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Anti-oxidative Effect of a Protein from *Cajanus indicus* L against Acetaminophen-induced Hepato-nephro Toxicity

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Overdoses of acetaminophen cause hepato-renal oxidative stress. The present study was undertaken to investigate the protective effect of a 43 kDa protein isolated from the herb *Cajanus indicus*, against acetaminophen-induced hepatic and renal toxicity. Male albino mice were treated with the protein for 4 days (intraperitoneally, 2 mg/kg body wt) prior or post to oral administration of acetaminophen (300 mg/kg body wt) for 2 days. Levels of different marker enzymes (namely, glutamate pyruvate transaminase and alkaline phosphatase), creatinine and blood urea nitrogen were measured in the experimental sera. Intracellular reactive oxygen species production and total antioxidant activity were also determined from acetaminophen and protein treated hepatocytes. Indices of different anti-oxidant enzymes (namely, superoxide dismutase, catalase, glutathione-S-transferase) as well as lipid peroxidation end-products and glutathione were determined in both liver and kidney homogenates. In addition, Cytochrome P450 activity was also measured from liver microsomes. Finally, histopathological studies were performed from liver sections of control, acetaminophen-treated and protein pre- and post-treated (along with acetaminophen) mice. Administration of acetaminophen increased all the serum markers and creatinine levels in mice sera along with the enhancement of hepatic and renal lipid peroxidation. Besides, application of acetaminophen to hepatocytes increased reactive oxygen species production and reduced the total antioxidant activity of the treated hepatocytes. It also reduced the levels of antioxidant enzymes and cellular reserves of glutathione in liver and kidney. In addition, acetaminophen enhanced the cytochrome P450 activity of liver microsomes. Treatment with the protein significantly reversed these changes to almost normal. Apart from these, histopathological changes also revealed the protective nature of the protein against acetaminophen induced necrotic damage of the liver tissues. Results suggest that the protein protects hepatic and renal tissues against oxidative damages and could be used as an effective protector against acetaminophen induced hepato-nephrotoxicity.

Keywords: Acetaminophen, Cajanus indicus, Hepatic and renal damages, 43 kDa protein, Hepato-nephro protector, Oxidative stress

Introduction

Acetaminophen (APAP), also known as paracetamol is a commonly used analgesic and antipyretic drug. The drug is safe at therapeutic levels, but an acute APAP overdose can lead to potentially fatal hepatic and renal necrosis in humans and experimental animals (Proudfoot and Wright, 1970; Cobden *et al*., 1982; Thomas, 1993; Eguia and Materson, 1997). It is becoming clear that reactive oxygen (ROS) and nitrogen (RNS) species take an important part in the development of hepatotoxicity caused by APAP (Nakae *et al*., 1990; Michael *et al*., 1999; Knight *et al*., 2001). The initial step of its toxicity is cytochrome P450 (CYP) metabolism of APAP to the reactive intermediate N-acetyl-p-benzoquinone imine (NAPQI) (Dahlin *et al*., 1984). At therapeutic doses this metabolite is removed by conjugation with glutathione (GSH).

Abbreviations: APAP-Acetaminophen, ROS-Reactive oxygen species, RNS-Reactive nitrogen species, NAPQI-N-acetyl-p-benzoquinone imine, CYP-Cytochrome P450, GSH-Glutathione, GPT-glutamate pyruvate transaminase, ALP-Alkaline phosphatase, BUN-blood urea nitrogen, SOD-superoxide dismutase, CAT-catalase, GST-glutathione-S-transferase, EDTA- Ethylene diamine tetra acetic acid, PMT-Phenazine methosulphate, NBT-Nitro blue tetrazolium, CDNB- chloro dinitro benzoic acid, NADH-reduced nicotinamide adenine dinucleotide, DTNB-Dithio nitro benzoic acid, TPTZ-Tripyridyltriazine, H_2DCFDA-2',7'-dichlorodihydrofluorescein di acetate, TBARS-Thio barbituric acid reactive substances, FRAP-Ferric reducing antioxidant potential

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However at large doses of APAP, conjugation with GSH leads to its depletion (Mitchell *et al*., 1973). In case of nephrotoxicity tubular cell loss is the characteristic feature of both acute and chronic renal failure.

Studies are going on throughout the world for the search of protective molecules that would provide maximum protection of the liver, kidney as well as other organs and practically very little or no side effects would be exerted during their function in the body (Montilla *et al*., 2005; Mansour *et al*., 2006). A number of herbs are traditionally used in different countries during drug or toxin induced hepatic and renal disorders (El-Beshbishy, 2005). *Cajanus indicus* is one such medicinal plant that has been in use for a long time for treating jaundice and hepatomegaly (Kirtikar and Basu, 1935; Ghosh and Biswas, 1973). Work in our laboratory led to the isolation and purification of a 43 kDa protein molecule (Sarkar K *et al*., 2006) that has been found to be effective in reducing hepatotoxicity induced by toxins such as fluoride, galactosamine, cadmium chloride, chloroform, thioactamide, etc. both *in vivo* and *in vitro* (Sarkar *et al*., 2005; Ghosh *et al*., 2006; Ghosh and Sil, 2006; Sarkar and Sil, 2006; Manna *et al*., 2007a; Manna *et al*., 2007b; Sinha *et al*., 2007a; Sinha *et al*., 2007b; Sinha *et al*., 2007c).

The aim of this particular study was to compare the hepatotoxic potential of APAP with its nephrotoxic property and how both these toxicities can be allayed by the administration of this particular protein. The dose and time dependent effects of the protein in APAP induced toxicity was evaluated by determining the level of serum marker enzymes glutamate pyruvate transaminase (GPT). Alkaline phosphatase (ALP) creatinine and blood urea nitrogen (BUN) were also measured from the serum in all the experimental mice groups. In addition, intracellular ROS production and total antioxidant power was measured from APAP and protein (both pre and post) along with APAP treated hepatocytes. The activity of endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST) were measured from liver and kidney homogenates. Further the level of non-protein thiol, glutathione (GSH) was also estimated from hepatic and renal samples. In addition, the extent of lipid peroxidation was measured from both livers and kidneys of treated mice. Besides, the activity of CYP was measured from the liver microsomal fraction of APAP and protein treated mice. To assess the ultrastructural changes of the liver, histopathological studies were conducted from liver sections of normal mice, mice treated with APAP, and mice treated with protein prior and post to APAP. Throughout the study the hepato-nephroprotective property of the protein was compared to that of Vitamin C, a well-known antioxidative agent.

Materials and Methods

Chemicals. Kits for the measurement of serum GPT, ALP, BUN and creatinine were purchased from Span diagnostics Ltd., India. Sodium pyrophosphate, Bradford reagent, Bovine sera albumin (BSA), acetaminophen (APAP), 2',7'-dichlorodihydro fluorescien di-acetate (H_2-DCFDA) were made available from Sigma chemical company. Chemicals like ethylene diamine tetra acetic acid (EDTA), phenazine methosulphate (PMT), nitro blue tetrazolium (NBT), chloro dinitro benzoic acid (CDNB), nicotinamide adenine dinucleotide (NADH), dithio nitro benzoic acid (DTNB) and tripyridyltriazine (TPTZ) used in the study were obtained from Sisco research laboratory, India.

Animals. Swiss albino male mice of body wt 25 ± 2 grams 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 plants (Sarkar *et al*., 2006). Briefly, the leaves were homogenized in 20 mM tris-HCl buffer, pH 7.2 and the supernatant was brought to 60% $(NH_4)_2SO_4$ saturation. The pellet was reconstituted and dialyzed in tris-HCl buffer, passed through DEAE Sephadex column and eluted using a linear gradient of 0-1 M NaCl in tris buffer. The protein fractions having maximum hepatoprotective activity was concentrated, dialyzed against the same buffer and applied on a Sephadex G-50 column. Elution was carried out in 20 mM tris buffer pH 7.2. The bioactive fraction was subjected to a C18 hydrophobic column for reverse phase chromatography and rechromatography using HPLC.

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

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

Pretreatment with the protein (preventive group). The pretreatment group was divided into 3 sub-groups each consisting of six mice. The first group (group I) served as normal control; mice in the 2nd group (group II) were fed orally with 300 mg/kg body wt of APAP for 2 days and served as toxin control and mice in the 3rd group (group IIIA) were intraperitoneally injected with the protein at a dose of 2 mg/kg body wt for 4 days followed by oral application of APAP for 2 days. After 24 h of the final treatment, mice were sacrificed and blood, liver as well as kidney samples were collected separately from each animal. A positive control group (group IV) was kept in which six mice were pretreated with 250 mg/kg body wt of Vitamin C for 4 days (Weng *et al*., 2007) followed by APAP treatment as in the protein treated models.

Dose dependent preventive effect of the protein. To determine the optimum dose of the protein that can provide maximum protective

effect, a dose response study was conducted. Five different groups of mice were separately treated with five different doses (0.1, 0.5, 1, 2 and 3 mg/kg body wt) of the protein for 4 days prior to APAP administration (300 mg/kg body wt) for 2 days. Twenty-four hrs later mice were sacrificed and serum GPT levels were measured. In the negative control group (group VA) mice were treated with varying concentration of BSA in the same way as that of the protein.

Time dependent curative effect of the protein. 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 (group IIIB) were injected with the protein (2 mg/kg body wt) for 1, 2, 3 and 4 days after the administration of a single dose of APAP (300 mg/kg body wt) for 2 days. Mice were sacrificed after 3rd, 4th, 5th and 6th day respectively after protein treatment in each case and then serum GPT levels were measured. In another set, six mice in each group were treated with APAP only for 2 days, kept untreated for 1, 2, 3 and 4 days and sacrificed after 3rd, 4th, 5th, and 6th day. In addition, six mice were kept as normal control. In the negative control group (group VB) mice were treated with 2 mg/kg body wt of BSA in the same way as that of the protein.

Assessment of liver and kidney functions. Blood samples collected from puncturing mice heart were kept overnight to clot and then centrifuged at 3,000 g for 10 min. Serum GPT was measured by 2,4 DNPH method of Rietman and Frankel (1957), ALP was estimated by Kind and King's method (1954). Blood urea nitrogen (BUN) was determined spectrophotometrically from serum samples using the method of diacetylmonooxime, DAM (Marsh *et al*., 1965; Crocker, 1967). Creatinine was measured according to the method of Bonses and Taussky (1945).

Hepatocyte isolation. Hepatocytes were isolated from mice liver (Ghosh *et al*., 2006) 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 ~2×10^6 cells/ml. About 1 ml of hepatocyte suspension (~2×10^6 cells) was incubated with the protein in varying concentrations (0.001, 0.01, 0.05, 0.1, 0.5 and 1 mg/ml) for different sets of experiment for different time intervals. APAP (5 mM) was added and incubated for varying periods after and before protein incubation in pre-treatment and post-treatment experiments respectively. The normal control cells were kept in culture medium only.

Measurement of intracellular ROS production. Intracellular ROS production was detected using the fluorescent intensity of the oxidant sensitive probe 2',7'-dichlorodihydrofluorescein di acetate (H_2DCFDA) to form the fluorescent compound dichloroflouorescein DCF (Kanno *et al*., 2006). Cells loaded with 5 mM APAP and APAP pre and post-treated with varying concentration of the protein were incubated with 20 μM DCFDA for 30 min at 37°C. The culture medium was removed, the cells were washed with PBS and 2 ml of PBS was added to each tube. The fluorescence intensity of the cell suspension was measured using a spectrophotometer with excitation at 488 nm and emission at 525 nm. The untreated groups were used as control and the results are expressed as percent over control.

Assay of antioxidant power of hepatocytes: FRAP assay. The FRAP assay measures the change in absorbance at 593 nm due to the formation of a blue colored Fe^{II}-tripyridyltriazine (Fe-TPTZ) compound from the colorless oxidized Fe^{III} form by the action of electron donating antioxidants (Benzie and Strain, 1999). Briefly, 50 μl (nearly 1×10^5 cells) of hepatocyte (normal as well as experimental cells) suspension was added to 1.5 ml freshly prepared and prewarmed (37°C) FRAP reagent (300 mM acetate buffer, pH 3.6, 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ in the ratio of 10 : 1 : 1) and incubated at 37°C for 10 min. The absorbance of the sample was read against reagent blank (1.5 ml FRAP reagent + 50 μl distilled water) at 593 nm.

Estimation of lipid peroxidation product from liver and kidney homogenates. Lipid peroxidation in terms of thio barbituric acid reactive substances (TBARS) formation was measured using the method of Esterbauer and Cheeseman (1990). Sample containing 1 mg protein was mixed with 1 ml TCA (20 %), 2 ml TBA (0.67%) and heated for 1 h at 100°C. After cooling, the precipitate was removed by centrifugation. The absorbance of the sample was measured at 535 nm using a blank containing all the reagents except the sample. TBARS concentration of the liver and kidney samples were calculated using the extinction coeffecient of MDA which is 1.56×10^{-5} $mmol^{-1}$ cm^{-1} since 99% of TBARS exists as MDA.

Assay of anti-oxidant enzymes

Estimation of CAT activity. The activity was measured in different homogenates by the method of Bonaventura (Bonaventura *et al*., 1972). The enzyme CAT converts H_2O_2 formed via the action of SOD on superoxide radical into water. About 5 μg protein from each sample was mixed with 2.1 ml of 7.5 mM H_2O_2 and a time scan was performed for 10 min at 240 nm at 25°C. The disappearance of peroxide depending on the CAT activity was observed. One unit of CAT activity is defined as the amount of enzyme, which reduces 1 μmol of H_2O_2 per minute.

Estimation of SOD activity. 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). The sample containing 5 μg protein was mixed with sodium pyrophosphate buffer, phenazine methosulphate (PMT) and nitro blue tetrazolium (NBT). The reaction was started by the addition of NADH. Reaction mixture was then incubated at 30°C for 90 seconds and stopped by the addition of 1 ml of glacial acetic acid. The absorbance of the chromogen formed was measured at 560 nm. One unit of SOD activity is defined, as the enzyme concentration required inhibiting chromogen production by 50% in one minute under the assay condition.

Estimation of GST activity. GST catalyzes the conjugation reaction with glutathione in the first step of mercapturic acid synthesis. In all the experimental samples, the activity of the enzyme was measured according to the method of Habig and

Jakoby (1974). The reaction mixture contained suitable amount of enzyme (25 µg of protein in tissue homogenate), KH_2PO_4 buffer, EDTA, CDNB and GSH. The reaction was carried out at 37°C and monitored spectrophotometrically at 340 nm for 5 min. A blank was run in absence of the enzyme. One unit of GST activity is defined as 1 µmol product formation per minute.

Estimation of GSH. Glutathione concentration was determined by the method of Tietze (1969). Liver and kidney homogenates were deproteinated with trichloroacetic acid (TCA) by centrifugation and GSH released in the supernatant were derivatized with 5,5' dithiobis-2-nitrobenzoic acid (DTNB). The development of colour was measured at 412 nm. A standard curve was drawn using different known concentrations of GSH solution. With the help of this standard curve, GSH contents in all the experimental samples were calculated.

Estimation of CYP activity from liver microsomes. The reaction mixture contained 100 µg microsomal protein in a100 µl reaction system containing 0.4 mM p-nitrophenol and 1 mM NADPH. The reaction was incubated at 37°C and stopped after 60 min by addition of 30 ml 20% TCA and placed on ice. Briefly after centrifugation the sup was taken and mixed with 2 M NaOH and the absorbance measured at 546 nm. 4-Nitrocatechol formation was quantitated by using an extinction coefficient of 10.28 mM^{-1} cm^{-1} (Patten *et al*., 1992).

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 µm thickness were stained with haematoxylin and eosin to study the general structure of the liver.

Statistical analysis. All the values are represented as mean ± S.D (n = 6). Students *t*-test was applied for detecting the significance of difference between groups. P values of 0.05 or less were considered significant.

Results

Effect of the protein on dose dependent preventive activity. The dose dependent preventive activity of the protein on APAP induced elevation in serum GPT levels are shown in Fig. 1. As seen from the figure, the dose of 0.10 mg/kg body wt had very little effect on APAP induced toxicity. When applied for 0.5, 1, 2 and 3 mg/kg body wt, the protective activity of the protein increased linearly and at the dose of 2 mg/kg body wt the protein significantly prevented the elevation of serum GPT. At the dose of 3 mg/kg body wt the effect of the protein was more or less similar to 2 mg/kg body wt. Similar effect was seen in mice pretreated with Vitamin C at a dose of 250 mg/kg body wt prior to APAP administration. However mice treated with BSA (negative control group) failed to show any positive result on APAP induced increase in GPT values.

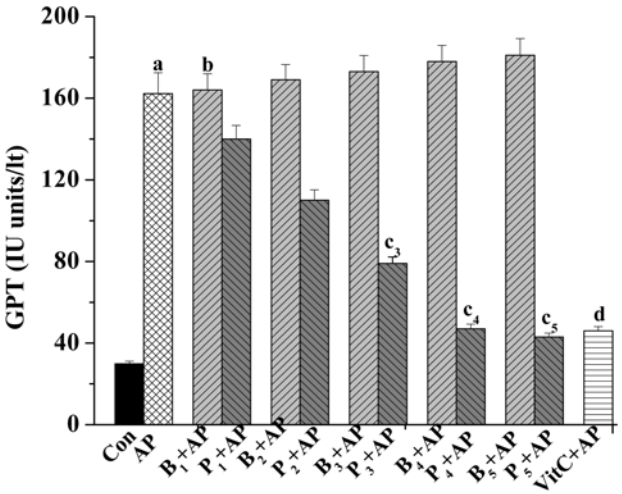

Fig. 1. Dose-dependent effect of the protein on serum GPT level against APAP induced toxicity. Con: GPT level in normal-control mice, AP: GPT level in APAP treated mice, P-1 + AP, P-2 + AP, P-3 + AP, P-4 + AP, P-5 + AP and Vit C + AP: GPT levels in the protein treated mice applied for 4 days at a dose of 0.1, 0.5, 1, 2.0 and 3 mg/kg body wt and 250 mg/kg body wt of Vitamin C respectively before APAP administration (300 mg/kg body wt). Similarily B-1 + AP, B-2 + AP, B-3 + AP, B-4 + AP, B-5 + AP: GPT levels in BSA treated mice applied for 4 days at the above mentioned doses before APAP administration (300 mg/kg body wt). Each column represents mean ± SD, n = 6 (P^a, P^b, P^{c3}, P^{c4}, P^{c5} and $P^d < 0.05$). P^a indicates statistically significant difference of the GPT values of APAP-treated mice with respect to normal control; P^b indicates statistically significant difference of the GPT values of BSA-treated mice with respect to normal control. P^{c3}, P^{c4} and P^{c5} indicates the same with respect to the GPT values of different doses of protein treated mice with respect to APAP-treated mice and P^d indicates the same for the GPT values of vitamin C treated mice with respect to APAP-treated mice.

Effect of the protein on time dependent curative activity. Fig. 2 shows the time dependent curative effect of the protein on APAP induced toxicity. As seen from the graph, after two days of APAP application the damage starts to recover of its own but the process was augmented when the protein was administered and maximum recovery was observed when the protein was applied for 4 days after APAP application for 2 days. Henceforth, all the post treatment studies were carried out for 4 days of protein treatment after APAP administration.

Effect of the protein on ALP, creatinine and BUN level. As seen from Table 1, the serum ALP, urea (BUN) and creatinine levels of mice treated with APAP was higher than the corresponding normal values. Administration of the protein before and after toxin application lowered the levels of ALP, BUN and creatinine respectively.

Effect of the protein on ROS production. After 6 h of

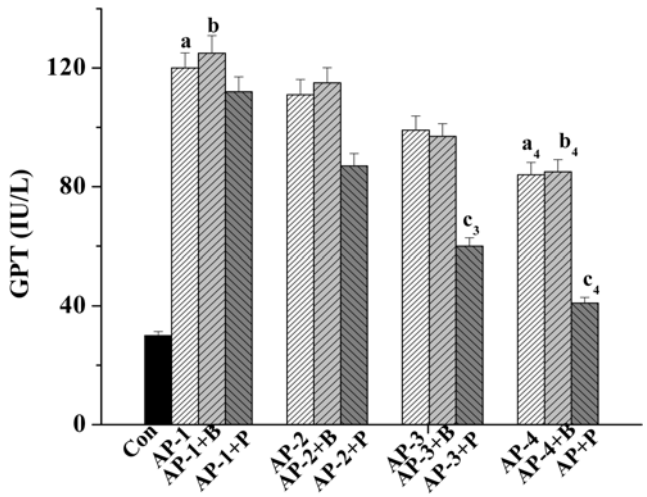

Fig. 2. Time-dependent effect of the protein on GPT level against APAP induced toxicity. Con: GPT level in normal mice, AP-1, AP-2, AP-3, AP-4 treated: APAP administration at a dose of 300 mg/kg body wt for 2 days, kept for 1, 2, 3, 4 days and then sacrificed after total of 3, 4, 5 and 6 days respectively. AP + P-1, AP + P-2, AP + P-3, AP + P-4: protein administration at a dose of 2 mg/kg body wt respectively for 1, 2, 3 and 4 days after APAP treatment for 2 days. AP + B-1, AP + B-2, AP + B-3, AP + B-4: BSA administration at a dose of 2 mg/kg body wt respectively for the above mentioned period after APAP treatment for 2 days. Each column represents mean ± SD, n = 6 (P^a, P^{a4}, P^b, P^{b4}, P^{c3} and $P^{c4} < 0.05$). P^a and P^b indicate statistically significant difference of the GPT values of APAP and APAP + BSA treated mice respectively with respect to normal control; P^{a4} indicates statistically significant difference of the GPT values of APAP treated mice sacrificed after 4 days from APAP application for 2 days with respect to GPT values of APAP-treated mice sacrificed after 1 day from APAP application for 2 days. P^{b4} indicates statistically significant difference of the GPT values of BSA treated mice for 4 days from APAP application for 2 days with respect to GPT values of APAP-treated mice sacrificed after 1 day from APAP application for 2 days. P^{c3}, P^{c4} indicates the same with respect to the GPT values of protein treated mice with respect to APAP-treated mice.

APAP application the flourescence intensity of the dye DCFDA in treated hepatocytes was 5.7 fold higher compared to control hepatocytes (Fig. 3a) indicative of increased ROS production induced by APAP. However hepatocytes treated with the protein before and after APAP application dose-dependently reduced the APAP induced generation of ROS (Fig. 3b).

Effect of the protein on Ferric Reducing/antioxidant Power (FRAP). APAP caused a significant reduction in FRAP value in a time dependent manner in isolated hepatocytes compared to that level in normal hepatocytes as shown in Fig. 4a. Incubation of the cells with the protein both prior and post to APAP addition increased the antioxidant power (Fig. 4b)

Effect of the protein on products of lipid peroxidation. The level of MDA in group I, group II, group IIIA and IIIB mice from liver homogenates are shown in Table 2. The MDA levels in group II mice were found to be elevated compared to group I mice. Group IIIA mice treated with 2 mg/kg body wt of protein, showed significant reduction in the MDA level. In group IIIB mice, the reduction of MDA upon protein treatment was much more significant compared to group IIIA. Similarly, as shown in Table 3 in kidney tissue homogenates, the MDA level of group II mice was also higher than the normal and there was reversal of MDA content upon protein administration both before and after APAP administration. MDA level in group IV mice was almost close to group I mice.

Effect on antioxidant enzymes

Effect of the protein on CAT activity. The CAT activity levels in all the mice groups of liver and kidney are shown in Table 2 and Table 3 respectively. The CAT activity in liver and kidney homogenates of group II mice was considerably lower than that of group I. Treatment of the mice with the protein for 4 days prior to APAP treatment increased the CAT activity compared to the APAP treated mice. Protein post-treatment for 1 and 2 days after APAP application practically had no effect on the reduced CAT activity (data not shown). However as shown in Table 2 and Table 3, CAT activity was found to be significantly increased when the animals were post-treated with the protein for 4 days, compared to APAP treated mice. CAT level in group IV mice was almost close to group I mice.

Effect of the protein on SOD activity. The activity of SOD in the liver and kidney homogenates of all mice groups are shown in Table 2 and Table 3 respectively. The SOD activity in group II mice was reduced compared to group I. In group IIIA mice there was an enhancement of SOD value, compared to group II mice. Mice in group IIIB also showed an augmented SOD value compared to its corresponding APAP control. Similar results were obtained from the kidney homogenates. SOD activity in group IV was almost close to group I mice.

Effect of the protein on GST activity. GST activity as measured from liver and kidney tissue homogenates of all experimental mice is shown in Table 2 and Table 3 respectively. As seen from the tables there is a reduction in GST activity of group II mice in both liver and kidney homogenates. In pretreated group IIIA mice, GST activity was higher compared to group II both in liver and kidney of treated mice, although it was less than group I. In group IIIB mice, the GST activity was elevated to almost normal levels in liver and kidney homogenates. GST activity in group IV mice was almost close to group I mice.

Table 1. Effect of the protein pre and post-treatment on ALP, BUN and creatinine level in blood serum against APAP intoxication (300 mg/kg body wt)

Serum Parameters	Control	AP treated	P+AP treated	AP+P treated	Vit C+P treated
Alkaline Phosphatase (KA units/ml serum)	17 ± 0.8	42 ± 1.5[a]	23 ± 1.1[b]	21 ± 0.9 [c]	24 ± 1.0 [d]
Blood urea nitrogen (Urea/100 ml serum)	13.2 ± 0.72	21 ± 1.1[a]	18.5 ± 0.92[b]	17.8 ± 0.86[c]	19 ± 0.86[d]
Creatinine (mg/L serum)	4.2 ± 0.12	5.3 ± 0.08[a]	4.8 ± 1.9[b]	4.5 ± 2.0[c]	4.9 ± 2.1[d]

Control: level in normal-control mice, AP: levels in APAP treated mice, P + AP: levels in which the protein was given at a dose 2 mg/kg body wt for 4 days prior to APAP administration. AP + P: levels in which the protein was given at a dose 2 mg/kg body wt for 4 days after APAP administration for 2 days. VitC + APAP: levels in which vitamin C was given at a dose 250 mg/kg body wt for 4 days prior to APAP administration Data represents mean ± SD, n = 6 ($P^{a,}$ $P^{b,}$ P^{c} and $P^{d} < 0.05$). P^{a} indicates statistically significant difference of the values of serum markers of APAP-treated mice with respect to normal control; P^{b}, P^{c} indicate the same with respect to the values of serum markers of protein pre and post-treated mice respectively with respect to APAP-treated mice and P^{d} indicates the same for the values of serum markers of vitamin C treated mice with respect to APAP-treated mice.

Effect of the protein on GSH level. As seen from Table 2 and Table 3 there was a 74% and 41% reduction of GSH in APAP treated liver and kidney homogenates respectively compared to normal controls. However, the protein treatment restored the GSH levels both in group IIIA and IIIB mice. In case of kidney homogenates, APAP treatment did not lower the GSH levels to the same extent as seen in liver homogenates. Administration of the protein though brought back the GSH level near the normal value. GSH level in group IV mice was almost close to group I mice.

Effect of the protein on CYP activity. Figure 5 shows that when mice were administered with APAP for 2 days and sacrificed the next day, there was a nearly 40% induction of CYP activity compared to normal controls. Mice treated with APAP for 2 days, kept untreated for 4 days and then sacrificed the next day showed a reduction in CYP activity that was not very significant. However, intraperitoneal application of the protein for 4 days after APAP administration for 2 days reduced the APAP induced increase in CYP activity close to the normal range.

Effect of the protein on liver histopathology. Histopathological analyses showed prominent changes in tissues treated with APAP compared to protein treated tissues. APAP induced necrosis along the central vein and disorganization of normal radiating pattern of cell plates around it. Besides, there was elongation of the central vein and distribution of polymorphonuclear infiltrates surrounding the central vein in APAP treated liver sections (Fig. 6B). However administration of the protein prior and after APAP administration showed a considerable improvement in liver morphology (Fig. 6C and 6D).

Discussion

Research in our laboratory has showed that the 43 kDa protein isolated from the leaves of the plant *Cajanus indicus* has a potent hepatoprotective activity against various toxins like sodium fluoride, galactosamine, carbon tetrachloride, chloroform, thioacetamide etc. (Sarkar *et al*., 2005; Ghosh *et al*., 2006; Ghosh and Sil, 2006; Sarkar and Sil, 2006; Manna *et al*., 2007a; Manna *et al*., 2007b; Sinha *et al*., 2007a; Sinha *et al*., 2007b; Sinha *et al*., 2007c).

The present study was conducted to find out the anti-oxidative effect of the protein in hepatic and renal dysfunctions imposed by APAP and also to compare the hepatoprotective property with its nephroprotective actions. The study provides evidence that the application of the protein both prior and post to APAP administration caused considerable changes in serum marker enzymes and in the oxidant-antioxidant status of hepatic and renal cells *in vivo*.

We found that treatment of mice with APAP increased serum GPT, ALP, creatinine and BUN although the increase in creatinine and BUN were considerably less compared to GPT and ALP. Protein treatment of mice both before and after APAP administration, however, reduced all these parameters to almost the normal range. This indicates that APAP at a dose of 300 mg/kg bd-wt is capable of inducing hepatotoxicity more severely than nephrotoxicity. A dose higher than this was found to be fatal to the mice though it may have an acute nephrotoxic potential.

Dose dependent studies showed that the protein exerts its maximum hepatoprotective activity when it was applied at a dose of 2 mg/kg body wt for duration for 4 days prior to APAP administration for 2 days (Fig. 1). Comparative study of the protective effect of the protein with respect to the self-

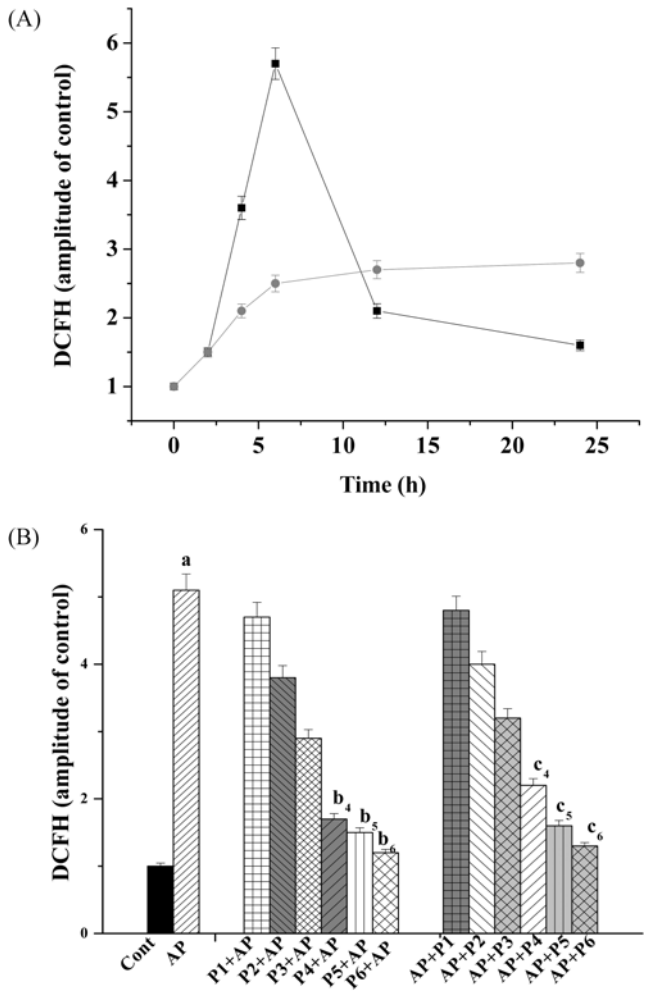

Fig. 3. (A) Time dependent effect of the protein (0.1 mg/ml) on APAP (5 mM) induced generation of ROS. Squares: APAP treatment groups. Closed circles: protein plus APAP treated groups. (B) Dose dependent effect of the protein on APAP (5 mM) induced generation of ROS for 6 h. Left panel: cont: ROS generation in untreated cell. AP: ROS generation in AP treated cell for 6 h. P-1 + AP, P-2 + AP, P-3 + AP, P-4 + AP, P-5 + AP and P-6 + AP: ROS generation in protein treated (0.001, 0.01, 0.05, 0.1, 0.5 and 1 mg/ml of protein respectively) hepatocytes for 6 h before APAP application for 6 h. Right panel: represents the same only the data is presented for post-treatement in which the protein at above mentioned doses was given for 6 h after ROS generation by APAP for 6 h. Each column represents mean ± SD, n = 6 (P^a, P^{b4}, P^{b5}, P^{b6}, P^{c4}, $P^{c5}$and $P^{c6} < 0.05$). P^a indicates statistically significant difference of ROS generation in APAP-treated mice with respect to normal control; $P^{b4,}$ P^{b5}, P^{b6} indicates the same with respect to the ROS generation of protein pre-treated mice at a dose of 0.1, 0.5 and 1 mg/ml of protein respectively with respect to APAP-treated mice and P^{c4}, $P^{c5}$and P^{c6} indicates the same for ROS generation in protein post-treated mice with respect to APAP-treated mice.

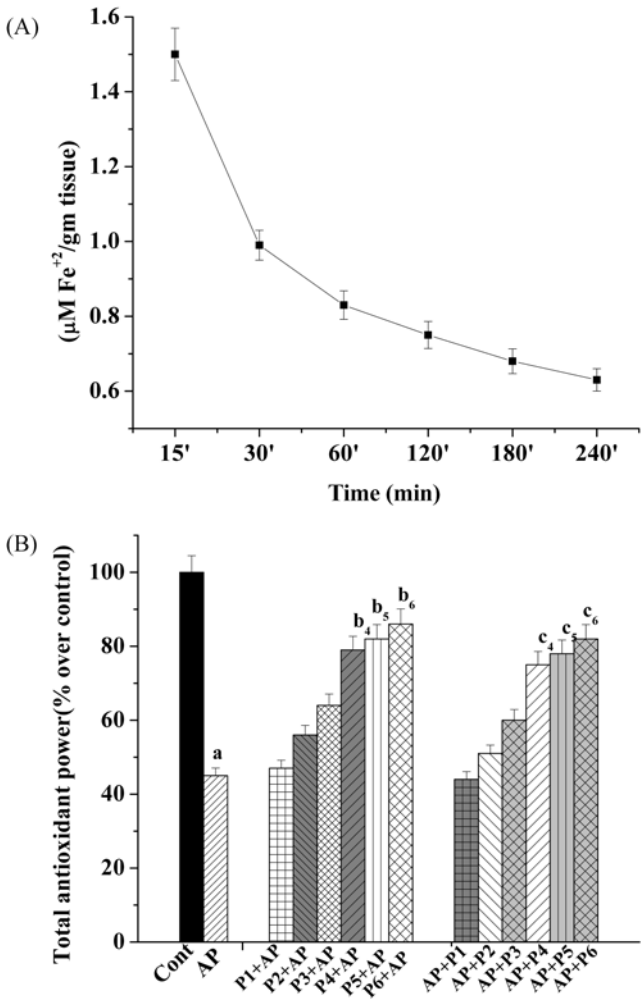

Fig. 4. (A) Time dependent effect of the protein (0.1 mg/ml) on APAP (5 mM) induced reduction in total antioxidant power (FRAP values) of hepatocytes. Squares: APAP treatment groups. (B) Dose dependent effect of the protein on APAP (5 mM) induced reduction in total antioxidant power of hepatocytes. Left panel: cont: FRAP values in untreated cell. AP: FRAP values in AP treated cell for 1 h. P-1 + AP, P-2 + AP, P-3 + AP, P-4 + AP, P-5 + AP and P-6 + AP: FRAP values in protein treated (0.001, 0.01, 0.05, 0.1, 0.5 and 1 mg/ml of protein respectively) hepatocytes for 1 h before APAP application for 1 h. Right panel: represents the same only the data is presented for post-treatement in which the protein at above mentioned doses was given for 1 h after APAP treatment for 1 h. Each column represents mean ± SD, n = 6 (P^a, P^{b4}, P^{b5}, P^{b6}, P^{c4}, $P^{c5}$and $P^{c6} < 0.05$). P^a indicates statistically significant difference of FRAP values in APAP-treated mice with respect to normal control; $P^{b4,}$ P^{b5}, P^{b6} indicates the same with respect to the FRAP values of protein pre-treated mice at a dose of 0.1, 0.5 and 1 mg/ml of protein respectively with respect to APAP-treated mice and P^{c4}, $P^{c5}$and P^{c6} indicates the same for FRAP values in protein post-treated mice with respect to APAP-treated mice.

recovery process of the liver after 2 days of APAP treatment has been shown in Fig. 2. Mice treated with APAP for 2 days and sacrificed sequentially after 3, 4, 5 and 6 days showed a gradual reduction (with respect to no of days) in APAP-induced serum GPT level. However, mice treated with APAP followed by the treatment with the protein for 1, 2, 3 and 4

Table 2. Effect of the protein pre and post-treatment on oxidative stress indices such as MDA content, CAT activity, SOD activity, GST activity and GSH level in liver homogenates.

	PRE-TREATMENT				POST-TREATMENT			
	N	AP	P+AP	C+AP	N	AP	AP+P	AP+C
MDA (nmoles/gm tissue)	41 ± 1.2	91 ± 3.6^a	55 ± 2.1^b	50 ± 2.0^c	41 ± 1.2	72 ± 3.2^a	43 ± 1.8^b	36 ± 2.0^c
CAT (Units/mg protein)	110 ± 5.1	41 ± 1.8^a	83 ± 3.9^b	78 ± 3.2^c	110 ± 5.1	66 ± 3.1^a	108 ± 4.1^b	101 ± 4.4^c
SOD (Units/mg protein)	295 ± 12	211 ± 7.1^a	267 ± 11.4^b	278 ± 12.6^c	290 ± 11.9	220 ± 9.1^a	272 ± 11^b	278 ± 12.6^c
GST (Units/mg protein)	6.2 ± 0.29	2.9 ± 0.13^a	5.6 ± 0.22^b	5.9 ± 0.2 ^{c}	6.2 ± 0.29	3.4 ± 0.14^a	5.8 ± 0.27^b	6 ± 0.21^c
GSH (mg/total protein)	46 ± 2.1	12.9 ± 0.67^a	34 ± 1.4 ^{b}	31 ± 1.43 ^{c}	46 ± 2.1	12 ± 0.4 ^{a}	35 ± 1.6 ^{b}	30 ± 1.3^c

N: values in normal-control mice, AP: levels in APAP treated mice, P + AP: values in which the protein was given at a dose 2 mg/kg body wt for 4 days prior to APAP administration. C + AP: values in which vitamin C was given at a dose 250 mg/kg body wt prior to APAP administration for 4 days. AP + P: levels in which the protein was given at a dose 2 mg/kg body wt for 4 days after APAP administration. AP + C: values in which vitamin C was given at a dose of 250 mg/kg body wt for 4 days after APAP administration. Data represents mean ± SD, n = 6 (P^a, P^b and $P^c < 0.05$). P^a indicates statistically significant difference of the indices of oxidative stress of APAP-treated mice with respect to normal control; P^b indicates the same with respect to the indices of oxidative stress of protein pre and post-treated mice with respect to APAP-treated mice and P^c indicates the same for the indices of oxidative stress of vitamin C treated mice with respect to APAP-treated mice.

Table 3. Effect of the protein pre and post-treatment on oxidative stress indices such as MDA content, CAT activity, SOD activity, GST activity and GSH level in kidney homogenates.

	PRE-TREATMENT				POST-TREATMENT			
	N	AP	P+AP	C+AP	N	AP	AP+P	AP+C
MDA (nmoles/gm tissue)	38 ± 1.5	82 ± 3.2^a	43 ± 1.9^b	40 ± 1.3^c	38 ± 1.5	67 ± 2.9^a	40 ± 1.6^b	42 ± 1.3^c
CAT (Units/mg protein)	73 ± 3.3	38 ± 1.2^a	64 ± 2.9^b	61 ± 2.4^c	73 ± 3.3	41 ± 1.9^a	69 ± 3.2 ^{b}	65 ± 2.4^c
SOD (Units/mg protein)	243 ± 9.6	197 ± 8.1^a	222 ± 7.1^b	238 ± 9.5 ^{c}	243 ± 9.6	207 ± 9.3 ^{a}	238 ± 8.2^b	241 ± 8.6^c
GST (Units/mg protein)	2.8 ± 0.13	1.1 ± .03^a	2.2 ± 0.09^b	2.4 ± 0.13 ^{c}	2.8 ± 0.13	1.7 ± 0.11^a	2.5 ± 0.13^b	2.6 ± 0.09^c
GSH (mg/total protein)	14 ± 0.55	8.3 ± 0.7^a	12.5 ± 0.63^b	11.3 ± 0.43^c	14 ± 0.55	9 ± 0.4 ^{a}	12.3 ± 0.7^b	10.8 ± 0.5^c

Legend is the same as that for the legend of table 2, except that the results in this table are the presentation of the parameters for renal samples. Data represents mean ± SD, n = 6 (P^a, P^b and $P^c < 0.05$). P^a indicates statistically significant difference of the indices of oxidative stress of APAP-treated mice with respect to normal control; P^b indicates the same with respect to the indices of oxidative stress of protein pre and post-treated mice with respect to APAP-treated mice and P^c indicates the same for the indices of oxidative stress of vitamin C treated mice with respect to APAP-treated mice.

days showed significantly better reduction in serum GPT values compared to natural recovery. Moreover, mice post-treated with the protein for 4 days after 2 days of APAP treatment showed a serum GPT value closer to the normal although natural recovery could not do so.

A number of evidence pointed to the potential involvement of oxidative stress via production of ROS in APAP-induced organ toxicity (Botta *er al*., 2006; Sener *et al*., 2006; James *et al*., 2003). We also measured the APAP induced generation of ROS and found that it peaked at 6 h, (as evidenced in DCFDA fluorescence). This increase, however, was attenuated by the administration of the protein for 6 h before and after APAP admistration for the same time. Similarly, the total antioxidant power of hepatocytes was increased when cells were incubated with the protein both before and after APAP addition than APAP alone. Thus, the results suggest that the protein possesses an antioxidative role in APAP induced generation of ROS and it increases the FRAP as well.

Our experimental data demonstrated that treatment of mice with a toxic dose of APAP increased the level of hepatic and

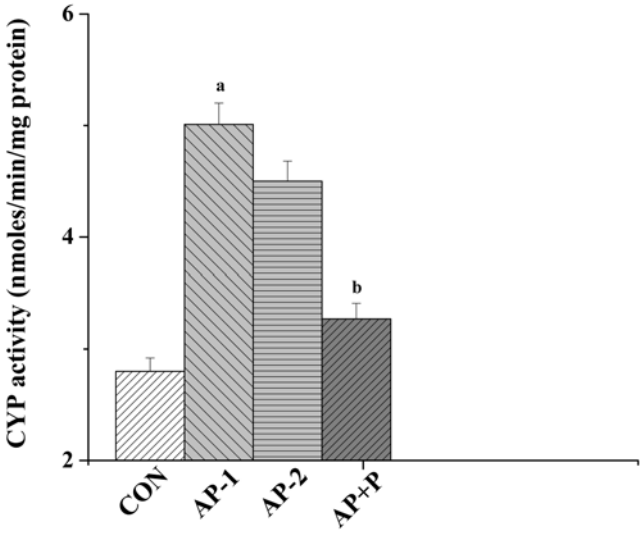

Fig. 5. Effect of the protein treatment on the CYP activity in APAP induced hepatic damage in mice. CON: CYP activity in normal mice, AP-1: CYP activity in mice treated with APAP for 2 days and sacrificed the next day, AP-2: CYP activity in mice treated with APAP for 2 days, kept for 4 days and sacrificed the next day. AP + P: CYP activity in which the protein was given for 4 days at a dose 2 mg/kg body wt after APAP administration for 2 days. Each column represents mean ± SD, n = 6; (P^a & $P^b < 0.05$). P^a indicates statistically significant difference of the CYP activity of APAP-treated mice with respect to normal control; P^b indicates the same with respect to the CYP activity of protein treated mice with respect to APAP-treated mice.

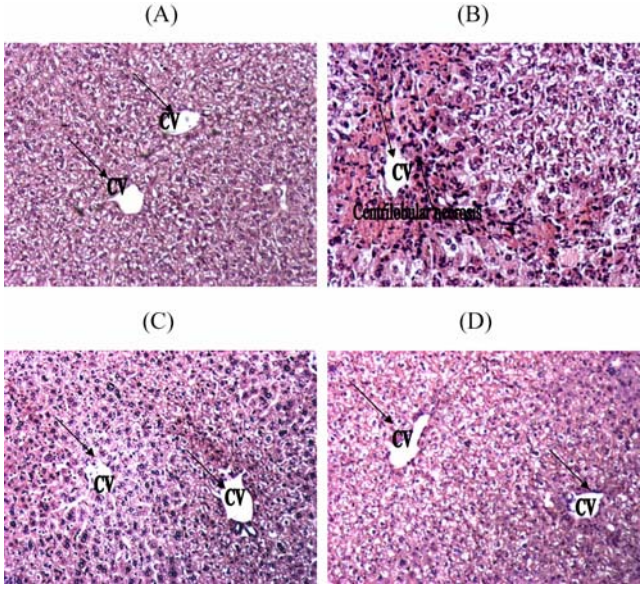

Fig. 6. Histopathology of normal liver section stained with haematoxylin-eosin stain. (A) Histopathology of normal liver section. (B) Histopathology of liver treated with APAP for 2 days and sacrificed on the 3rd day. (C) Histopathology of liver treated with the protein for 4 days and then treated with APAP for 2 days. (D) Histopathology of liver treated with APAP for 2 days and then treated with the protein for 4 days. CV indicates the central vein.

renal lipid peroxidation and that could be inhibited by protein administration. Results suggest that the protein might have some effect on inhibiting the ROS induced membrane damages. Moreover, SOD, CAT and GST activities were decreased in livers and kidneys of APAP treated mice. The reduced activity of SOD, CAT, GST could be due to enhanced lipid peroxidation or inactivation of the antioxidative enzymes. When mice were treated with the protein prior to APAP administration, the reduction of SOD, CAT and GST activity was inhibited. APAP administration also caused a significant diminution in GSH content. Protein treatment helped to uplift the GSH depletion induced by APAP. Similar results were obtained when the protein was applied after the application of the toxin. Comparing the values in Table 2 and Table 3, it is evident that the basal levels of CAT, GST and GSH in kidney homogenates were in general lower than liver homogenates. APAP application reduced their levels further but protein pre and post treatment showed a positive effect in uplifting the level of these antioxidant enzymes. Combining the results it can be said that the protein significantly prevented the alterations of the hepato-nephrotoxic damages caused by APAP probably by enhancing the activities of endogenous antioxidants.

In human liver the first step of biotransformation is mediated mainly by CYPs. CYPs are involved in biotransformation of over 50% of drugs in use today (Xinsheng *et al*., 2006). Moreover, there are some plant extracts that are known to alter the level of CYPs as their antioxidative mechanism of action (Rajesh and Lata, 2004). To determine whether CYPs influence the toxicity of APAP and whether the protein could alter its effect, we measured the activities of CYPs from liver microsomal fraction. Mice treated with the toxic dose of APAP enhanced CYP activity that could be altered by the protein treatment. Thus it can be speculated that the protein might have some inhibitory activity on APAP induced increment of CYP levels in microsomes. Histological studies of APAP treated mice showed severe necrosis along the central vein. APAP treated liver sections had massive inflammation (redness) and out flow of polymorphonuclear infiltrates along the central vein. On the other hand, the inflammations in the livers of both protein pre- and post-treated mice were less necrotic. These results also confirm the protective response of the protein against the hepatic damage caused by APAP. However histological sections from APAP treated kidney did not show much demarcation from either the normal or protein treated slides (data not shown). This again corroborates the fact that the hepatotoxicity induced by APAP at a dose of 300 mg/kg body wt is more severe than nephrotoxicity.

Taken together, the results demonstrate that the protein isolated from *Cajanus indicus* has considerable protective effect on hepatic and renal damages induced by APAP. The protein not only possesses effective protective action; it also

possesses curative action. The protein acts and combats the toxicity of APAP at three different levels; a) it could manage the acute oxidative stress imposed by APAP by decreasing ROS generation, lipid peroxidation and increasing the total antioxidant potential of hepatocytes along with the levels of antioxidant enzymes like SOD, CAT and GST; b) it helped to abrogate the negative GSH balance in cells imposed by APAP by uplifting the reduced thiol level and finally c) the APAP induced increase in CYP content was blunted when mice were treated with the protein. Further studies are needed to find out its exact mechanism of action during the hepato-nephro protection and currently are in progress.

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References

Benzie, I. F. F. and Strain J. J. (1999) Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Methods Enzymol.* **299**, 15-27.

Bonaventura, J., Schroeder, W. A. and Fang, S. (1972) Human erythrocyte catalase:an improved method of isolation and a revaluation of reported properties. *Arch. Biochem. Biophys.* **150**, 606-617.

Botta, D., Shi, S., White, C. C., Dabrowski, M. J., Keener, C. L., Srinouanprachanh, S. L., Farin, F. M., Ware, C. B., Ladiges, W. C., Pierce, R. H., Fausto. N. and Kavanagh, T. (2006) Acetaminophen-induced liver injury is attenuated in male glutamate-cysteine ligase transgenic mice. *J. Biol. Chem.* **281**, 28865-28875.

Bonsnes, R. W. and Taussky, H. H. (1945) On the colorimetric determination of creatinine by the Jaffe reaction. *J. Biol. Chem.* **158**, 581-591.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Cobden, I., Record, C. O., Ward, M. K. and Kerr, D. N. S. (1982) Paracetamol induced acute renal failure in the absence of fulminant liver damage. *Br. Med. J.* **284**, 21-22.

Crocker, C. L. (1967) Rapid determination of urea nitrogen in serum or plasma without deproteinisation. *Am. J Med. Technol.* **33**, 361-365.

Dahlin, D. C., Miwa, G. T., Lu, A. Y. and Nelson, S. D. (1984) N-acetyl p-benzoquinimine: a cytochrome P450 mediated oxidation product of acetaminophen. *Proc. Natl. Acad. Sci. USA* **81**, 1327-1331.

Eguia, L. and Materson, B. J. (1997) Acetaminophen related acute renal failure without fulminant hepatic failure. *Pharmacotherapy* **17**, 363-370.

El-Beshbishy, H. A. (2005) Hepatoprotective effect of green tea (Camellia sinensis) extract against tamoxifen-induced liver injury in rats. *J. Biochem. Mol. Biol.* **38**, 300-306

Esterbauer, H and Cheeseman, K. H. (1990) Determination of aldehydic lipid peroxidation products: Malonaldehyde and 4-hydroxynonenal. *Methods Enzymol.* **186**, 407-421.

Ghosh, A. and Biswas, K. (1973) (Ed) A. Chatterjee, Calcutta: Calcutta University Press Bhartiya Banausadhi **2**, 332-334.

Ghosh, A., Sarkar, K. and Sil, P. C. (2006) Protective effect of a 43 kDa protein from the leaves of the herb, *Cajanus indicus* L on chloroform induced hepatic-disorder. *J. Biochem. Mol. Biol.* **39**, 197-207.

Ghosh, A. and Sil, P. C. (2006) A 43 kDa protein from the leaves of the herb, *Cajanus indicus* L, modulates chloroform induced hepatotoxicity *in vitro*. *Drug Chem. Toxicol.* **29**, 397-413.

Habig, W. H. and Jakoby, W. B. (1974) Glutathione S-Transferases. The first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* **249**, 7130-7139.

James, L. P., Mayeux, P. R. and Hinson, J. A. (2003) Acetaminophen induced hepatotoxicity. *Drug. Metab. Dispos.* **31**, 1499-1506.

Kakkar, P., Das, B. and Viswanathan, P. N. (1984) A modified spectrophotometric assay of superoxide dismutase. *Indian J. Biochem. Biophys.* **21**, 130-132.

Kanno, S., Tomizawa, A., Hiura, T., Osanai, Y., Kakuta, M., Kitajima, Y., Koiwai, K.,Ohtake, T., Ujibe, M and Ishikawa, M. (2006). Melatonin Protects on Toxicity by Acetaminophen But Not on Pharmacological Effects in Mice. *Biol. Pharm. Bull.* **29**, 472-476.

Kirtikar, K. R. and Basu, B. D. (1935) Papilionacea: *Cajanus indicus*; in *Indian Medicinal Plants*, Blatter, E., Caius, J. F. and Mhaskarr, R. S. (eds.), pp. 809-11.2.2, Probasi Press, Calcutta, India.

Kind, P. R. N. and King, E. J. (1954) Estimation of plasma phosphatase by determination of hydrolyzed phenol with anti-pyrine. *J. Clin. Path.* **7**, 322-326.

Knight, T. R., Kurtz, A., Bajt, M. L., Hinson, J. A. and Jaeschkae, H. (2001) Vascular and hepatocellular peroxonitrite formation during acetaminophen toxicity: Role of mitochondrial oxidant stress. *Toxicol. Sci.* **62**, 212-220.

Manna, P., Sinha, M. and Sil, P. C. (2007a) A 43 kDa protein isolated from the herb Cajanus indicus L attenuates sodium fluoride-induced hepatic and renal disorders in vivo. *J. Biochem. Mol. Biol.* **40**, 382-395.

Manna, P., Sinha, M. and Sil, P. C. (2007b) Galactosamine-induced hepatotoxic effect and hepatoprotective role of a protein isolated from the herb Cajanus indicus L in vivo. *J. Biochem. Mol. Toxicol.* **21**, 13-23.

Mansour, H. H., Hafez, H. F. and Fahmy, N. M. (2006) Silymarin modulates Cisplatin-induced oxidative stress and hepatotoxicity in rats. *J. Biochem. Mol. Biol.* **39**, 656-661.

Marsh, W. H., Fingerhut, B. and Miller, H. (1965) Automated and manual direct methods for the determination of blood urea. *Clin. Chem.* **11**, 624-627.

Michael, S. L., Pumford, N. R., Mayeux, P. R., Niesman, M. R. and Hinson, J. A. (1999) Pretreatment of mice with macrophage inactivators decreases acetaminophen hepatotoxicity and the formation of reactive oxygen and nitrogen species. *Hepatology* **30**, 186-195.

Mitchell, J. R., Jollow, D. J., Potter, W. Z., Gillete, J. R. and Brodie, B. B. (1973) Acetaminophen induced hepatic necrosis. IV.

Protective role of glutathione. *J. Pharmacol. Exp. Ther.* **187**, 211-217.

Montilla, P., Barcos, M., Munoz, M. C., Bujalance, I., Munoz-Castaneda, J. R. and Tunez, I. (2005) Red wine prevents brain oxidative stress and nephropathy in streptozotocin-induced diabetic rats. *J. Biochem. Mol. Biol.* **38**, 539-544.

Nakae, D., Yamamoto, K., Yoshij, H., Kinugasa, T., Marayuma, H., Farber, J. L. and Konishi, Y. (1990) Liposome-encapsulated superoxide dismutase prevents liver necrosis induced by Acetaminophen. *Am. J. Pathol.* **136**, 787-795.

Nishikimi, M., Rao, N. A. and Yagi, K. (1972) The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. *Biochem. Biophys. Res. Commun.* **46**, 849-854.

Patten, C. J., Ishizaki, H., Aoyama, T., Lee, M., Ning, S. M. and Huang, W. (1992) Catalytic properties of human cytochrome P450 2E1 produced by cDNA expression in mammalian cells. *Arch. Biochem. Biophys.* **299**, 163-171.

Proudfoot, A. T. and Wright, N. (1970) Acute Paracetamol poisoning. *Br. Med. J.* **3**, 557-558.

Rajesh, M. G. and Latha, M. S. (2004) Protective activity of *Glycyrrhiza glabra* Linn. on Carbon-tetrachloride-induced peroxidative damage. *Indian J. Pharmacol.* **36**, 284-287.

Reitman, S. and Franke, S. A. (1957) A colorimetric method for the determination of serum glutamic oxaloacetic and glutamic pyruvic transminases. *Am. J. Clin. Path.* **28**, 56-63.

Sarkar, K., Ghosh, A. and Sil, P. C. (2005) Preventive and curative role of a 43 kDa protein from the leaves of the herb *Cajanus indicus* L on thioacetamide induced hepatotoxicity *in vivo*. *Hepatol. Res.* **33**, 39-49.

Sarkar, K. and Sil, P. C. (2006) A 43 kDa protein from the herb Cajanus indicus L. protects thioacetamide induced cytotoxicity in hepatocytes. *Toxicol. In Vitro* **20**, 634-640.

Sarkar, K., Ghosh, A., Kinter, A., Mazumder, B. and Sil, P. C. (2006) Purification and characterization of a 43 kDa hepatoprotective protein from the herb Cajanus indicus L. *Protein. J.* **25**, 411-421.

Sener, G., Omurtag, G. Z., Sehirli, O., Tozan, A., Yuksel, M., Ercan, F. and Gedik, N. (2006). Protective effects of ginkgo biloba against acetaminophen-induced toxicity in mice. *Mol. Cell. Biochem.* **283**, 39-45.

Sinha, M., Manna, P. and Sil, P. C. (2007a) Amelioration of galactosamine-induced nephrotoxicity by a protein isolated from the leaves of the herb, Cajanus indicus L. *BMC. Complement. Altern. Med.* **7**, 11.

Sinha, M., Manna, P. and Sil, P. C. (2007b) A 43 kDa protein from the herb, Cajanus indicus L., protects against fluoride induced oxidative stress in mice erythrocytes. *Pathophysiology* **14**, 47-54.

Sinha, M., Manna, P. and Sil, P. C. (2007c) Attenuation of cadmium chloride induced cytotoxicity in murine hepatocytes by a protein isolated from the leaves of the herb Cajanus indicus L. *Arch. Toxicol.* **81**, 397-406.

Soni, M. G., Raniah, S. K., Mumtaz, M. M., Clewell, H. and Mehendale, H. M. (1999) Toxicant inflicted injury and stimulated tissue repair are opposing toxicodynamic forces in predictive toxicology. *Regulatory Pharmacol. Toxicol.* **19**, 165-174.

Thakore, K. N. and Mehendale, H. M. (1994) Effect of Phenobarbital and mirex pretreatments on carbon tetrachloride auto protection. *Toxicol. Pathol.* **22**, 291-299.

Thomas, S. H. L. (1993) Paracetamol (Acetaminophen) poisoning. *Pharmacol. Ther.* **60**, 91-120.

Tietze, F. (1969) Enzymic method for quantitative determination of nanogram amount of total and oxidizes glutathione: applications to mammalian blood and other tissues. *Anal. Biochem.* **27**, 502-522.

Weng, D., Lu, Y., Wei, Y., Liu, Y. and Shen, P. (2007) The role of ROS in microcystin-LR-induced hepatocyte apoptosis and liver injury in mice. *Toxicology* **232**, 15-23.

Xinsheng, G., Sui, K., Duan, L., Tao, S., Paul, T., Arnold, R., Michael, G., Wen, X. and Yanan, T. (2006) Role of NF-κB in Regulation of PXR-mediated Gene Expression: A mechanism for suppression of cytochrome P-450 3A4 by peoinflammatory agents. *J. Biol. Chem.* **281**, 17882-17889.