



AMELIORATION OF PARACETAMOL-INDUCED HEPATOTOXICITY BY A PROTEIN ISOLATED FROM THE LEAVES OF THE HERB *CAJANUS ACUTIFOLIUS* LINN

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ABSTRACT

The hepatoprotective effects of a protein isolated from *Cajanus acutifolius* on paracetamol (PCM) induced hepatotoxicity and the probable mechanism(s) involved in this protection were investigated in rats. Both preventive as well as curative effect of the protein was investigated in the study. Liver damage was induced in Wistar rats by administering PCM (750 mg/kg, i.p). Pretreatment and post treatment with protein at 500 mg/kg and 1000 mg/kg, and the reference drug silymarin (100 mg/kg) were done intraperitoneally in PCM treated rats. Levels of marker enzymes (SGOT, SGPT and SALP), uric acid, total albumin (Alb) and total proteins (TP) were assessed in serum. The effects of protein on lipid peroxidation (LPO), reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) were assayed in liver homogenates to evaluate antioxidant activity. Protein (500 and 1000 mg/kg) and silymarin elicited a significant hepatoprotective activity by lowering the levels of serum marker enzymes and lipid peroxidation and elevated the levels of GSH, SOD, CAT, Alb and TP in a dose dependant manner, which was confirmed by the decrease in the total weight of the liver and histopathological examination. The present findings suggest that the hepatoprotective effect of protein in PCM induced oxidative damage may be related to its antioxidant and free radical scavenging activity.

Keywords: *Cajanus acutifolius*; Hepatoprotective; Antioxidant enzymes; Protein; Lipidperoxidation; Silymarin; Catalase.

INTRODUCTION

Liver is the vital organ responsible for drug metabolism and appears to be sensitive target site for substances modulating biotransformation¹. Liver diseases are mainly caused by toxic chemicals, excess consumption of alcohol, infections and autoimmune disorders. Most of the hepatotoxic chemicals damage liver cells mainly by inducing lipid peroxidation and other oxidative stress in liver².

Paracetamol (PCM) was discovered in Germany at the end of the 19th century, but was not widely used until mid way through the 20th century. PCM is probably the most versatile and widely used analgesic and antipyretic drug worldwide³. Its potential hepatotoxicity was not suspected until the first clinical reports of severe and fatal liver damage following over dosage was reported by Davidson and Eastham⁴. PCM taken in over doses results in hepatotoxicity and nephrotoxicity in men and in experimental animals⁵. The chemical structure of PCM is N-acetyl-p-aminophenol (APAP). It has an excellent safety profile in therapeutic doses, but hepatotoxicity can develop with overdoses. The major target organ in PCM poisoning is the liver and the primary lesion is acute centrilobular hepatic necrosis. Paracetamol being converted into reactive toxic metabolites by hepatic microsomal cytochrome P-450 in turn causes hepatotoxicity⁶.

Liver diseases are considered as one of the serious health problems. Steroids, vaccines and antiviral drugs that are employed as therapy for liver diseases have potential adverse effects especially when administered for long periods⁷. An important factor for avoiding complications in PCM poisoning is early administration of the antidotes. However with the emerging scientific researches on herbal medicines together with the global trend of increasing consumption of natural products, a naturally presenting antidote may already be present in the human system through dietary intake to converse a protective effect against drug toxicity. Herbal medicines have been used traditionally worldwide for the prevention and treatment of various diseases. According to the WHO, 80% of the world population uses plant-based remedies as their primary form of healthcare⁸. Earlier studies reported that medicinal herbs play a protective role against PCM induced liver damage. Although, a big list of hepatoprotective phytochemicals is reported in the scientific literature, only few were potent against various types of liver damages. Of these, silymarin^{9,10},

andrographolide¹¹, neoandrographolide, curcumin¹², picoside¹³, kutkoside¹³, phyllanthin^{14, 15}, hypophyllanthin, and glycyrrhizin have largely attracted the scientific community¹⁶.

The herb *Cajanus acutifolius*, an important medicinal plant, has been widely used in folklore medicine in India and many other countries for the treatment of various hepatic disorders along with other *Cajanus species*. A 43kDa protein was isolated from *Cajanus indicus* plant showed hepatoprotective effects against thioacetamide induced^{17, 18}, chloroform induced¹⁹ and acetaminophen induced²⁰ hepatotoxicity in experimental animals. There is no evidence available about the in vivo therapeutic effects of this protein in the treatment of paracetamol induced hepatotoxicity. Based on the evidence available from *Cajanus indicus*, the experiment was carried out to isolate 43kDa protein from the leaves of plant *Cajanus acutifolius* and to find out whether the protein of our interest could ameliorate the PCM induced hepatic damage. Experiments were designed accordingly to evaluate both the preventive as well as curative role of this protein against PCM induced hepatic toxicity in vivo.

MATERIALS AND METHODS

Plant Material

Cajanus acutifolius is a shrub belonging to the family Leguminosae and subfamily Papilionaceae. Fresh young leaves were procured from a commercial supplier and cultivator Rati Ram who had cultivated *C. acutifolius* in his nursery at village Khurrampur, district Saharanpur (Uttar Pradesh, India).

Chemicals

All the chemicals and solvents were of analytical grade and were procured from Ranbaxy Fine Chemicals Ltd., Mumbai, India. Paracetamol (PCM) was procured from E.Merck (India) Ltd, Mumbai. The standard drug silymarin was obtained from Cadila Pharma Ltd, India. Standard kits for SGOT, SGPT, SALP, uric acid and bilirubin were obtained from Span Diagnostics Ltd, India.

Animals

Swiss albino rat (male, body weight 150 ± 20 g) was used for the experiments. The animals were acclimatized under standard laboratory conditions for a fortnight before starting the experiments. They were provided with standard diet and water ad libitum. They were maintained under standard conditions of

temperature (30°C) and humidity (50%) with an alternating 12 hours light/dark cycles. All experimental protocols were reviewed and approved by the Institutional Animal Ethical Committee (IAEC) prior to the initiation of the experiment and the care of the laboratory animals was taken as per the CPCSEA regulations.

Preparation of the homogeneous protein from the leaves of *Cajanus acutifolius*

The protein was purified from the leaves of *Cajanus acutifolius* according to the method described by Sarkar¹⁸. The leaves were homogenized in 20 mM tris-HCl buffer, pH 7.4 and the supernatant was brought to 60% (NH₄)₂SO₄ saturation. The pellet was reconstituted and dialyzed in tris-HCl buffer, passed through DEAE Sephadex column and eluted in linear gradient of 0–1 M NaCl in tris buffer. The active fraction eluted at 0.2 M NaCl was concentrated and applied on a Sephadex G-50 column. The bioactive fraction obtained was subjected to a C₁₈ hydrophobic column for reverse phase column chromatography. The homogeneity of preparation was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Pre treatment with the protein

The pre-treatment group was divided into five sub-groups each consisted of six rats. Group I received only single daily dose of vehicle i.e normal saline (5ml/kg), served as normal control. Group II served as toxin control, in which PCM was administered intraperitoneally at a dose of 750mg/kg suspended in sucrose solution (40% w/v) on 7th day after 6 days of vehicle treatment. Group III, and IV was pre-treated with the protein by giving intraperitoneal injection at a dose of 500 and 1000mg/kg body weight once daily and the treatment was carried out for 7 days followed by the toxin treatment for another 7 days. A well-known hepatoprotective agent Silymarin, (at a dose of 50mg/kg body weight) was used as the positive control for the study in group V. After silymarin treatment for 7 days, the animals were treated with PCM for another 7 days. The animals were then sacrificed under anesthesia and liver were collected.

Post-treatment with the protein

The post-treatment group was divided into five sub-groups each consisted of six rats. Group I and II received the similar treatment as in pretreatment method. Group III, and IV of rat was intoxicated with PCM (750 mg/kg body weight, once daily) for 3 days followed by intraperitoneal administration of the protein at a dose of 500 and 1000mg/kg respectively, once daily for 4 days. In group V, a positive control group was kept in which rats were treated with 50 mg/kg body weight Silymarin for 4 days after PCM intoxication for 3 days. Rats were then sacrificed under light ether anesthesia and liver was collected.

Assessment of hepatoprotective activity

The animals were sacrificed by cervical decapitation; blood was withdrawn by intracardiac puncture, blood was allowed to coagulate for 30 min and serum was separated by centrifugation at 2500 rpm

for 10 min and stored at 4°C until use. The serum was used to estimate serum glutamate oxaloacetate transaminases²¹ (SGOT), serum glutamate pyruvate transaminase²¹ (SGPT), serum alkaline phosphatase²² (SALP), uric acid²³, total protein²⁴ and total bilirubin content²⁵. After collection the blood the liver was immediately excised and rinsed in ice cold normal saline, blotted with filter paper and weighed.

Histopathological studies

Portions of the liver from all the experimental groups were fixed in 10% neutral formalin, dehydrated in graded alcohol and then embedded in paraffin. Microtome sections (5 µm thick) were prepared from each liver sample and stained with haematoxylin - eosin (H&E) dye. The sections were examined for the pathological findings of hepatotoxicity²⁶.

Measurement of antioxidant activity

From all the experimental groups, the portion of the liver was collected and rinsed with 0.15 M Tris- HCl (pH-7.4). A 10% w/v of liver homogenate was prepared in 0.15 M Tris-HCl buffer and processed for the estimation of lipid peroxidation in the form of malondialdehyde (MDA) in liver by measuring the thiobarbituric acid reactive substance (TBARS)²⁷. From part of the homogenate, after precipitating proteins with 20% trichloro-acetic acid containing 1 mM EDTA, the supernatant was used for reduced glutathione (GSH) estimation²⁸. The rest of the homogenate was centrifuged at 2000 rpm for 10 min at 4°C. The cell free supernatant thus obtained was used for the estimation of super oxide dismutase (SOD)²⁹ and catalase (CAT) activity³⁰.

Statistical analysis

The data were represented as mean ± SEM, (n = 6). Data were analyzed using one way analysis of variance (ANOVA) followed by the Tukey's multiple comparison post hoc test (SPSS 10 software was used). Difference were considered significant at p<0.05.

RESULTS

Effect of protein on serum marker enzyme levels:

There was significant elevation in the levels of serum marker enzymes like SGOT, SGPT and SALP content of PCM intoxicated animals. In contrast pretreatment and post treatment with protein (500 and 1000 mg/kg) and silymarin (50 mg/kg) exhibited an ability to counteract the hepatotoxicity by decreasing by decreasing serum marker enzyme levels in a dose dependent manner (P<0.05) (Table 1).

Effect of protein on biochemical parameters

In PCM treated groups, there was a significant increase in total bilirubin and significant reduction in uric acid and total protein content. Whereas, pretreatment and post treatment with protein caused significant reduction in total bilirubin and significant increase in the activities of uric acid and total protein content dose dependently (Table 1).

Table 1: Effect of protein on serum enzyme and biochemical parameters in PCM induced hepatic damage in rats

Groups (n=6)	Dose (mg/kg)	Biochemical parameters (mean±S.E.M), n=6					
		SGOT (U/L)	SGPT (U/L)	SALP (U/L)	Uric acid (mg/dl)	Total protein (mg/dl)	Total bilirubin (mg/dl)
I Vehicle control	5 ml	40 ±1.43	32.27 ± 4.1	36 ±0.43	2.42 ± 0.32	6.38 ±0.22	1.02 ± 0.18
II PCM control	750	127±1.5 4 ^a	95.08±2.54 ^a	104±0.23 ^a	1.32 ± 0.26 ^a	5.12 ±0.14 ^a	2.22 ± 0.26 ^a
III Protein +PCM (Pre-treated)	500	94 ± 1.22 ^d	73 ± 1.32 ^d	81 ±0.47 ^d	1.76 ± 0.16 ^b	5.71 ± 0.22 ^b	1.86 ± 0.19 ^b
Protein+PCM (Post-treated)	500	99 ± 0.4 ^d	78 ± 1.14 ^d	88 ± 0.36 ^c	1.58 ± 0.24 ^b	5.28 ± 0.12 ^b	1.99 ± 0.21 ^b
IV Protein +PCM (Pre-treated)	1000	60 ±1.43 ^d	48 ±2.56 ^d	61±0.98 ^d	2.14 ± 0.23 ^c	6.04 ± 0.16 ^d	1.24 ± 0.14 ^d
Protein +PCM (Post-treated)	1000	62 ±0.98 ^d	53 ±1.43 ^d	68±1.43 ^d	2.04 ± 0.26 ^d	5.76 ± 0.18 ^d	1.48 ± 0.16 ^d
V Silymarin+PCM (Pre-treated)	50	48 ±1.51 ^d	39 ±1.78 ^d	43±1.20 ^d	2.34 ±0.15 ^d	6.26 ±0.8 ^d	1.09 ±0.28 ^d
Silymarin+PCM (Post-treated)	50	56 ± 1.23 ^d	48 ± 1.26 ^d	52 ± 1.67 ^d	2.26± 0.22 ^d	6.02 ± 0.9 ^d	1.28 ± 0.34 ^d

One way ANOVA followed by Tukey's multiple comparison post hoc test. ^a P< 0.001 when compared with vehicle treated control group, ^b P< 0.05, ^c P< 0.01, ^d P< 0.001 when compared with PCM treated control group.

Effect of protein on antioxidant activity

There was significant increase in MDA content and reduction in GSH, SOD and CAT activities of PCM intoxicated animals (Table 2). Pretreatment and post treatment with protein (500 and 1000 mg/kg) and silymarin significantly ($P < 0.05$) prevented the increase in MDA levels and brought them near to normal levels, whereas GSH, SOD and CAT levels were significantly ($P < 0.05$) raised, thus providing protection against PCM toxicities.

Effect of protein on liver weight

The weight of the liver was significantly increased in PCM intoxicated animal groups, but it was normalized in silymarin and

protein treated groups of animals (Table 2). A significant reduction in ($P < 0.05$) in liver weight supports this finding.

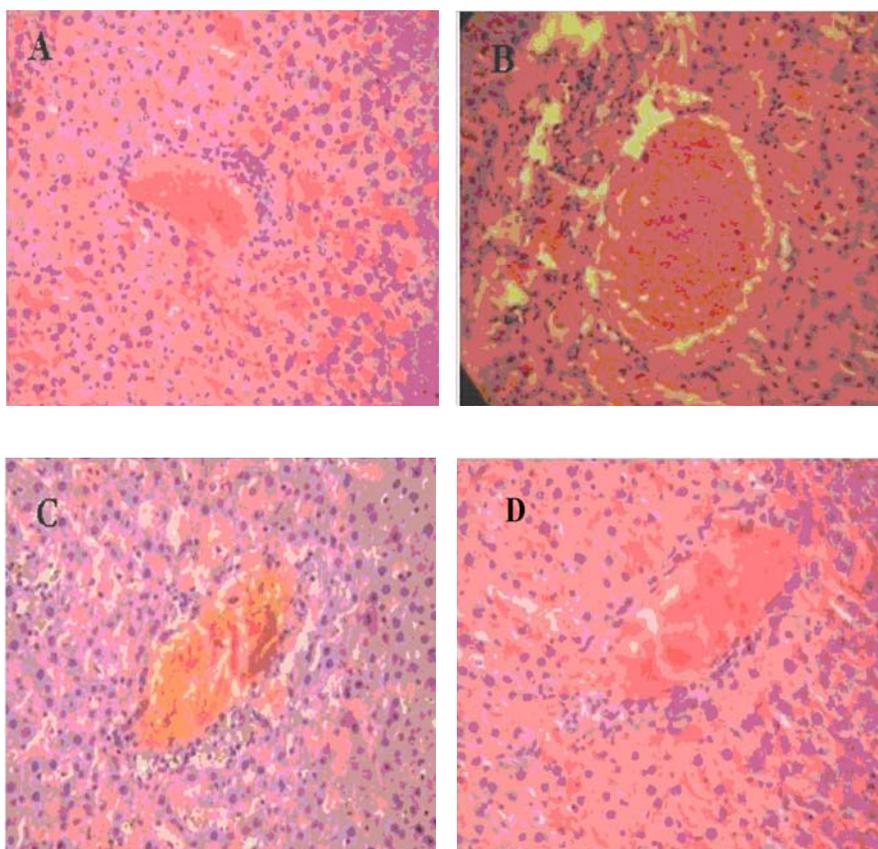
Histopathology

Histopathological studies also provided a supportive evidence for biochemical analysis. The liver sections of the rats of PCM intoxicated groups showed hepatic cells with severe toxicity characterized by inflammatory infiltration and necrosis in many areas. Treatment with silymarin and protein exhibited significant liver protection against PCM induced liver damage, which is evident by the presence of more or less normal hepatocytes and reduced inflammatory infiltration and necrosis (Fig 1 A-E). The effect is more pronounced for protein at dose level of 1000 mg/kg.

Table 2: Effect of protein on lipid peroxidation (LPO), glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and liver weight in PCM induced hepatic damage in rats.

Groups (n=6)	Dose (mg/k g)	Measurement of antioxidant activity (mean±S.E.M), n=6				
		LPO (nM MDA/mg protein bw)	GSH (µg/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)	Liver weight (Wt/100g)
I Vehicle control	5 ml	0.96 ± 0.12	5.43 ± 0.13	98.51 ± 1.37	368.41 ± 3.92	3.41 ± 0.12
II PCM control	750	7.12 ± 1.78 ^a	0.72 ± 0.10 ^a	58.07 ± 1.14 ^a	254.72 ± 2.92 ^a	6.28 ± 0.32 ^a
III Protein +PCM (Pre-treated)	500	3.98 ± 0.16 ^d	3.64 ± 0.11 ^d	63.72 ± 1.64 ^d	316.06 ± 1.34 ^b	5.43 ± 0.22 ^b
Protein+PCM (Post-treated)	500	4.12 ± 0.19 ^d	3.24 ± 0.10 ^d	56.34 ± 1.44 ^c	306.14 ± 1.23 ^b	5.65 ± 0.19 ^b
IV Protein +PCM (Pre-treated)	1000	1.56 ± 0.12 ^d	4.96 ± 0.12 ^d	84.24 ± 1.29 ^d	346.27 ± 2.44 ^c	4.18 ± 0.12 ^c
Protein +PCM (Post-treated)	1000	1.86 ± 0.16 ^d	4.67 ± 0.16 ^d	74.22 ± 1.24 ^d	326.24 ± 2.57 ^d	4.58 ± 0.16 ^d
V Silymarin+PCM (Pre-treated)	50	1.20 ± 0.86 ^d	5.36 ± 0.11 ^d	95.64 ± 1.74 ^d	364.30 ± 1.72 ^d	3.58 ± 0.14 ^d
Silymarin+PCM (Post-treated)	50	1.47 ± 0.56 ^d	4.98 ± 0.15 ^d	88.43 ± 1.46 ^d	348.24 ± 2.45 ^d	4.05 ± 0.23 ^d

One way ANOVA followed by Tukey's multiple comparison post hoc test. ^a $P < 0.001$ when compared with vehicle treated control group, ^b $P < 0.05$, ^c $P < 0.01$, ^d $P < 0.001$ when compared with PCM treated control group.



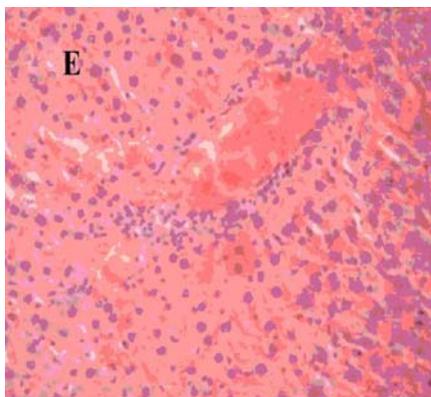


Fig. 1: Effect of ethanolic extract of the roots of *Cajanus acutifolius* on PCM induced liver damage in rats. (A) Photomicrograph of vehicle treated group- showing normal architecture of hepatic cells; (B) PCM treated group – showing inflammatory infiltration, fatty changes and necrosis; (C) Liver of rats pretreated with silymarin prior to PCM administration- showing normal architecture with less fatty changes; (D) Photomicrograph of liver of rats pretreated with protein 500 mg/kg prior to PCM administration- moderate accumulation of fatty lobules and cellular necrosis were observed; (E) Photomicrograph of liver of rats pretreated with protein 1000 mg/kg prior to PCM administration- showing a pattern of reduced inflammatory infiltration. Significant and more pronounced liver protection as evident by the presence of normal hepatic cords, absence of necrosis and fatty infiltration.

DISCUSSION

The aim of the present study was to investigate whether the protein isolated from the herb *Cajanus acutifolius* possesses any preventive as well as curative role against PCM induced hepatic damages. PCM induced hepatic injuries are commonly used models for the screening of hepatoprotective drugs and the extent of hepatic damage is assessed by the level of released cytoplasmic alkaline phosphatase and transaminases in circulation. It is well documented that PCM is biotransformed under the action of microsomal cytochrome-P450 of liver to reactive metabolites^{31, 32}. These free radicals bind covalently to unsaturated lipid membrane, provoking a sharp increase of lipid peroxides followed by pathological changes such as elevated levels of serum marker enzymes like SGOT, SGPT and SALP, depletion of GSH, decreased protein synthesis, triglyceride accumulation, increased lipid peroxidation, destruction of Ca^{2+} homeostasis and finally hepatocyte damage³³. This suggests that PCM induces liver injury by sharing a common property of free radical mechanism.

Hepatocellular necrosis or membrane damage leads to very high levels of serum GOT and GPT released from liver to circulation. Among the two, GPT is a better index of liver injury, since SGPT catalyses the conversion of alanine to pyruvate and glutamate, and released in a similar manner, thus liver GPT represents 90% of the total enzyme present in the body³⁴. The elevated levels of serum marker enzymes are indicative of cellular leakage and loss of functional integrity of cellular membrane and liver³⁵. SALP activities on the other hand are related to functioning of hepatocytes, its increase in serum is due to increased synthesis in the presence of increased biliary pressure³⁶. It is well known that toxicants like PCM produce sufficient injury to hepatic parenchyma cells to cause elevation in serum bilirubin, and in contrast decrease the level of total plasma protein content³⁷.

In the present study protein at a dose of 500 and 1000 mg/kg (Post & Pre treated) caused a significant inhibition in the levels of SGOT and SGPT towards the respective normal range and this is an indication of stabilization of plasma membrane as well as repair of hepatic tissue damage caused by PCM. On the other hand suppression of elevated SALP activities with concurrent depletion of raised bilirubin level and an increase in the total plasma protein content suggests the stability of biliary dysfunction in rat liver during hepatic injuries with toxicants³⁸. These results indicate that protein preserved the structural integrity of the hepatocellular

membrane and liver cell architecture damaged by PCM which was confirmed by histopathological examination (Fig 1A-E).

PCM caused a significant increase in the liver weight, which is due to blocking of secretion of hepatic triglycerides into the plasma³⁹. The reduced level of uric acid in PCM induced hepatotoxicity is probably due to the increased utilization of uric acid against increased production of free radicals, which is a characteristic feature of cancer and tissue necrosis. The results from the present study suggests that altered uric acid level to nearly normal in protein treated animals could be due to strong antioxidant property of the extract.

Lipid peroxidation has been postulated to be the destructive process in liver injury due to PCM administration⁴⁰. In the present study, an elevation in the levels of MDA in liver of animals treated with PCM was observed. The increase in MDA levels of liver suggest enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radicals. Pretreatment with protein significantly reduced the levels of lipid peroxidation. Hence it may be possible that the mechanism of hepatoprotection by protein is due to its antioxidant potential.

Glutathione (GSH) is one of the most abundantly naturally occurring tripeptide, non-enzymatic biological antioxidant present in liver⁴¹. Its functions are concerned with the removal of free radicals such as H_2O_2 and superoxide radicals, maintenance of membrane protein, detoxification of foreign chemicals and biotransformation of drugs⁴². In the present study, the decreased levels of GSH have been associated with an enhanced level of lipid peroxidation in PCM intoxicated group of rats. Pretreatment and Post treatment with protein significantly increased the level of glutathione in a dose dependent manner. Thus protein may act by inducing the detoxifying enzymes and these enzymes may detoxify the reactive oxygen species (ROS) following administration of toxicants.

Serum activities of superoxide dismutase (SOD) and catalase (CAT) are the most sensitive enzymatic index in liver injury caused by ROS and oxidative stress. SOD is one of the most abundant intracellular antioxidant enzymes present in all aerobic cells and it has an antitoxic effect against ROS⁴³. CAT is a haemoprotein; it protects the cells from the accumulation of H_2O_2 and O_2 ⁴⁴. Therefore reduction in the activities of these enzymes may indicate the toxic effects of ROS produced by toxicants. In the present study, it was observed that pre treatment with protein caused a significant rise in

hepatic SOD and CAT activities. This suggests that protein can reduce ROS that may lessen the oxidative damage to the hepatocytes and improve the activities of the liver antioxidant enzymes thus protecting the liver from PCM.

CONCLUSION

It can be concluded that data obtained in the present study suggest that the protein possess significant hepatoprotective and antioxidant activities on PCM induced hepatic damage in rats. These results reveal that the hepatoprotective effect of the protein may be due to its ability to block the bioactivation of toxicant and its potent antioxidant activity, and/or by scavenging the free radicals and inhibiting lipid peroxidation. PCM administration caused oxidative insult in the liver. Protein treatment both prior and post to the toxin administration normalize that stress in the organ. As PCM induced liver failure seems to occur at the end stage of liver cirrhosis, the protective role of the protein against PCM induced renal damages is likely to be an indirect effect probably comes to play via the protection of hepatic disorders. Further works are however needed to define the exact mechanisms by which the protein exhibits this protective action and are currently in progress.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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