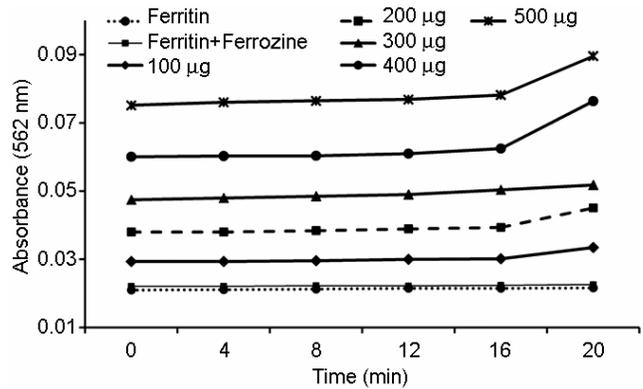


Fig. 3—Dose dependent (100-500 µg) formation of the $[\text{Fe}(\text{ferrozine})_3]^{2+}$ complex following release of Fe^{2+} from ferritin by CLME with time (0-20 min).

Table 3—Effect of *C. cajan* leaf extract on intrinsic liver antioxidants' levels
[Values are mean ± SD from 6 animals each. Figures in parentheses are % change.]

Treatments	SOD Unit/mg protein	CAT Unit/mg protein	GST Unit/mg protein	GSH µg/mg protein
B	0.64 ± 0.09 ---	21.97 ± 1.50 ---	8.15 ± 0.79 ---	0.48 ± 0.02 ---
C	0.08 ± 0.6 ^{X3} (87.03)	9.89 ± 0.84 ^{X3} (54.98)	1.88 ± 0.55 ^{X3} (76.93)	0.31 ± 0.02 ^{X3} (36.49)
S50	0.12 ± 0.04 ^{X3} (81.25)	9.90 ± 0.21 ^{X3} (54.94)	3.30 ± 0.11 ^{X3} (59.50)	0.33 ± 0.04 ^{X3Y2} (31.45)
S100	0.13 ± 0.07 ^{X3} (80.39)	10.36 ± 0.65 ^{X3Y2} (52.84)	3.80 ± 0.69 ^{X3Y3} (53.35)	0.37 ± 0.02 ^{X3Y2} (22.95)
S200	0.13 ± 0.07 ^{X3} (80.45)	10.60 ± 0.55 ^{X3} (51.75)	3.77 ± 0.81 ^{X3Y2} (53.75)	0.40 ± 0.03 ^{X2Y3} (18.32)
D	0.40 ± 0.07 ^{X3Y2} (38.28)	18.97 ± 0.81 ^{X1Y3} (13.65)	3.13 ± 0.53 ^{X3Y1} (61.57)	0.39 ± 0.05 ^{X3Y3} (18.88)

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: $P < 0.001$); SOD-superoxide dismutase; CAT-catalase; GST-glutathione S-transferase; GSH-reduced glutathione

Table 4—Effect of *C. cajan* leaf extract on serum marker levels of hepatocellular injury:
[Values are mean ± SD from 6 animals. Figures in parentheses are % change.]

Treatment	ALAT (Unit/L)	ASAT (Unit/L)	ALP (Unit/L)	Bilirubin (Unit/L)	Serum ferritin (µg/g protein)
B	21.50 ± 3.86 ---	25.72 ± 2.79 ---	105.36 ± 12.39 ---	1.27 ± 0.10 ---	39.48 ± 3.90 ---
C	49.47 ± 7.63 ^{X1} (130.14)	57.29 ± 6.53 ^{X1} (122.70)	341.46 ± 8.41 ^{X2} (224.09)	5.01 ± 0.47 ^{X2} (293.26)	136.04 ± 3.90 ^{X3} (244.57)
S50	45.94 ± 1.56 ^{X1} (113.72)	55.27 ± 2.09 ^{X2} (114.86)	322.86 ± 3.53 ^{X2Y1} (206.42)	4.94 ± 0.26 ^{X2} (287.60)	107.71 ± 13.52 ^{X3Y1} (172.83)
S100	37.33 ± 1.89 ^{X1} (73.65)	53.78 ± 1.37 ^{X2} (109.05)	322.55 ± 15.10 ^{X2} (206.13)	4.66 ± 0.09 ^{X3} (265.50)	83.25 ± 8.50 ^{X3Y3} (110.88)
S200	36.70 ± 2.71 ^{X1} (70.76)	51.20 ± 2.12 ^{X2} (99.02)	307.55 ± 13.78 ^{X2} (191.89)	4.46 ± 0.12 ^{X3} (250.13)	53.64 ± 6.25 ^{X1Y3} (35.87)
D	23.55 ± 1.39 ^{Y1} (9.57)	44.67 ± 5.52 ^{X1} (73.68)	151.58 ± 9.92 ^{X1Y2} (43.87)	1.59 ± 0.12 ^{X2Y2} (25.34)	54.48 ± 1.80 ^{X2Y3} (37.99)

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: $P < 0.001$); ALAT-alanine aminotransferase; ASAT-aspartate aminotransferase; ALP-alkaline phosphatase

whereas that of iron overloaded group exhibited generalized hepatic necrosis, ballooning degeneration, severe periportal fibroplasias and neutrophilic cellular infiltration (Fig. 5B). CLME treated mice revealed marked reduction in hepatic lesions. Histopathological alterations were substantially removed in CLME treated groups (Fig. 5C-E) thus

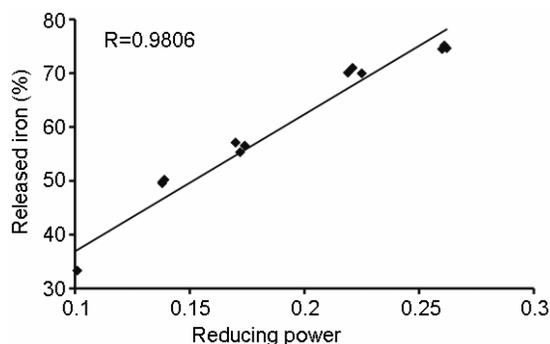


Fig. 4—Correlation of reducing power versus released ferritin iron (%) by CLME.

presenting protection against iron overload induced hepatic damage. The liver sections prepared from desirox treated group showed that the liver tissue restored its structure to almost the normal picture with few inflammatory cell infiltrations in between (Fig. 5F).

Discussion

Iron mediated uncontrolled overproduction of free radicals gives rise to a wide spectrum of pathogenic conditions that is not always effectively antagonized by the intrinsic physiological antioxidant system. On the other hand, excess iron presence in body systems due to ominous xenobiotics and dietary overload gives rise to hepatic siderosis, resulting free radical generation, thus forming the vicious cycle.

The present study demonstrated the protective effect of 70% methanolic extract of *Cajanus cajan* leaf 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

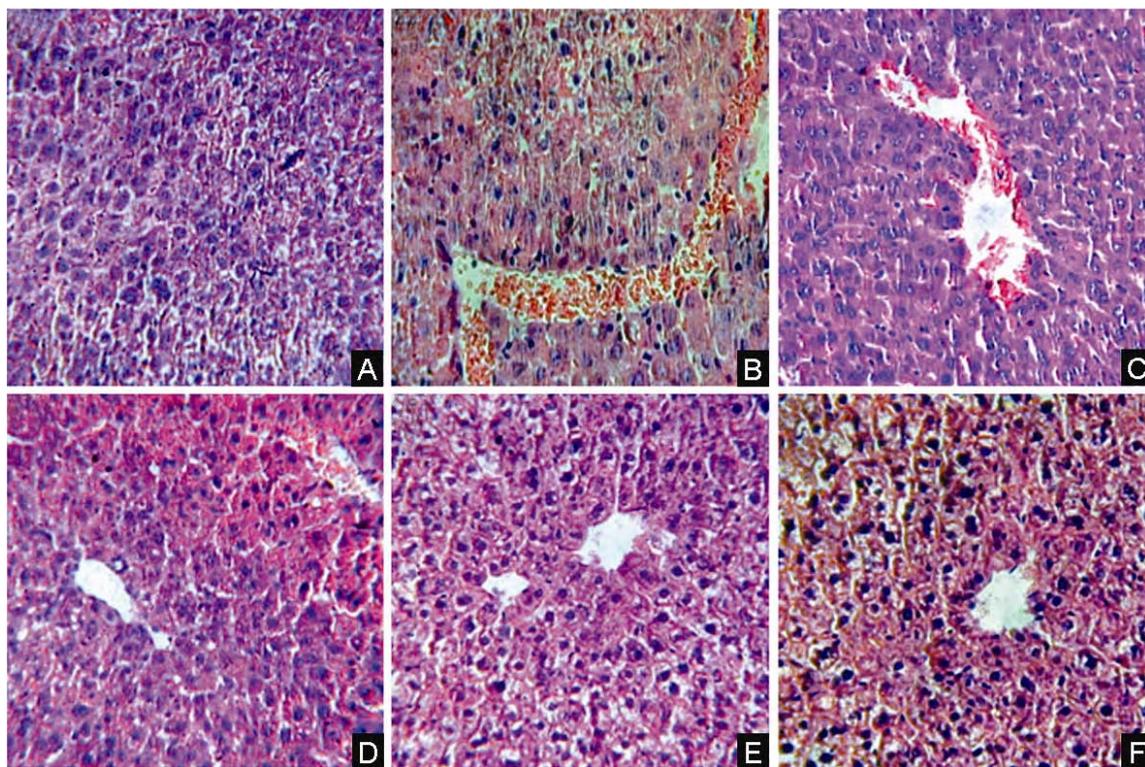


Fig. 5—Photomicrograph of H&E (haematoxylin and eosin) stained mice liver sections at x40; (A) Normal mice liver. (B) Iron overloaded (iron-dextran, 100 mg/kg b.w.) liver section showing fatty change, ballooning degeneration and necrosis in most of the hepatic parenchyma. (C) Liver section treated with iron-dextran + 50 mg/kg b.w. CLME. (D) Liver section treated with iron-dextran + 100 mg/kg b.w. CLME. (E) Liver section treated with iron-dextran + 200 mg/kg b.w. CLME. All three groups (C), (D) and (E) shows marked reduction in hepatic lesions with reduced hepatocellular necrosis dose dependently. (F) Liver section treated with iron-dextran + 20 mg/kg b.w. desirox also shows that the liver tissue restored its structure to almost the normal picture with few inflammatory cell infiltrations in between.

components present in the plant are needed to be well extracted in a medium of moderate polarity, e.g., methanol-water (70:30) system, such that the most effective extraction of the polar as well as non-polar compounds from the plant material can take place. Moreover, with the removal of residual methanol from the extract upon evaporation under reduced pressure and subsequent lyophilization, the results obtained are devoid of the effect of methanol. As is also evident from the phytochemical screening and the HPLC standardization of the sample, the likely presence of bioactive compounds also justifies the procedure for extraction of the plant material.

Various dose- and time-dependent studies showed optimum protective activity of the extract against hepatic oxidative damages. For most of the parameters studied, the values retained significantly the normal levels as achieved by treatment with standard drug desirox, after oral treatment with CLME. The extract has also been shown to possess free-radical scavenging activity, which can be attributed to the phenolic and flavonoid contents of the plant^{10,14,15}. The *in vitro* studies revealed effective scavenging of DPPH radical by CLME alongside the ability to dose-dependently protect pUC-18 DNA scission. This observation concretizes the anti-oxidative ability of the plant extract.

Intraperitoneal iron injection, to resemble *in vivo* hemochromatosis, is meant to avoid direct interruption of plant extract on intestinal iron absorption. The oral administration of CLME significantly reduced hepatic iron content compared to iron overloaded control group, thus supporting its iron chelating potency. Iron mediated lipid peroxidation is an initial step by which iron causes cellular injury. In the present study, the CLME has been found to be an effective lipid peroxidation inhibitor, which is corroborated by corresponding decrement in diffused ballooning degeneration found from histopathological studies. A significant factor in the pathogenesis of iron overload disease, iron mediated protein oxidation is successfully reduced by CLME more effectively than the standard desirox, as reflected in lowering of protein carbonyl level. Similarly, treatment with CLME significantly reduced hydroxyproline content, which is the marker of hepatic fibrogenesis. Correspondingly, the liver sections of the extract treated mice from histological data showed revival from severe hepatic lesions and neutrophilic cellular infiltrations thus gradually approaching normal cytoarchitecture with increasing

CLME concentration per kg body weight. Added to that, the present results suggest that CLME arrested the iron induced depletion of endogenous cellular antioxidants and thus reduced the iron overload damage, in a comparable manner with that of standard desirox, if not better.

Iron overloaded hepatic damage induces leakage of cellular enzymes into the bloodstream, resulting increased levels of serum ALAT, ASAT, ALP and bilirubin³³. Dose dependent decrease in their levels along that of with serum ferritin, which raises with increment of liver iron content, in CLME treated mice signified the iron releasing capacity of the plant extract. The results from CLME treated mice approached the values achieved in those treated with standard iron chelator drug desirox. Ferritin, a ubiquitous intracellular protein, stores iron in a non-toxic form since no physiological mechanism can remove toxic levels of iron³⁴. Since inaccessibility to ferritin-bound iron is the one drawback of most iron chelators used, chelation therapy depends on reductive release of iron from ferritin, which is successfully done *in vitro* by CLME. Moreover, the previously determined reducing capability of CLME¹⁵ has been found to be significantly correlated to the ferritin iron release capacity of the extract.

Conclusively, the present results suggest that oxidative stress in iron-overloaded induced liver damage could be prevented by 70% methanolic extract of *Cajanus cajan* leaf. This activity along with the free radical scavenging potential of the plant extract can be attributed to its significant phytochemical constituents. Indeed, to have a clear picture of the aforementioned activity, further investigation is needed to study the partaking mechanism, which is in progress.

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