Protective Effect of a 43 kD Protein from the Leaves of the Herb, *Cajanus indicus* L on Chloroform Induced Hepatic-disorder

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Cajanus indicus is a herb with medicinal properties and is traditionally used to treat various forms of liver disorders. Present study aimed to evaluate the effect of a 43 kD protein isolated from the leaves of this herb against chloroform induced hepatotoxicity. Male albino mice were intraperitoneally treated with 2 mg/kg body weight of the protein for 5 days followed by oral application of chloroform (0.75 ml/kg body weight) for 2 days. Different biochemical parameters related to physiology and pathophysiology of liver, such as, serum glutamate pyruvate transaminase and alkaline phosphatase were determined in the murine sera under various experimental conditions. Direct antioxidant role of the protein was also determined from its reaction with Diphenyl picryl hydrazyl radical, superoxide radical and hydrogen peroxide. To find out the mode of action of this protein against chloroform induced liver damage, levels of antioxidant enzymes catalase, superoxide dismutase and glutathione-S-transferase were measured from liver homogenates. Peroxidation of membrane lipids both *in vivo* and *in vitro* were also measured as malonaldehyde. Finally, histopathological analyses were done from liver sections of control, toxin treated and protein pre- and post-treated (along with the toxin) mice. Levels of serum glutamate pyruvate transaminase and alkaline phosphatase, which showed an elevation in chloroform induced hepatic damage, were brought down near to the normal levels with the protein pretreatment. On the contrary, the levels of antioxidant enzymes such as catalase, superoxide dismutase and glutathione-S-transferase that had gone down in mice orally fed with chloroform were significantly elevated in protein pretreated ones. Besides, chloroform induced lipid peroxidation was effectively reduced by protein treatment both *in vivo* and *in vitro*. In cell free system the protein effectively quenched diphenyl picryl hydrazyl radical and superoxide radical, though it could not catalyse the breakdown of

hydrogen peroxide. Post treatment with the protein for 3 days after 2 days of chloroform administration showed similar results. Histopathological studies indicated that chloroform induced extensive tissue damage was less severe in the mice livers treated with the 43 kD protein prior and post to the toxin administration. Results from all these data suggest that the protein possesses both preventive and curative role against chloroform induced hepatotoxicity and probably acts by an anti-oxidative defense mechanism.

Keywords: Antioxidant, Hepatotoxin, Hepatoprotection, Liver damage, Oxidative stress, 43 kD protein

Introduction

Herbal plants have been recently popularized in modern medicine, since many therapeutically important compounds are derived from them. Extracts prepared from these plants are used in the treatment of liver diseases like hepatitis, cirrhosis etc. *Cajanus indicus* is one such plant, which is used extensively in folklore medicine to treat hepatic disorders (Chopra *et al.*, 1986). The aqueous extract of this plant is used to treat several forms of liver disorders, including jaundice and hepatomegaly (Kirtikar and Basu, 1935; Ghosh and Biswas, 1973). Despite of being aware of its beneficial actions on liver, the exact mode of action of this plant as a hepatoprotective agent is still not known. Moreover, therapies could not be devised due to insufficient standardization of the plant extract. In our laboratory, a 43 kD protein has been isolated and purified to homogeneity from the leaves of this herb and is believed to be its major hepatoprotective component. This protein has been found to be effective in reducing hepatotoxicity induced by toxins such as carbon tetrachloride, thioacetamide etc (Sarkar *et al.*, 2005).

Among the various halogenated toxicants, chloroform (CHCl₃) is one of the most well known liver damaging agents (Kluwe *et al.*, 1978; Smith *et al.*, 1983). It finds its way into

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our body through chlorine-contaminated drinking water. CHCl_3 acts as a central nervous system depressant and at large doses causes anesthesia, analgesia and necrosis (Arena and Drew, 1986). The organs in our body that are worst affected are liver and kidney (Larson *et al.*, 1993). The acute toxicity demonstrated by CHCl_3 is due to its bio-transformation to a nucleophilic bi-functional metabolite phosgene that reacts with glutathione to form diglutathionyl dithiocarbonate or is directly metabolized to carbon dioxide and chloride free radicals (Constan *et al.*, 1999). Cytochrome P450-2E1 is the enzyme that catalyses the conversion process. Phosgene, formed in the biotransformation process of CHCl_3 binds covalently to tissue macromolecules resulting in impairment of cellular vital function. CHCl_3 , like most other liver damaging agents, such as acetaminophen, alcohol or viral hepatitis release reactive oxygen species (ROS) at the site of injury (Stevens and Anders, 1981; Tomasi *et al.*, 1985). At the molecular level, the stress caused by ROS is implicated by the activation of several inflammatory cytokines such as tumor necrosis factor- α (TNF- α), interleukin-1, arachidonic acid, metabolites such as thromboxane A_2 , prostaglandins and leukotrienes (Ljungman *et al.*, 1991). Since CHCl_3 toxicity is basically mediated by free radicals, therefore free radical scavengers such as dimethyl sulphoxide (DMSO) and amino-benzotriazole (ABT) apart from Vitamin-E and Vitamin-C have been in use in CHCl_3 insults (Achdume, 1991).

Present study investigated the efficacy of the homogeneously prepared 43 kD protein against CHCl_3 induced liver damage *in vivo*. The hepatic damage caused by CHCl_3 and the reverse role played by the protein were evaluated by determining the i) levels of the enzymes maintaining liver integrity such as serum glutamate pyruvate transaminase (serum GPT) and alkaline phosphatase (ALP); ii) levels of the antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione-S- transferase (GST) and iii) by measuring the level of *in vivo* and *in vitro* hepatic malonaldehyde (MDA). Moreover, direct antioxidant potential of this protein was determined from its ability to scavenge diphenyl dipicryl hydrazyl (DPPH) radical and superoxide (O_2^-) radical and also by its ability to react with hydrogen peroxide (H_2O_2). In addition, histopathological studies were conducted from liver sections of a) normal mice, b) mice treated with CHCl_3 , and c) treated with protein prior and post to CHCl_3 administration to assess the ultrastructural changes of the liver.

Materials and Methods

Material. *Cajanus indicus* is a shrub belonging to the family leguminosae and, sub family papilionacea. Fresh plant leaves were obtained from Bose institute Experimental farm.

Chemicals. Kits for the measurement of serum GPT and ALP were purchased from Span diagnostics Ltd., India. CHCl_3 was purchased from Sisco research laboratories (SRL), India. Beside this Sodium pyrophosphate, Collagenase type I, Dulbecco's modified eagle's

medium (DMEM), Fetal bovine sera (FBS) and DPPH radical, purified catalase (from murine liver) and superoxide dismutase (from bovine liver) enzymes were made available from Sigma chemical Co. All other chemicals used in the study were of analytical grade of highest quality.

Animals. Swiss albino male mice of body wt 25 ± 2 g were used in the experiments. The animals were kept for two weeks prior to the experiment to acclimatize with the lab conditions. All of them had free access to standard diet and water ad libitum. The study was conducted in conformity with standard experimental animals study ethical protocols.

Preparation of homogeneous protein from the leaves of *Cajanus indicus*. The protein was purified from the leaves of young *Cajanus indicus* (manuscript submitted for publication). The leaves were homogenized in 20 mM tris-HCl buffer, pH 7.4 and the clear homogenate was saturated with 60% ammonium sulphate. The pellet was reconstituted and dialysed in Tris-HCl buffer, passed through DEAE Sephadex column and eluted in linear gradient of 0-1 M NaCl in tris buffer. The fraction showing hepatoprotective activity was desalted, concentrated and applied on a Sephadex G-50 column. The protein fraction showing biological activity was finally subjected to reverse phase chromatography on a C-18 hydrophobic column attached to HPLC. The molecular weight of this fraction was determined on a SDS polyacrylamide gel.

Protein estimation. Protein concentration was measured according to the method of Bradford (Bradford MM, 1976) using crystalline bovine serum albumin as standard.

Liver injury models. In order to compare the efficacy of the hepatoprotective protein in providing protection and cure against CHCl_3 induced liver damage, we divided the experimental mice broadly into two groups. The first group is the pre-treatment (protective) group and the second group is the post-treatment (curative) group.

Protective group. The pretreatment group was divided into 3 sub-groups each consisted of six mice. The first one served as corn oil control and received 0.75 ml/kg body wt of corn oil for 5 days; mice in the 2nd group were fed orally with 0.75 ml/kg body wt of corn oil for 5 days followed by 0.75 ml/kg body wt of CHCl_3 in corn oil at 1 : 1 ratio for 2 days and the mice in the 3rd were intraperitoneally injected with the protein at a dose of 2 mg/kg body wt for 5 days followed by oral feeding of CHCl_3 for two days. Mice groups were then sacrificed after 48 h of CHCl_3 administration since hepatic necrosis caused by CHCl_3 becomes visible as early as 10 h and reaches peak at 48 h (Lind *et al.*, 2000). Blood and liver samples were collected separately for each animal. A positive control group was kept in which six mice were pretreated with 200 mg/kg body wt of Vitamin E for 5 days (Gokalp *et al.*, 2003) followed by CHCl_3 treatment as in the protein treated models.

Curative group. It is known that liver can regenerate itself after two days of toxin injury (Thakore and Mehendale, 1994; Soni *et al.*, 1999). To ascertain whether the protein can cause the healing of the liver faster than its natural regeneration, a time course study was

conducted where six mice in each group were injected with the protein for 1, 2, 3 and 4 days after the administration of a single dose of the toxin for 2 days. Mice were sacrificed after 2nd, 3rd, 4th and 5th day respectively. In another set, six mice in each group were treated with the toxin only for 2 days and sacrificed after 2nd, 3rd, 4th and 5th day. In addition, six mice were kept as normal control.

Assessment of liver functions. Blood samples collected from puncturing mice heart were kept overnight to clot and then centrifuged at $3,000 \times g$ for 10 min. Serum GPT was measured by 2,4 dinitro phenyl hydrazine (DNPH) method of Rietman and Frankel (Rietman and Frankel, 1957) and ALP was estimated by Kind and King's method (Kind and King, 1954).

Determination of antioxidant activity in cell-free systems

Quenching of DPPH radical. The free radical activity of the protein was measured using the DPPH radical (Blois, 1958). The DPPH radical in ethanol (100 mM, 2 ml) was added to 2 ml of various concentrations (5-50 mg/ml) of the protein. The solution was then shaken vigorously and held for 30 min at room temperature in dark. The decrease in absorbance of DPPH was measured at 517 nm. Ethanol was used as the blank solution and DPPH solution in ethanol was used as control. Percent inhibition was calculated by comparing the absorbance values of the control and the protein.

Scavenging effect on H₂O₂. For the assay, different concentration of the protein (0.001 mg/ml, 0.005 mg/ml, 0.01 mg/ml, 0.05 mg/ml) was mixed separately with 700 μ l, 5 mM H₂O₂ and incubated at 37°C. Disappearance of peroxide was observed at 240 nm for 10 min. 1 unit of CAT activity is that which reduces 1 μ mole of H₂O₂ per minute. Purified catalase (50 mU/ml) was used as a standard.

Scavenger effect on O₂⁻ radical. Superoxide anion was generated *in vitro* as described by Paoletti (Paoletti *et al.*, 1986). The assay mixture contained in a total volume of 1 ml, 100 mM triethanolamine-diethanolamine buffer (pH 7.4), 3 mM NADH, 25 mM/12.5 mM EDTA/MnCl₂, 10 mM β -mercaptoethanol, the 43 kD protein of different concentrations (0.001 mg/ml, 0.005 mg/ml, 0.01 mg/ml, 0.05 mg/ml). After 20 min incubation at 25°C, the decrease in absorbance was measured at 340 nm. Purified SOD (80 mU/ml) was used as a standard.

Estimation of lipid peroxidation products

In vivo (from liver homogenates). Lipid peroxidation in terms of thiobarbituric acid reactive substances (TBARS) formation, was measured using the method of Esterbauer and Cheeseman (Esterbauer and Cheeseman, 1990). TBARS concentration of the samples were calculated using the extinction coefficient of MDA which is $1.56 \times 10^{-5} \text{ M}^{-1} \text{ cm}^{-1}$ since 99% of TBARS exists as MDA.

In vitro (from hepatocytes). Hepatocytes were isolated from mice liver (Malhi *et al.*, 2002) by the perfusion technique with collagenase type I at 37°C. The cells were suspended in DMEM containing FBS and the suspension was adjusted to obtain $\sim 2 \times 10^6$ cells/ml. About 1 ml of hepatocyte suspension ($\sim 2 \times 10^6$ cells) was incubated with the protein in varying concentrations (0.001 mg/ml, 0.01 mg/ml and 0.1 mg/ml) for different sets of experiment for 30 min. CHCl₃ (10 mM) was then added and incubated for another 30

min. The normal control cells were kept in culture medium only. The toxin control was done by incubating the hepatocytes with CHCl₃ (10 mM) for 30 min. All the experiments were done in triplicates under the same conditions. For positive control, hepatocytes were incubated with α -tocopherol (Vitamin E at a concentration of 1 mg/ml) and then exposed to CHCl₃. Incubation was executed at 37°C with gentle shaking and at the end of the incubation period, hepatocyte suspensions were collected to assess the cell damage. Hepatocyte suspensions were centrifuged at $60 \times g$ and membrane lipid peroxidation was measured from the precipitate by a colorimetric reaction with thiobarbituric acid (TBA) (Esterbauer and Cheeseman, 1990).

Determination of the levels of anti-oxidant enzymes in the liver homogenates

Estimation of CAT. The enzyme CAT converts hydrogen peroxide formed via the action of SOD on superoxide radical into water. The activity was measured in liver homogenates by the method of Bonaventura (Bonaventura *et al.*, 1972).

Estimation of SOD. The activity of SOD was assayed following the method originally developed by Nishikimi (Nishikimi *et al.*, 1972) and then modified by Kakkar (Kakkar *et al.*, 1984).

Estimation of GST. GST catalyses the conjugation reaction with glutathione in the first step of mercapturic acid synthesis. It was measured according to the method of Habig and Jakoby (Habig and Jakoby, 1974).

Liver histopathology. Liver specimens from all the experimental groups were fixed in 10% buffered formalin and were processed for paraffin sectioning. Sections of about 5 μ m thickness were stained with haematoxylin and eosin to study the general structure of the liver.

Statistical analysis. All the values are represented as mean $\pm$ S.D (n = 6). Student's *t*-test was applied for detecting the significance of difference between groups. P values of 0.05 or less were considered significant.

Result

SDS-PAGE of the purified protein from the leaves of *Cajanus indicus*. Fig. 1 shows the SDS-PAGE pattern of the final step purified hepatoprotective protein from *Cajanus indicus*. The protein was purified to homogeneity by 5 different steps including homogenization, (NH₄)₂SO₄ precipitation, ion exchange chromatography, gel filtration and high performance liquid chromatography. A single symmetrical band appeared in the region of 43 kD, indicating the homogenous preparation of the protein. Present study was carried out using this purified protein.

To study the protective role (pretreatment) of the protein a dose-dependent study was carried out. The hepatoprotective protein was applied intraperitoneally at a dose of 150 μ g/kg, 500 μ g/kg, 1 mg/kg, 2 mg/kg and 2.5 mg/kg body wt prior to

Fig. 1. SDS-PAGE pattern of the purified protein from the leaves of *Cajanus indicus* L. The protein appeared as a single band with an approximate molecular mass of 43 kD.

Fig. 2. Dose dependent effect of the protein pretreatment on GPT activity in blood serum against CHCl₃ induced hepatic damage. Normal: corn oil treatment at a dose of 0.75 ml/kg body wt of mice. CHCl₃: CHCl₃ administration at a dose of 0.75 ml/kg body wt in 1:1 ratio of corn oil. P(1)+CHCl₃, P(2)+CHCl₃, P(3)+CHCl₃, P(4)+CHCl₃ and P(5)+CHCl₃: protein administration at a dose of 150 mg/kg body wt, 500 mg/kg body wt, 1,000 mg/kg body wt, 2,000 mg/kg body wt and 2,500 mg/kg body wt respectively for 5 days prior to CHCl₃ administration. Vit E: Vitamin E administration at a dose of 250 mg/kg body wt. Each value represents mean ± SD (**p* < 0.05, *n* = 6).

chloroform administration. A concentration of 2 mg/kg body weight of the protein showed maximum hepatoprotective action as evidenced from its effect on the levels of serum GPT (Fig. 2) and antioxidant enzyme, CAT, as will be shown later. This concentration of the hepatoprotective protein has been used throughout the study.

Fig. 3. Time course study of the protein post-treatment on serum GPT values against CHCl₃ damage. Normal: corn oil treatment at a dose of 0.75 ml/kg body wt of mice. CHCl₃ (1), CHCl₃ (2), CHCl₃ (3), CHCl₃ (4) treated: CHCl₃ administration at a dose of 0.75 ml/kg body wt in 1:1 ratio of corn oil for 2 days and sacrificed after 1, 2, 3 and 4 days respectively. CHCl₃+P(1), CHCl₃+P(2), CHCl₃+P(3), CHCl₃+P(4): protein administration at a dose of 2 mg/kg body wt respectively for 1, 2, 3 and 4 days after CHCl₃ treatment. Each value represents mean ± SD (**p* < 0.05, *n* = 6).

As described previously (materials and methods) a time course study was followed with the post-treated mice. After receiving the toxin once for 2 days, the protein was administered for 1, 2, 3 and 4 days and sacrificed on 2nd, 3rd, 4th and 5th day respectively. Fig. 3 shows that maximum response was observed on serum GPT when the protein post treated mice were sacrificed on the fourth day. This is also true for liver CAT levels as will be shown later. Results for all the other studies were, therefore, presented for the fourth day.

Effect of the protein on serum GPT level. CHCl₃ treatment of the mice increased the serum GPT level (78 ± 2 units/ml serum) by approximately 2.3 fold than the normal level, (34 ± 2 units/ml of serum). Pretreatment of the protein for 5 days prior to CHCl₃ administration lowered the level to 43 ± 2 units/ml serum, while mice treated with Vitamin E effectively reduced the serum GPT level to 40 ± 2 units/ml serum. This observation is shown in the Fig. 2. As seen from the figure protein administration at a dose of 2 mg/kg body wt showed the best protective effect of the protein.

The curative effect of the protein on serum GPT level is shown in Fig. 3. After 48 hrs of CHCl₃ administration, the serum GPT value increases to 96 ± 3 units/ml serum from the normal level of 36 ± 2 units/ml serum. A single dose of protein after CHCl₃ administration had no effect on serum GPT level. Protein injected for 2 days after CHCl₃ administration reduced the serum GPT level to 75 ± 2 units/ml. Protein administration for 3 and 4 days after CHCl₃ administration

Fig. 4. Pretreatment. Effect of the protein pretreatment on ALP activity in blood serum against CHCl_3 damage. Normal: corn oil treatment at a dose of 0.75 ml/kg body wt of mice. CHCl_3 : CHCl_3 administered at a dose of 0.75 ml/kg body weight in 1:1 ratio of corn oil. P + CHCl_3 : protein administration at a dose of 2 mg/kg body wt for 5 days prior to chloroform administration. Vit E: Vitamin E administration at a dose of 250 mg/kg body wt. Post-treatment. Effect of the protein post-treatment on ALP activity in blood serum against CHCl_3 damage. Normal same as that of the pretreatment. CHCl_3 : CHCl_3 administered at a dose of 0.75 ml/kg body wt in 1:1 ratio of corn oil for 2 days, left untreated for 3 days and sacrificed next day. CHCl_3 + P: protein administration at a dose of 2 mg/kg body wt for 3 days after CHCl_3 treatment for 2 days and sacrificed next day. Each value represents mean $\pm$ SD (* p < 0.05, n = 6).

significantly reduced the serum GPT to 40 ± 1 units/ml and 38 ± 1 units/ml respectively than their respective CHCl_3 controls, which were 55 ± 2 units/ml and 51 ± 2 units/ml of serum. Protein post-treatment of mice for 3 days and sacrificed on the 4th day had the most prominent curative effect on CHCl_3 toxicity.

Effect of the protein on ALP level. The ALP level in the serum of the normal control, CHCl_3 and protein treated prior to CHCl_3 intoxication are shown in the Fig. 4. ALP levels in the serum of the CHCl_3 treated mice increased to 58 ± 2 KA units/ml of serum indicating severe liver damage. This value was approximately 3.5 times of the normal value of 17 ± 1 KA units/ml serum. Protein pretreatment significantly reduced the level to 33 ± 1 KA units/ml serum, while Vitamin E treatment reduced the ALP value to 40 ± 1 KA units/ml serum (Pretreatment).

Post-treatment in Fig. 4 shows the ALP level in mice treated with the protein for 3 days after toxin treatment for 2 days. Best results were obtained when the protein was given for 3 days. Mice sacrificed on 4th day after protein post treatment for 3 days showed serum ALP level within the normal range of 22 ± 1 KA units/ml serum.

Fig. 5. DPPH radical scavenging activity of the protein. The curve is obtained by plotting concentration of the protein (mg/ml) against percent inhibition of DPPH radical.

Fig. 6. Scavenging effect of the protein at different concentration on H_2O_2 . Blank: absorbance of H_2O_2 without any protein at 240 nm. P(1), P(2), P(3) and P(4): absorbance of H_2O_2 and protein at concentrations of 0.001 mg/ml, 0.005 mg/ml, 0.01 mg/ml and 0.05 mg/ml respectively. Std: absorbance of H_2O_2 and the purified catalase (50 mU/ml).

Effect of the protein on products of lipid peroxidation. Fig. 8 (Pretreatment) shows the *in vivo* level of MDA in the normal control, CHCl_3 treated, protein pretreated and Vitamin E fed mice. The increase in lipid peroxidation in CHCl_3 treated mice (68 ± 2 nmoles/gm liver tissue) was 2.4 fold over the control group (28 ± 1 nmoles/gm liver tissue). Mice pretreated with the protein for 5 days, with 2 mg/kg body wt of protein, showed significant reduction in the MDA level (24 ± 1 nmoles/gm tissue). The value of MDA obtained in the protein pretreated mice was even less than the normal controls.

Fig. 7. Scavenging effect of the protein at different concentration on O_2^- . P(1), P(2), P(3) and P(4): reaction of generated super oxide and protein at concentrations of 0.001 mg/ml, 0.005 mg/ml, 0.01 mg/ml and 0.05 mg/ml respectively. Std: absorbance of generated super oxide and the purified Superoxide dismutase (80 mU/ml). Results are expressed as percentage inhibition of NADH oxidation. Each value represents mean $\pm$ SD (* $p < 0.05$).

Fig. 8. Pretreatment. Effect of the protein pretreatment on MDA activity from liver homogenates in $CHCl_3$ induced damage. Normal: corn oil treatment at a dose of 0.75 ml/kg body wt of mice. $CHCl_3$: $CHCl_3$ administered at a dose of 0.75 ml/kg body wt in 1:1 ratio of corn oil for 2 days. P + $CHCl_3$: protein administration intraperitoneally at a dose of 2 mg/kg body wt for 5 days prior to $CHCl_3$ administration. Vit E: Vitamin E administration at a dose of 250 mg/kg body wt. Post-treatment. Effect of the protein post-treatment on MDA activity from liver homogenates in $CHCl_3$ induced damage. Normal same as that of the pretreatment. $CHCl_3$: $CHCl_3$ administered at a dose of 0.75 ml/kg body wt in 1:1 ratio of corn oil for 2 days, left untreated for 3 days and sacrificed next day. $CHCl_3$ + P: protein administration at a dose of 2 mg/kg body wt for 3 days after $CHCl_3$ treatment for 2 days and sacrificed next day. Each value represents mean $\pm$ SD (* $p < 0.05$, $n = 6$).

Fig. 9. Effect of the 43 kD protein on lipid peroxidation in hepatocytes. $CHCl_3$: hepatocytes exposed to $CHCl_3$ (10 mM) for 30 min. P1 + $CHCl_3$, P2 + $CHCl_3$ and P3 + $CHCl_3$: hepatocytes incubated with the protein (0.001 mg/ml, 0.01 mg/ml and 0.1 mg/ml concentration respectively) for 30 min and then exposed to $CHCl_3$ for another 30 min. Vit E + $CHCl_3$: hepatocytes first incubated with Vitamin E (1 mg/ml) and then with $CHCl_3$. Results have been shown as percent over control. Each value represents mean $\pm$ S.D. (* $p < 0.05$).

Fig. 8 (Post-treatment) shows the effect of the protein on normal control, $CHCl_3$ treated and protein post treated mice. Mice treated with the toxin for 2 days showed MDA values of 68 ± 2 nmol/gm tissue. Post treatment of mice with the protein after toxin treatment reduced the production of MDA to almost normal level of 37 ± 2 nmol/gm tissue. This is the effect of the protein observed when the mice were treated with the toxin (once for 2 days) + protein administered for 3 days and sacrificed on the 4th day.

Fig. 9 shows the effect of the protein on $CHCl_3$ induced lipid peroxidation in hepatocytes. Lipid peroxidation was increased significantly (110 %) in $CHCl_3$ treated hepatocytes over normal control cells indicating membrane damage. Protein treatment of (0.1 mg/ml) prior to $CHCl_3$ administration, significantly prevented that damage by reducing the MDA formation to a level even less than that of the control value.

Effect of the protein on CAT level. The dose dependent effect of the protein on CAT levels has been shown in Fig. 10. CAT activity in $CHCl_3$ treated mice was lower (40 ± 1 units/mg total protein) than that of normal (108 ± 3 units/mg total protein). Treatment of the mice with the protein prior to $CHCl_3$ treatment increased the CAT activity to 81 ± 3 units/mg total protein as compared to the $CHCl_3$ treated mice. The CAT activity in normal mice liver (109 ± 3 units/mg total protein) was reduced when treated with $CHCl_3$ (35 ± 1 units/mg total protein). As seen from the figure a dose of 2 mg/kg body wt of the protein showed the maximum hepatoprotective activity.

Fig. 10. Dose dependent effect of the protein pretreatment on CAT activity in liver samples against CHCl_3 damage. Normal: corn oil treatment at a dose of 0.75 ml/kg body wt of mice. CHCl_3 treated: CHCl_3 administration at a dose of 0.75 ml/kg body wt in 1:1 ratio of corn oil. P(1)+ CHCl_3 , P(2)+ CHCl_3 , P(3)+ CHCl_3 , P(4)+ CHCl_3 and P(5)+ CHCl_3 treated: protein administration at a dose of 150 mg/kg body wt, 500 mg/kg body wt, 1000 mg/kg body wt, 2000 mg/kg body wt and 2500 mg/kg body wt respectively for 5 days prior to CHCl_3 administration. Vit E: Vitamin E administration at a dose of 250 mg/kg body wt. Each value represents mean $\pm$ SD (* p < 0.05, n = 6).

Protein post-treatment for 1 and 2 days after chloroform application practically had no effect on the reduced CAT activity (Fig. 11). The CAT activity elevated to 84 ± 2 and 86 ± 4 units/mg total protein when the animals were post-treated with the protein for 3 and 4 days compared to their respective CHCl_3 controls (62 ± 2 and 76 ± 2 units/mg total protein). Fig. 11 shows that maximum effect of the protein was seen on levels of CAT when the protein post-treated mice were sacrificed on the fourth day.

Effect of the protein on SOD level. The levels of SOD in the liver homogenates of normal control, CHCl_3 and protein treated prior to CHCl_3 are shown in Pretreatment of Fig. 12. The SOD activity in CHCl_3 treated liver homogenates was reduced by 1.7 fold (223 ± 5 units/mg total protein) compared to normal (381 ± 7 units/mg total protein). Mice receiving the protein prior to the toxin showed an increase in SOD value of 329 ± 4 units/mg total protein, compared to CHCl_3 treated mice.

Mice post treated with the protein for 3 days showed SOD level of 283 ± 6 units/mg total protein, while its corresponding toxin control showed a reduced value of 217 ± 6 units/mg total protein (Post-treatment of Fig. 12).

Fig. 11. Time course study of the protein post treatment on CAT values in liver samples against CHCl_3 damage. Normal: corn oil treatment at a dose of 0.75 ml/kg body wt of mice. CHCl_3 (1), CHCl_3 (2), CHCl_3 (3), CHCl_3 (4) treated: CHCl_3 administration at a dose of 0.75 ml/kg body wt in 1:1 ratio of corn oil for 2 days and sacrificed after 1, 2, 3 and 4 days respectively. CHCl_3 + P(1), CHCl_3 + P(2), CHCl_3 + P(3), CHCl_3 + P(4): protein administration at a dose of 2 mg/kg body wt respectively for 1, 2, 3 and 4 days after CHCl_3 treatment. Each value represents mean $\pm$ SD (* p < 0.05, n = 6).

Effect of the protein on GST level. The normal mice showed a GST level of 17.0 ± 0.7 units/mg total protein. This was reduced by 4.0 fold in CHCl_3 treated mice (4.0 ± 0.1 units/mg total protein), while the level increased to 9.0 ± 0.1 units/mg total protein in mice treated with the protein prior to CHCl_3 feeding (Pretreatment of Fig. 13).

Post-treatment of Fig. 13 shows the effect of the protein on GST activity in liver upon its administration after CHCl_3 treatment for 2 days. After 3 days of protein treatment, the GST activity was elevated to almost normal level of 7.3 ± 0.1 units/mg total protein while its respective chloroform control showed a value of 4.0 ± 0.1 units/mg total protein.

Effect of the protein on liver histopathology. Liver samples were taken from normal, CHCl_3 treated and protein treated (both pre- and post-) mice. Histopathological assessment showed prominent changes including centrilobular necrosis, ballooning of hepatocytes, distribution of polymorphonuclear infiltrates surrounding the central vein, disorganization of normal radiating pattern of cell plates around central vein etc. in tissues treated with CHCl_3 (Fig. 14b). Qualitatively, the inflammation in protein treated mice liver before and after CHCl_3 administration were less severe compared to that of CHCl_3 (Fig. 14c and Fig. 14d respectively). These observations show the protective role of the 43 kD protein against CHCl_3 induced hepatic damage.

Fig. 12. Pretreatment. Effect of the protein pretreatment on SOD activity from liver homogenates in CHCl₃ induced damage. Normal: corn oil treatment at a dose of 0.75 ml/kg body wt of mice. CHCl₃: CHCl₃ administered at a dose of 0.75 ml/kg body wt in 1 : 1 ratio of corn oil. P + CHCl₃: protein administration at a dose of 2 mg/kg body wt for 5 days prior to CHCl₃ administration. Vit E: Vitamin E administration at a dose of 250 mg/kg body wt. Post-treatment. Effect of the protein post-treatment on SOD activity from liver homogenates in CHCl₃ induced damage. Normal as that of the pretreatment. CHCl₃: CHCl₃ administered at a dose of 0.75 ml/kg body wt in 1 : 1 ratio of corn oil for 2 days, left untreated for 3 days and sacrificed next day. CHCl₃ + P: protein administration at a dose of 2 mg/kg body wt for 3 days after CHCl₃ treatment for 2 days and sacrificed next day. Each value represents mean $\pm$ SD (* p < 0.05, n = 6).

Discussion

It is well documented that exposure of living beings to any toxic metabolite constitutes stress which results in a surge of free radicals (Testai *et al.*, 1995; Diez-Fernandez *et al.*, 1996; Abraham *et al.*, 1999). These free radicals can trigger cell damage through mechanisms of covalent binding to tissue macromolecules, enhanced lipid peroxidation and depleting stores of antioxidant enzymes. Reduced activity of one or more antioxidant enzymes leads to oxidative stress and damage to the liver (Kyle *et al.*, 1987; Kurose *et al.*, 1997). The preliminary effect of hepatotoxicity is the enhancement in the serum levels of GPT, serum glutamate oxaloacetate transaminase (serum GOT) and ALP, which are required for maintaining the integrity of the liver. When the hepatocyte membrane is damaged, these enzymes located normally in the cytosol, are released in the blood stream. Estimation of these enzymes in the serum is a useful quantitative marker for the extent of liver damage (Ansari *et al.*, 1991).

The present study reveals the hepatoprotective activity of

Fig. 13. Pretreatment. Effect of the protein pretreatment on GST activity from liver homogenates in CHCl₃ induced damage. Normal: corn oil treatment at a dose of 0.75 ml/kg body wt of mice. CHCl₃: CHCl₃ administration at a dose of 0.75 ml/kg body wt in 1 : 1 ratio of corn oil. P + CHCl₃: protein administration at a dose of 2 mg/kg body wt for 5 days prior to CHCl₃ treatment. Vit E: Vitamin E administration at a dose of 250 mg/kg body wt. Post-treatment. Effect of the protein post-treatment on GST activity from liver homogenates in CHCl₃ induced damage. Normal same as that of the pretreatment. CHCl₃: CHCl₃ administered at a dose of 0.75 ml/kg body wt in 1 : 1 ratio of corn oil for 2 days, left untreated for 3 days and sacrificed next day. CHCl₃ + P: protein administration at a dose of 2 mg/kg body wt for 3 days after CHCl₃ treatment for 2 days and sacrificed next day. Each value represents mean $\pm$ SD (* p < 0.05, n = 6).

the herbal protein purified from *Cajanus indicus* against CHCl₃ toxicity. Mice treated with CHCl₃ showed elevated levels of the serum enzymes such as serum GPT and ALP. In cell free systems the protein shows an unique scavenging activity by reacting with DPPH which is a synthetic radical and also with superoxide radical. Moreover CHCl₃ treatment also produced significant elevation in the hepatic MDA level (*in vivo* and *in vitro*) and a decline in antioxidant enzymes CAT, SOD and GST. Administration of the protein at a dose of 2 mg/kg body wt prior and post to CHCl₃ treatment, normalized (reduced almost to the normal controls) the values of serum GPT, ALP and MDA formation both *in vivo* and *in vitro* and uplifted the level of CAT, SOD and GST.

The DPPH radical, because of its odd electron gives a strong absorption band at 517 nm in visible spectroscopy. As this electron becomes paired off in presence of a radical scavenger, its absorption vanishes and the resulting decolourisation is stoichiometric with respect to the number of electrons taken up. The 43 kD protein at various concentration was able to quench the synthetic DPPH radical. Having established a concentration based radical scavenging activity of the protein we then proceeded to investigate the ability of

Fig. 14. (a) Histopathology of normal liver section stained with haematoxyline-eosin stain. CV indicates the central vein. (b) Histopathology of liver treated with CHCl_3 for 2 days and sacrificed on the 3rd day. (c) Histopathology of liver treated with the protein for 5 days and then treated with CHCl_3 for 2 days. (d) Histopathology of liver treated with CHCl_3 for 2 days and then treated with the protein for 3 days.

the protein to react with O_2^- and H_2O_2 , both of which are potentially cytotoxic. Results showed that the protein could not react with H_2O_2 and potentiate its breakdown to water. However it exhibited a SOD-like effect, inhibiting O_2^- formation in a dose dependent manner.

As mentioned previously (Stevens and Anders, 1981) CHCl_3 damages the liver via production of ROS. Our results also suggest that treatment of mice with CHCl_3 caused an overall decrease in the levels of the antioxidant enzymes such as CAT, SOD and GST. CAT and SOD belong to preventive and chain breaking class of antioxidants respectively. Both these enzymes work in a coordinated manner to quench the oxygen free radicals produced in our body. Decreased levels of CAT and SOD suggest once again that CHCl_3 exerts its toxic effect by causing oxidative stress. The antioxidant defense system in our body cannot cope up with the rate of generation of these free radicals, when their levels increase beyond the optimum range. As a result, these antioxidant enzymes get used up and their content falls below the normal level. However, treatment of mice with the protein by intraperitoneal means bring back the levels of CAT and SOD

to near normal levels. This may happen due to two reasons. The protein may act directly and scavenge the ROS derived by oxidation-reduction cycle within the cell or it may work in unison with the existing antioxidant enzymes and help prevent their loss during the oxidative injury caused by CHCl_3 .

GST is an enzyme that participates in the detoxification process due to conjugation reaction between GSH and xenobiotics (Adang *et al.*, 1990). It plays a critical role in protection against reactive free radicals and products of oxidative stress (Hayes and Pulford, 1995). As seen with CAT and SOD, GST activity was also reduced in mice treated with CHCl_3 indicating the use of the enzyme in combating ROS formed in an oxidative process. This 43 kD protein increased the level of GST, again suggesting its antioxidant role.

Apart from generation of ROS at the site of injury, CHCl_3 also acts as a direct hepatotoxin by disrupting tissue membranes. The commonly used indicator of tissue damage is the peroxidation of lipids on the membrane of liver in the form of aldehydic products such as MDA. Ability of CHCl_3 to cause free radical damage was indicated by the production of increased hepatic lipid peroxides measured as MDA in CHCl_3

induced hepatotoxicity. This finding is similar to the findings as reported by Masuda [Masuda *et al.*, 1980] that halomethanes like CHCl_3 or CCl_4 increased the levels of MDA content in liver. However, pretreatment as well as post treatment of mice *in vivo* with the protein mitigated this CHCl_3 induced lipid peroxidation. Treatment of mice with the protein, prior to chloroform application had a profound effect on lipid peroxidation products as it lowered the MDA level even below the normal range. Extent of lipid peroxidation in CHCl_3 treated hepatocytes also showed a similar reduction in MDA value, upon protein treatment. Result suggests that the protein plays a positive role in minimizing lipid peroxidation and act through an antioxidant mechanism.

Liver histology of CHCl_3 treated mice showed severe necrosis along the central vein. The sizes of the hepatocytes were also larger and balloon like compared to the normal mice liver. The necrosis was mostly centrilobular and extending through the whole liver lobule. On the other hand, the inflammations in the livers of both protein pre- and post-treated mice were less necrotic. These results again show the protective nature of the protein against the hepatic damage caused by CHCl_3 .

In most studies on xenobiotic induced hepatotoxicity, the protective agent has been shown to be able to protect the liver when administered prior to the toxicant. Our study however reveals that the protein has got equal potential as a hepatoprotective agent in both pretreated (preventive) and post treated (curative) mice. Time course study of the protein as a curative agent showed that the efficacy of the protein increased proportionally with the number of days, with the peak effect reaching on the 4th day. Thus the results showed that post treatment of the toxin treated mice with the protein showed an additive effect and it actually accelerates the self-healing process of the liver. In the time course study when the protein was initially administered for 1 and 2 days, subtle changes were observed relative to the CHCl_3 control mice in the levels of serum GPT and ALP, but there were practically no changes in the levels of the antioxidant enzymes. This finding shows that the protein may not be able to induce antioxidant mechanism of the liver quickly (when the protein is administered for 2 days only) as it does so with the liver specific serum marker enzymes. This fact is also revealed from another study done in our laboratory by taking thioacetamide as the toxin (Sarkar *et al.*, 2005).

In conclusion, we would like to mention that the probable mechanism by which the protein exerts its hepatoprotective action against CHCl_3 induced toxicity could be attributed to the enhanced supply of antioxidant enzymes or by the acceleration of detoxification and excretion. Taken together, the results of this present study demonstrate that the protein might have a therapeutic role in preventing CHCl_3 induced hepatotoxicity possibly through its free radical scavenging properties. The results encourage further *in vitro* and *in vivo* studies to elucidate its efficacy as a hepatoprotective molecule.

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