

Protein isolate from the herb, *Phyllanthus niruri* L. (Euphorbiaceae), plays hepatoprotective role against carbon tetrachloride induced liver damage via its antioxidant properties

Rajesh Bhattacharjee, Parames C. Sil *

Department of Chemistry, Bose Institute, 93/1, Acharya Prafulla Chandra Road, Kolkata 700009, West Bengal, India

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Abstract

Phyllanthus niruri L. (Euphorbiaceae) (*P. niruri*) is a well-known hepatoprotective herbal plant. In the present study, hepatoprotective potential of the protein isolate of *P. niruri* was investigated against carbon tetrachloride (CCl₄) induced liver damage in vivo. Protein isolate of *P. niruri* was intraperitoneally injected in mice either prior to (preventive) or after the induction of toxicity (curative). Levels of different liver marker enzymes in serum and different anti-oxidant enzymes, as well as lipid peroxidation products and glutathione (GSH) in liver homogenates were measured in normal, control (toxicity induced) and protein isolate treated mice. Administration of CCl₄ increased the serum glutamate pyruvate transaminase (GPT) and alkaline phosphatase (ALP) levels of mice sera along with increased lipid peroxidation and reduced levels of antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) in the liver. Treatment with the protein isolate of *P. niruri* significantly altered these changes to almost normal. The protein isolate also showed protective properties as was evidenced in histopathological studies. Results suggest that the protein isolate of *P. niruri* protects liver tissues against oxidative damage and somehow helps stimulating repair mechanism present in liver. It could be used as an effective hepatoprotector against CCl₄ induced liver damage.

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Keywords: CCl₄-induced hepatotoxicity; *Phyllanthus niruri*; Protein isolate; Antioxidant; Hepatoprotection

1. Introduction

Herbal products have been used since the dawn of civilization on the earth to maintain human health and to get the remedies from various diseases by the vast majority of the world's population (Myagmar et al., 2004; Bei et al., 2004; Guerra et al., 2000; Chauhan et al., 1992; Gyamfi et al., 2002). Among these herbs, *Phyllanthus niruri*, has

been widely used for the treatment of jaundice and other hepatic disorders (Syamasundar et al., 1985; Unander et al., 1995; Lin et al., 2003). Venkateswaran et al. (1987) reported that there are one or more materials in *P. niruri*, which inhibit the replication of woodchuck hepatitis virus in vivo and decrease the pathological effect of the same virus on woodchuck liver. Moreover clinical studies on humans have also been carried out with *P. niruri* without any side effect (Thyagarajan et al., 1988). The herb has been reported to possess potent protective activity against various hepatic disorders like viral hepatitis and toxicity caused by different drugs and environmental toxicants (Unander et al., 1995; Naik and Juvekar, 2003; Padma and Setty, 1999; Sebastian and Setty, 1999). But mechanisms responsible for this hepatoprotective action of *P. niruri*, however are not well defined.

Abbreviations: ALP, alkaline phosphatase; CAT, catalase; CCl₄, carbon tetrachloride; GPT, glutamate pyruvate transaminase; GSH, glutathione; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances.

* Corresponding author. Tel.: +91 33 23506619/2402/2403x412(lab), 413(off); fax: +91 33 2350 6790.

E-mail addresses: parames@bosemain.boseinst.ac.in, parames_95@yahoo.co.in (P.C. Sil).

In recent years attention has been focused on the role of biotransformation of chemicals to highly reactive metabolites that initiate cellular toxicity. Many compounds including clinically useful drugs can cause cellular damage through metabolic activation of the compound to highly reactive substances such as free radicals. One such environmental toxicant is CCl_4 and its administration is widely used as different animal models of liver damage. Hepatotoxicity of CCl_4 is attributed to the formation of trichloromethyl and trichloromethyl peroxy radicals, initiating lipid peroxidation and resulting in fibrosis and cell necrosis (Recknagel et al., 1989; Kadiiska et al., 2000). CCl_4 is metabolically activated by Cyt-P450 into the trichloromethyl radical (Poyer et al., 1980; Noguchi et al., 1982) which in the presence of oxygen gets converted into the peroxy radical (Packer et al., 1978). The interaction of these free radicals with the biological system cause cellular damage by initiating lipid peroxidation, covalently binding to proteins, causing a rise in intracellular Ca^{2+} , depleting GSH or releasing iron. These events ultimately establish a condition of oxidative stress in which the defense capabilities of cells against ROS, like the superoxide anion, hydrogen peroxide and the hydroxyl radical, becomes insufficient.

Present study was focused on investigating the role of protein isolate of *P. niruri* against CCl_4 induced hepatic disorder and to find out its possible mode of action in hepatoprotection. Mice were injected with the protein isolate either prior to (preventive) or after the induction of toxicity (curative). Thereafter, serum levels of different marker enzymes related to hepatic integrity, like, GPT and ALP were determined. To evaluate whether the protein isolate of *P. niruri* has any antioxidant properties, its effect on the toxin-induced cellular levels of different antioxidant enzymes (like SOD, CAT), non-protein thiol GSH and lipid peroxidation in the liver were also determined. In addition, histopathological studies were done to prove its effectiveness in the preventive and curative role against CCl_4 induced toxicity in vivo.

2. Material and methods

2.1. Materials

Fresh *P. niruri* was collected from local market. $(\text{NH}_4)_2\text{SO}_4$, SDS, EDTA, PMSF, Na_2HPO_4 , MnCl_2 , 1-chloro-2,4 dinitro benzene (CDNB), 5, 5' dithiobis-2-nitrobenzoic acid (DTNB), EDTA, 2-mercaptoethanol, GSH, Trichloroacetic acid (TCA), Thiobarbituric acid, diethanol amine and triethanol amine were purchased from Sisco Research Laboratories (SRL). Kits for the determination of SGPT and ALP were purchased from Span Diagnostics Ltd., India.

2.2. Bioassay for measuring CCl_4 induced hepatotoxicity

Swiss albino mice (male, body weight 25 ± 2 g) were acclimatized under laboratory condition for two weeks before starting experiments. They were provided with standard pellet diet and water ad libitum. Research was conducted in accordance with the internationally accepted principles for laboratory animal use and care. The animals were divided into three groups consisting of six animals each for different experiments. First group served as controls and received paraffin oil orally instead of

CCl_4 and phosphate buffer was administered intraperitoneally instead of protein isolate. Second group received only CCl_4 orally at a dose of 10 mmol/kg of body weight for 2 days and injected with phosphate buffer (5 days) for respective experiments. Third group was post-treated with the experimental samples for 5 days by intraperitoneal injection at a dose of 5 mg/kg body weight after CCl_4 treatment at the same dose as above.

2.3. Determination of the effect of aqueous and methanol extract of the leaves *P. niruri* on CCl_4 induced hepatotoxicity

The air-dried leaf powder was extracted with methanol (100 gm/l) with constant shaking for 4 h. The extract was filtered and the filtrate was evaporated to dryness at 50 °C under reduced pressure in a rotary evaporator. About 5 gm of dried methanol extract was obtained from 100 gm dried leaf. The extract was suspended in 5% Tween -80 and was used for bioassay at a dose of 5 mg/kg body weight. To prepare the aqueous extract, the leaves were homogenized in phosphate buffer, pH 7.4, and centrifuged at 12,000g for 15 min. The supernatant was dialysed against water, concentrated by lyophilisation and resuspended in the same phosphate buffer. The aqueous extract was used for the bioassay using a concentration of 5 mg/kg body weight.

2.4. Determination of the effect of the aqueous extract administered orally and intraperitoneally on hepatotoxicity

The aqueous extract of the leaves of *P. niruri* was administered to two different groups of mice separately via two routes, orally and intraperitoneally for 5 days after CCl_4 administration at a dose of 5 mg/kg body weight. After 24 h of final dose of the sample, the mice were sacrificed and serum marker enzyme levels were measured.

2.5. Extraction of proteins from the leaves of *P. niruri*

Fresh leaves of *P. niruri* were extracted in 50 mM phosphate buffer, pH-7.4 containing 1 mM EDTA, 3 mM PMSF, 0.1 M sodium ascorbate and 1.5% (w/v) polyvinyl pyrrolidone (PVP) (Loomis, 1969), at 4 °C in a cyclomixer. The extract was then filtered through cheesecloth and the filtrate was centrifuged at 20,000g for 30 min at 4 °C. The resultant clear supernatant was brought to 60% $(\text{NH}_4)_2\text{SO}_4$ saturation and kept overnight. It was then centrifuged at 20,000g for 30 min at 4 °C and the pellet collected was reconstituted in 50 mM phosphate buffer, pH-7.4, and dialysed to remove the salt. All the experiments described here were carried out using this 60% $(\text{NH}_4)_2\text{SO}_4$ saturated protein isolate.

2.6. Determination of the effect of proteinous and non-proteinous aqueous fraction on hepatotoxicity

To separate the protein and non-protein part from the aqueous leaf extract, 60% $(\text{NH}_4)_2\text{SO}_4$ precipitation was done. The precipitated protein was collected by centrifugation at 12,000g for 30 min and dialysed to remove excess $(\text{NH}_4)_2\text{SO}_4$. The protein concentration was measured by the method of Bradford (1976) and administered to mice at a dose of 5 mg/kg body weight. With the remaining supernatant 100% $(\text{NH}_4)_2\text{SO}_4$ precipitation was done. Supernatant non-proteinous part was dialysed to remove excess $(\text{NH}_4)_2\text{SO}_4$. The non-proteinous aqueous fraction was dried, reconstituted in phosphate buffer and administered at a dose of 5 mg/kg body weight in mice.

2.7. Determination of the effect of heat treatment on the hepatoprotective activity of the protein fraction

To determine the effect of heat on the biological activity of the protein isolate, it was heated at 90 °C for 5 min, cooled and then intraperitoneally injected (5 mg/kg body weight) into mice after CCl_4 intoxication

(10 mmol/kg body weight). A control group was kept in which the biologically active protein isolate was administered at the same dose. After 24 h of the last dose of the protein, all the mice were sacrificed. From blood serum, GPT and ALP levels were measured.

2.8. Determination of the effect of protease treatment on the hepatoprotective protein

To determine the effect of protease trypsin on the biological activity of the protein isolate, the protein isolate and trypsin were incubated at 37 °C for 1 h and then it was administered to mice intraperitoneally for 5 days after CCl₄ intoxication (10 mmol/kg body weight). Two control groups were kept. In the first one only trypsin was administered along with phosphate buffer and another control group was kept where the bioactive protein isolate was administered.

2.9. Protein estimation

Protein concentrations were measured following the method of Bradford (1976).

2.10. Animal experiments

Swiss albino mice (male, body weight 25 ± 2 g) were acclimatized under laboratory condition for two weeks before starting experiments. The animals were divided into several groups of six in different models of experiments.

First group served as controls and received paraffin oil orally for CCl₄ and phosphate buffer intraperitoneally for protein. Second group received only CCl₄ orally at a dose of 10 mmol/kg of body weight for 2 days and injected with phosphate buffer for respective experiments. Third group was pretreated with the protein isolate for 5 days by intraperitoneal injection at a dose of 5 mg/kg body weight of mice and followed by CCl₄ at the same dose as above. The concentration of protein isolate to be used was determined by carrying out a dose response study with the protein isolate pretreated group using different doses of protein concentrations which were 2.5 mg/kg, 5 mg/kg, 10 mg/kg, 20 mg/kg and 40 mg/kg of body weight.

Fourth group were post treated with the protein isolate for 5 days in the same way as above after CCl₄ treatment. With this group a time course study was done by injecting *P. niruri* protein isolate for 1, 2, 3, 4, 5 and 6 days. Optimum result was obtained when protein was administered for 5 days.

Fifth group was treated with protein isolate (5 mg/kg body weight) by intraperitoneal injection and served as the protein isolate control group in all the experiments.

2.11. Evaluation of liver function

Blood samples collected from puncturing mice hearts were kept overnight to clot and then centrifuged at 3000g for 10 min. About 200 mg liver tissue was homogenized in 10 volumes of appropriate buffer and centrifuged at 10,000g for 20 min at 4 °C. The supernatant and blood serum were used to estimate the hepatotoxicity produced by CCl₄.

2.12. GPT and ALP measurement

Serum GPT and ALP were measured by the methods of Reitman and Frankel (1957) and Kind and King (1954), respectively.

2.13. CAT and SOD assays

The CAT activity was measured in liver homogenates by the method of Bonaventura et al. (1972). For the assay, the liver homogenates containing 5 µg total protein was mixed separately with 700 µl, 5 mM hydrogen peroxide and incubated at 37 °C. The disappearance of peroxide was

observed at 240 nm for 15 min. One unit of catalase activity is that which reduces 1 µmol of hydrogen peroxide per minute.

The principle of measuring SOD is based on the oxidation of NADH mediated by super oxide radical (Paoletti et al., 1986). The presence of super oxide dismutase in the reaction mixture inhibits the rate of oxidation of NADH by scavenging the super oxide radicals. Triethanolamine–diethanolamine (TDB) buffer, NADH solution, EDTA–MnCl₂ solution and sample (2 µg total protein) were mixed thoroughly and read at 340 nm for a stable baseline. Finally 2-mercaptoethanol was added, mixed and the decrease in absorbance was monitored at 340 nm for 20 min. A blank was run in the absence of the sample. The amount of superoxide dismutase activity was calculated with respect to the blank value. One unit of SOD is the amount of enzyme required to inhibit NADPH oxidation of blank by 50%.

2.14. Lipid peroxidation assay

Thiobarbituric acid reactive substances (TBARS), the last product in lipid peroxidation pathway, were measured using the modified method of Esterbauer and Cheeseman (1990). Liver tissue (200 mg) was homogenized in 10 volumes of ice-cold 50 mM Phosphate buffer (pH 7.4) and the homogenates were centrifuged at 12,000g for 15 min at 4 °C. The supernatant was used for the assay. Protein concentrations of different homogenates were measured according to the method of Bradford. Protein (1 mg) was incubated at 37 °C for 1 h and then 1 ml 20% TCA and 2 ml 0.67% TBA was added and heated for 30 min at 100 °C. Precipitate was removed by centrifugation at 1000g for 10 min. The absorbance of the samples was measured at 535 nm against a blank that contains all the reagents except the sample. TBARS concentration of the samples were calculated using the extinction co-efficient of MDA which is $1.56 \times 10^5 \text{ mmol}^{-1} \text{ cm}^{-1}$ as 99% of TBARS is MDA.

2.15. Measurement of hepatic GSH level

Hepatic GSH level was determined by the method of Ellman (1959) with slight modification. Briefly, 720 µl of the liver homogenate in 200 mM tris buffer, pH 7.2, was diluted to 1440 µl with the same buffer. Five percent TCA (160 µl) was added to it and mixed thoroughly. The samples were then centrifuged at 10,000g for 5 min at 4 °C. Supernatant (330 µl) was taken in a tube and 660 µl of Ellman's reagent (DTNB solution) was added to it. Finally the absorbance was taken at 405 nm.

2.16. Liver histopathology

Mice were divided into four groups and treated with CCl₄ and protein isolate as described earlier. 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 effect of *P. niruri* protein isolate on the CCl₄ treated mice liver.

2.17. Statistical analysis

All the values are represented as mean ± S.D. ($n = 6$). The statistical differences among different groups were analyzed by Student's *T*-test.

3. Results

3.1. Effect of aqueous and methanol extract of *P. niruri* on CCl₄ induced hepatotoxicity

Fig. 1A shows the effect of aqueous and methanol extracts of the leaves of *P. niruri* on CCl₄ induced hepatotoxicity. The increased SGPT level due to CCl₄ intoxication was significantly reduced when treated with aqueous

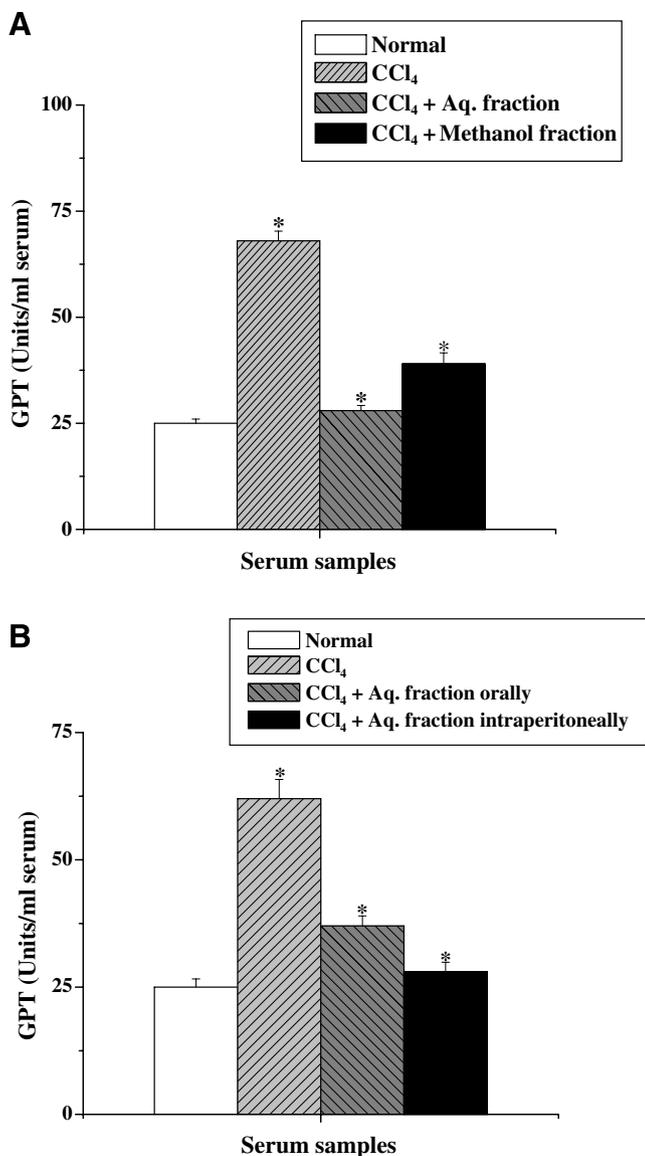


Fig. 1. A. Effect of aqueous and methanol extract of *P. niruri* leaves on CCl₄ induced hepatotoxicity. Normal mice (control); CCl₄ – CCl₄ treated mice at a dose of 10 mmol/kg body weight; CCl₄ + Aq. fraction – CCl₄ treated at a dose of 10 mmol/kg body weight for 2 days + Aqueous fraction of *P. niruri* post-treated at a dose of 5 mg/kg body weight for 5 days; CCl₄ + Methanol fraction – CCl₄ treated at a dose of 10 mmol/kg body weight for 2 days + Methanol fraction of *P. niruri* post-treated at a dose of 5 mg/kg body weight for 5 days. Each value represents mean \pm SD ($n = 6$). (* $P < 0.05$). B. Hepatoprotective activity of aqueous extract of *P. niruri* administered orally and intraperitoneally. Normal mice (control); CCl₄ – CCl₄ treated mice at a dose of 10 mmol/kg body weight; CCl₄ + Aq. fraction orally – CCl₄ treated at a dose of 10 mmol/kg body weight for 2 days + Aqueous fraction of *P. niruri* given orally at a dose of 5 mg/kg body weight for 5 days; CCl₄ + Aq. fraction intraperitoneally – CCl₄ treated at a dose of 10 mmol/kg body weight for 2 days + Aqueous fraction of *P. niruri* intraperitoneally injected at a dose of 5 mg/kg body weight for 5 days. Each value represents mean \pm SD ($n = 6$). (* $P < 0.05$).

extract of the leaves. The methanol extract of the leaves also had considerable lowering effect on the elevated SGPT level, but less when compared with the aqueous extract treated mice group. The vehicle, parafin oil had no hepatoprotective effect.

3.2. Hepatoprotective activity of aqueous extract of *P. niruri* administered orally and intraperitoneally

Fig. 1B shows the effect of aqueous extract of the leaves of *P. niruri* administered orally and intraperitoneally in two separate groups of mice, previously intoxicated with CCl₄. The hepatotoxicity, as revealed from SGPT levels, was lower in the mice treated with aqueous extract intraperitoneally than those treated with the aqueous fraction orally.

3.3. Effect of protein and non-protein aqueous fraction of *P. niruri* on CCl₄ induced hepatotoxicity

Fig. 2 shows the hepatoprotective effect of proteinous and non-proteinous aqueous fractions of *P. niruri* leaves on CCl₄ induced hepatotoxicity. From the SGPT values it was evident that the protein isolate obtained from the aqueous extract showed more hepatoprotective activity than the non-proteinous fraction when treated separately after CCl₄ intoxication.

3.4. Effect of heat treatment on the hepatoprotective activity of the protein

The preheated protein administration did not alter the elevated SGPT level in mice intoxicated with CCl₄

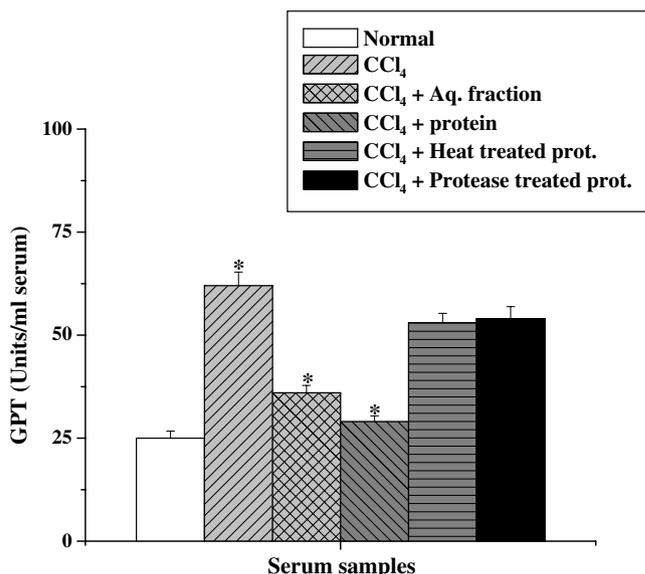


Fig. 2. Effect of different extracts of *P. niruri* leaves on CCl₄ induced hepatotoxicity. Normal mice (control); CCl₄ – CCl₄ treated mice at a dose of 10 mmol/kg body weight; CCl₄ + Aq. fraction – CCl₄ treated at a dose of 10 mmol/kg body weight for 2 days + Aqueous fraction of *P. niruri* post-treated at a dose of 5 mg/kg body weight for 5 days; CCl₄ + Protein – CCl₄ treated at a dose of 10 mmol/kg body weight for 2 days + Protein isolate of *P. niruri* post-treated at a dose of 5 mg/kg body weight for 5 days; CCl₄ + Heat treated prot. – CCl₄ treated at a dose of 10 mmol/kg body weight for 2 days + Heat treated protein isolate of *P. niruri* post-treated at a dose of 5 mg/kg body weight for 5 days; CCl₄ + Protease treated prot. – CCl₄ treated at a dose of 10 mmol/kg body weight for 2 days + Protease treated protein isolate of *P. niruri* post-treated at a dose of 5 mg/kg body weight for 5 days. Each value represents mean \pm SD ($n = 6$). (* $P < 0.05$).

compared to the effect of bioactive protein. Hence it may be concluded that the protein lost its hepatoprotective activity during heating. The result is shown in Fig. 2.

3.5. Effect of protease treatment on the hepatoprotective activity of the protein

Trypsin digested protein administration after CCl₄ intoxication failed to recover from the hepatotoxicity caused by CCl₄ as revealed from the SGPT values. The control where only trypsin was administered after CCl₄ had no effect on the hepatotoxicity. The bioactive protein administration significantly cured the damages caused by CCl₄. Hence, trypsin digestion resulted in loss of hepatoprotective activity of the protein. The result is shown in Fig. 2.

3.6. Effect of protein isolate of *P. niruri* against CCl₄-induced hepatotoxicity

3.6.1. Dose response study

A dose response study was done by injecting different concentrations of protein prior to CCl₄ administration. The doses used were, 2.5 mg/kg body weight, 5 mg/kg body weight, 10 mg/kg body weight, 20 mg/kg body weight and 40 mg/kg body weight of protein. Serum GPT levels and liver SOD levels of all the mice were measured. The results are shown in Fig. 3A and B. It is evident from the graphs that optimum results were obtained at a dose of 5 mg/kg body weight of protein, which was used for all the other experiments.

3.6.2. Time dependent study

A time dependent study was done by injecting protein isolate for 1, 2, 3, 4, 5 and 6 days after 2 days of CCl₄ administration. Serum GPT levels and liver SOD levels of these mice were measured. The results are shown in Fig. 4A and B. From the results it is evident that 5 days protein isolate injection gave optimum results. So, further experiments were carried out by injecting the protein isolate for 5 days.

3.6.3. Serum GPT and ALP levels

Effect of CCl₄ alone and effect of treatment with the protein isolate of *P. niruri* prior to and followed by CCl₄ administration on serum GPT and ALP levels have been shown in Fig. 5A and B, respectively. Increased GPT and ALP levels in the serum of the CCl₄ treated mice indicates severe liver damage. Protein isolate pretreatment as well as post-treatment for 5 days at a dose of 5 mg/kg body weight significantly reduced the levels of GPT and ALP levels in both the cases.

3.6.4. Liver histopathology

Histopathological samples were taken from normal, CCl₄ treated and protein isolate treated (both pre and post) mice liver. Histopathological assessment represented in

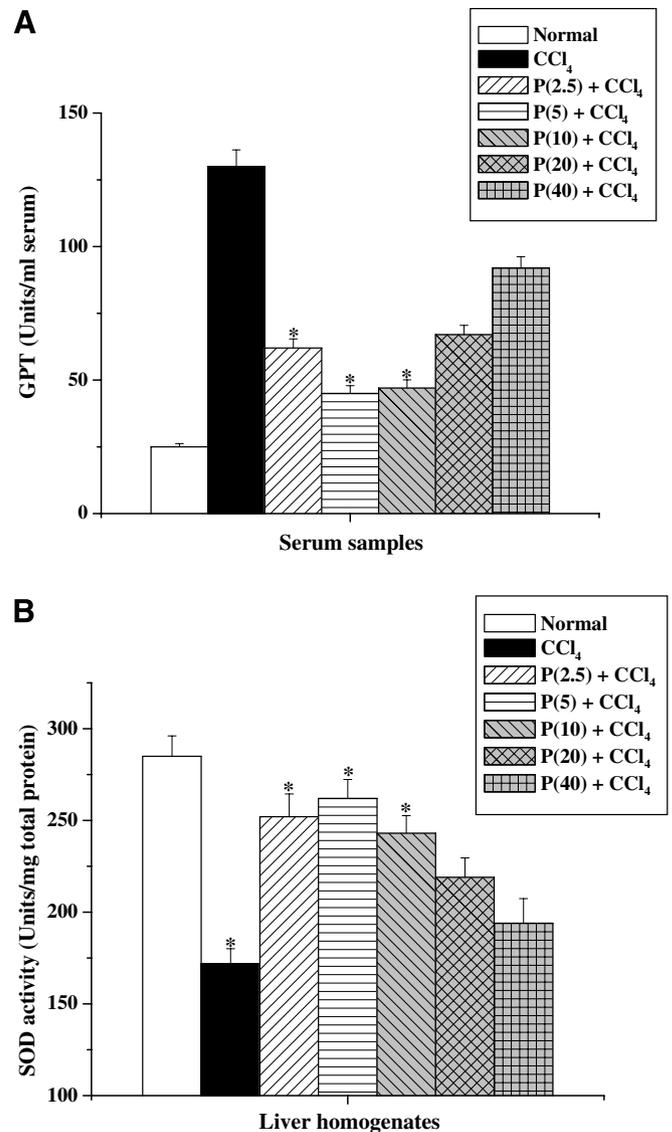


Fig. 3. Dose response study of the effects of protein isolate of *P. niruri* against CCl₄ toxicity. A. GPT levels – Normal mice (control); CCl₄ – CCl₄ treated mice at a dose of 10 mmol/kg body weight; P(2.5) + CCl₄ – protein pretreated at a dose of 2.5 mg/kg body weight + CCl₄ treated; P(5) + CCl₄ – protein pretreated at a dose of 5 mg/kg body weight + CCl₄ treated; P(10) + CCl₄ – protein pretreated at a dose of 10 mg/kg body weight + CCl₄ treated; P(20) + CCl₄ – protein pretreated at a dose of 20 mg/kg body weight + CCl₄ treated; P(40) + CCl₄ – protein pretreated at a dose of 40 mg/kg body weight + CCl₄ treated. Mice were pretreated with the protein fraction for 5 days as it gave optimum results. Each value represents mean ± SD (*n* = 6) (**P* < 0.05). B. Super oxide dismutase (SOD) levels – Legends of this figure is identical as that described in the legend of Fig. 1A.

Fig. 6, showed prominent changes including centrilobular necrosis, bile duct proliferation, disorganization of normal radiating pattern of cell plates around central vein etc., in tissues treated with CCl₄. Qualitatively, the inflammation in protein isolate treated mice liver before and after CCl₄ administration were less severe compared to that with CCl₄. There were no significant differences in the histology of CCl₄ controls sacrificed after 5 days. These observations

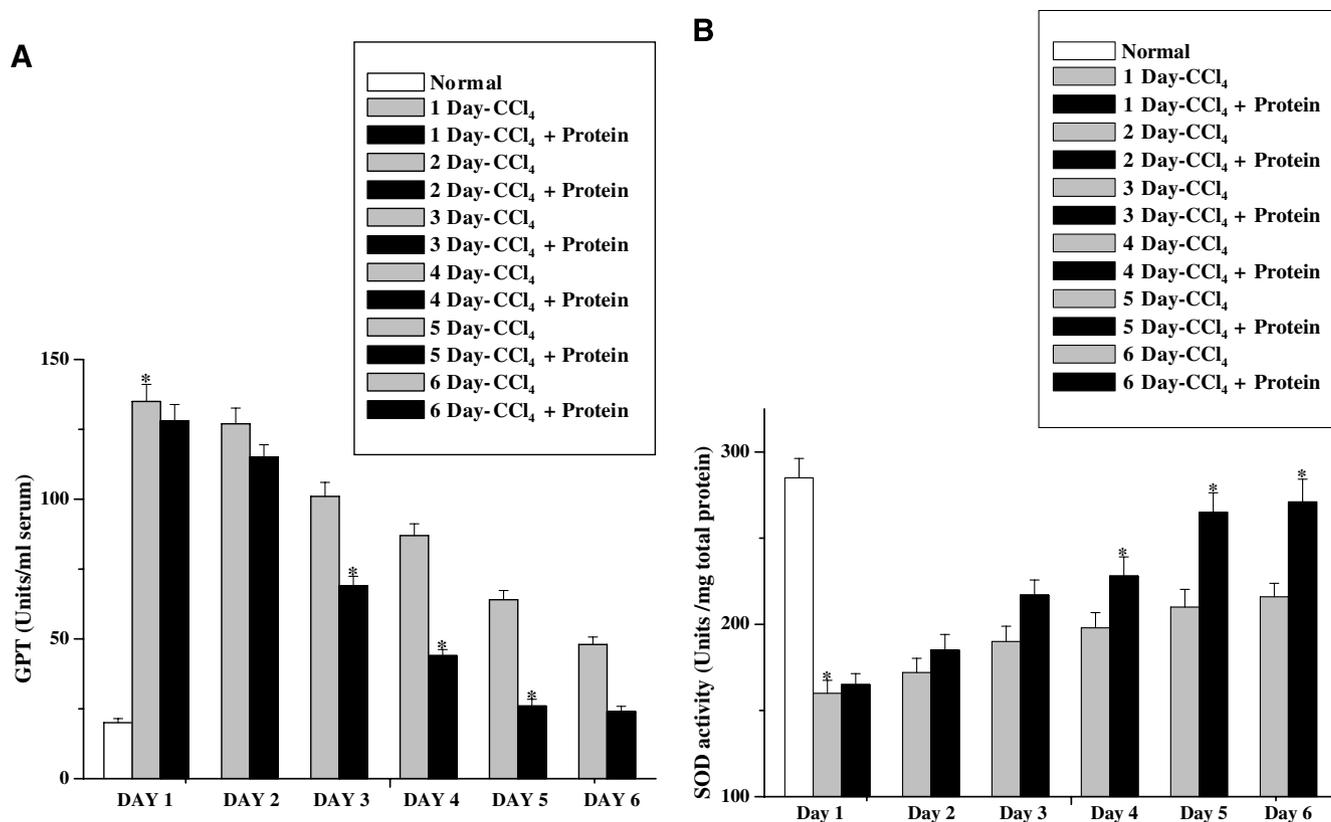


Fig. 4. Time dependent study of the effects of protein isolate of *P. niruri* against CCl₄ toxicity. A. GPT levels – Normal mice (control); 1 day CCl₄ – CCl₄ treated mice at a dose of 10 mmol/kg body weight and sacrificed after 1 day; 1 day CCl₄ + Protein – CCl₄ treated + protein post treated at a dose of 5 mg/kg body weight for 1 day; 2 day CCl₄ – CCl₄ treated mice at a dose of 10 mmol/kg body weight and sacrificed after 2 days; 2 day CCl₄ + Protein – CCl₄ treated + protein post treated at a dose of 5 mg/kg body weight for 2 days; 3 day CCl₄ – CCl₄ treated mice at a dose of 10 mmol/kg body weight and sacrificed after 3 days; 3 day CCl₄ + Protein – CCl₄ treated + protein post treated at a dose of 5 mg/kg body weight for 3 days; 4 day CCl₄ – CCl₄ treated mice at a dose of 10 mmol/kg body weight and sacrificed after 4 days; 4 day CCl₄ + Protein – CCl₄ treated + protein post treated at a dose of 5 mg/kg body weight for 4 days; 5 day CCl₄ – CCl₄ treated mice at a dose of 10 mmol/kg body weight and sacrificed after 5 days; 5 day CCl₄ + Protein – CCl₄ treated + protein post treated at a dose of 5 mg/kg body weight for 5 days. 6 day CCl₄ – CCl₄ treated mice at a dose of 10 mmol/kg body weight and sacrificed after 6 days; 6 day CCl₄ + Protein – CCl₄ treated + protein post treated at a dose of 5 mg/kg body weight for 6 days. Each value represents mean \pm SD ($n = 6$) (* $P < 0.05$). B. Super oxide dismutase (SOD) levels – Legends of this figure is identical as that described in the legend of Fig. 2A.

significantly prove the protective role of the protein isolate from *P. niruri*.

3.6.5. SOD and CAT activity

SOD and CAT activities in mice liver homogenates are shown in Fig. 7A and B, respectively. SOD and CAT activities in liver homogenates of CCl₄ treated group were found to be significantly lower than the normal group. Treatment of the mice with the protein isolate prior to and after CCl₄ treatment increased the activities of both the enzymes as compared to the CCl₄ treated mice.

3.6.6. Cellular lipid peroxidation

Lipid peroxidation, measured as MDA in the liver homogenates of experimental mice has been shown in Fig. 8A. MDA contents in liver homogenates of CCl₄ treated mice was increased significantly compared to the normal mice group. MDA level of protein isolate pre-treated group was lowered close to normal levels. In case of post-treatment MDA contents in liver homogenates of

CCl₄ treated mice was increased compared to the normal mice group. MDA level of protein isolate post-treated group was almost lowered to normal levels. So protein post-treatment inhibited lipid peroxidation caused by CCl₄ treatment.

3.6.7. Cellular GSH levels

Changes in GSH content of different liver homogenates has been shown in Fig. 8B. GSH level was reduced in CCl₄ treated mice compared to normal mice group. GSH level was increased in protein isolate pretreated mice. When post-treated with protein GSH contents in liver homogenates of CCl₄ treated mice were decreased as usual compared to the normal mice group. GSH level of protein isolate post-treated group was increased.

4. Discussion

The liver is a major target organ for toxicity of xenobiotics and drugs, because most of the orally ingested

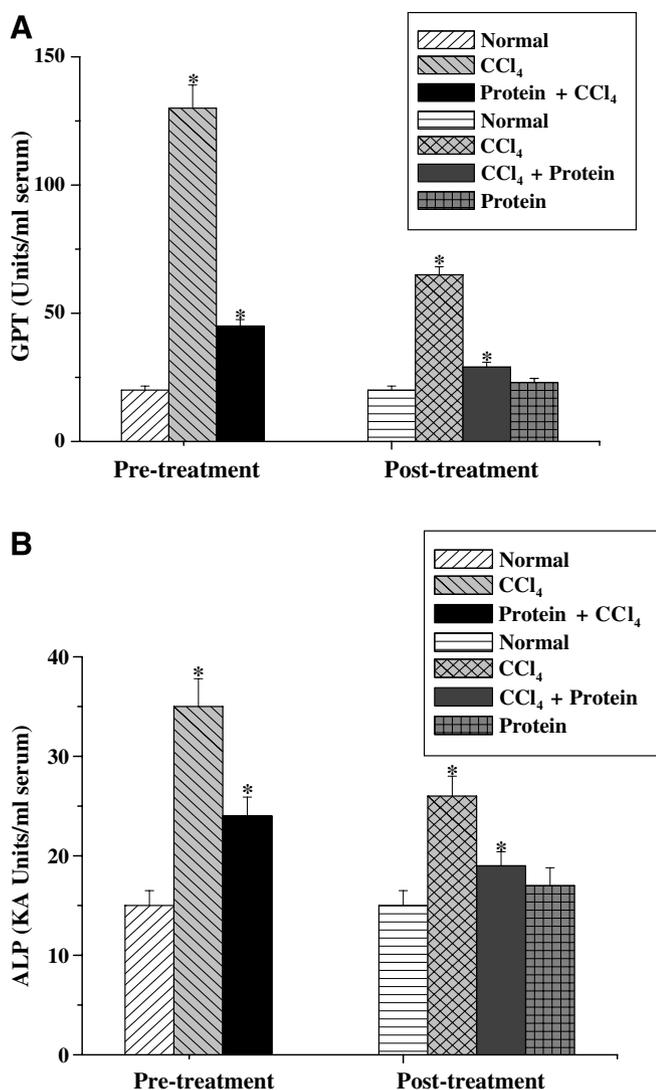


Fig. 5. A. Effect of *P. niruri* protein isolate on serum GPT level – Normal mice (control); CCl₄ – CCl₄ treated mice at a dose of 10 mmol/kg body weight; Protein + CCl₄ – protein pretreated at a dose of 5 mg/kg body weight for 5 days + CCl₄ treated at a dose of 10 mmol/kg body weight for 2 days; CCl₄ + Protein – CCl₄ treated at a dose of 10 mmol/kg body weight for 2 days + protein post-treated at a dose of 5 mg/kg body weight for 5 days; Protein – protein treated at a dose of 5 mg/kg body weight for 5 days. Each value represents mean \pm SD ($n = 6$). (* $P < 0.05$). B. Effect of *P. niruri* protein isolate on serum ALP level – Legends of this figure is identical as that described in the legend of Fig. 3A. Each value represents mean \pm SD ($n = 6$) (* $P < 0.05$).

chemicals and drugs first go to liver where they are metabolized into toxic intermediates. CCl₄ is one of the most extensively studied hepatotoxiant. The hepatotoxicity of CCl₄ results from its metabolic conversion to free radical product CCl₃ by Cyt P-450 (Noguchi et al., 1982). Once CCl₃ has been formed it reacts very rapidly with O₂ to produce CCl₃OO \cdot , a much more reactive radical than CCl₃ (Packer et al., 1978). These free radicals attack microsomal lipids leading to its peroxidation and also covalently bind to microsomal lipids and proteins. This results in the generation of reactive oxygen species (ROS), which

includes the super-oxide anion O₂⁻, H₂O₂ and the hydroxyl radical. Although various enzymatic and non-enzymatic systems have been developed by cell to cope up with the ROS and other free radicals, when a condition of oxidative stress establishes, the defense capacities against ROS becomes insufficient (Wei, 1998). ROS also affects the antioxidant defense mechanisms, reduces the intracellular concentration of GSH, and decreases the activity of SOD and CAT. It has also been known to decrease the detoxification system produced by GST (Halliwell and Gutteridge, 2000). Increasing evidence indicates that oxidative stress causes liver injury, cirrhosis development and carcinogenesis (Yamamoto et al., 1998; Yamamoto and Yamashita, 1999; Stal and Olson, 2000).

Present study was focused on investigating the role of *P. niruri* against CCl₄ induced hepatotoxicity and to find its possible mode of action in hepatoprotection. During the extraction procedure of the responsible active principle, we experimented with different fractions and tested those fractions for the hepatoprotective activity. At first, effect of the aqueous and methanol extracts of the leaves of *P. niruri* on CCl₄ induced hepatotoxicity was determined. Major hepatoprotective activity was found in the aqueous fraction. The idea of the protein molecules having this activity came from a preliminary experiment in which the same amount of aqueous extract was administered orally and intraperitoneally in two different groups of animals and the intraperitoneal administration showed more activity than the oral administration. We confirmed this idea by performing an experiment in which the proteinous aqueous fraction showed better activity against CCl₄ induced hepatotoxicity than the non-proteinous fraction. Finally, another set of experiments showed that heat treatment and enzymatic digestion destroyed the biological activity of the protein isolate. Results from all the experiments clearly suggested that the protein isolate was responsible, at least partly, for the hepatoprotective activity of *P. niruri*.

To demonstrate that the protein isolate of the herb *P. niruri* has vital hepatoprotective properties against hepatic damage induced by a common hepatotoxicant like CCl₄, we treated mice with CCl₄ at a dose of 10 mmol/kg body weight. We observed that this resulted in severe hepatic toxicity as evident from the increased levels of liver marker enzymes GPT and ALP and histopathological studies. It also induced a significant depletion of several antioxidant enzymes like SOD and CAT. Moreover, CCl₄ treatment enhanced lipid peroxidation significantly and reduced hepatic levels of GSH. Decrease in the levels of antioxidant enzymes as well as GSH and enhancement of lipid peroxidation suggest that CCl₄ causes liver toxicity by establishing a condition of oxidative stress in liver by generating ROS. As a result, the cellular defense capabilities against ROS become insufficient.

In order to elucidate the protective role of *P. niruri*, we examined the preventive and curative role of the protein isolate of *P. niruri* against CCl₄ induced hepatotoxicity

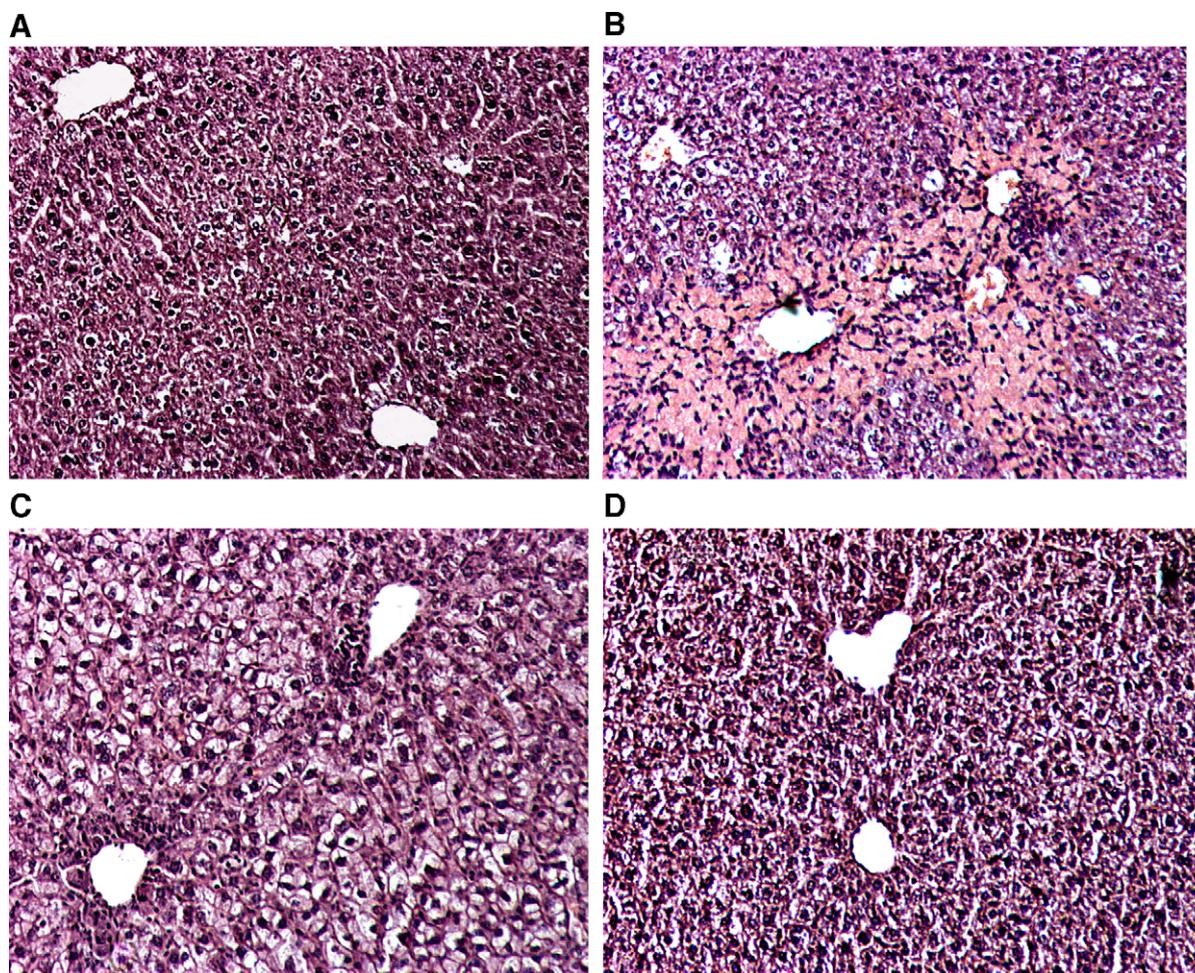


Fig. 6. Effect of *P. niruri* protein isolate on liver histopathology. A. Normal liver. B. Liver from mice treated with CCl_4 at a dose of 10 mmol/kg body weight. C. Liver from mice pretreated with protein isolate for 5 days and then CCl_4 treated at a dose of 10 mmol/kg body weight for 2 days. D. Liver from mice treated with CCl_4 at a dose of 10 mmol/kg body weight for 2 days and then post-treated with protein isolate.

in mice. It was observed that *P. niruri* was able to reduce the increased levels of liver marker enzymes GPT and ALP, significantly. In histopathological studies, damage around the lobular region of the liver from protein isolate treated mice liver before and after CCl_4 administration was less severe compared to that with CCl_4 . It also restored the levels of antioxidant enzymes like SOD and CAT almost back to the normal levels. Among these enzymes SOD plays an important role in the elimination of ROS derived from the peroxidative process in liver tissues. SOD removes superoxide by converting it to H_2O_2 , which can be rapidly converted to water by CAT (Halliwell et al., 1992). The observed increase in SOD and CAT activity suggests that the *P. niruri* protein isolate has an efficient protective mechanism in response to ROS. The non-protein thiol, GSH serves as a scavenger of different free radicals and is one of the major defenses against oxidative stress (Halliwell and Gutteridge, 2000). Treatment with the protein isolate of *P. niruri* restored the depleted GSH levels by a small amount. Moreover, it reduced cellular lipid peroxidation.

Present study indicates that *P. niruri* may be associated with decreased oxidative stress and free radical-mediated tissue damage and helps in rejuvenating antioxidative defense mechanism. Very recently, we have also shown that the protein isolate of *P. niruri* was effective against oxidative stress induced by a non-steroidal anti-inflammatory drug, nimesulide (Chatterjee et al., 2006). These findings indicate that *P. niruri* protects the liver through its preventive action on lipid peroxidation and also helps in scavenging ROS.

When mice were pretreated with the protein isolate of *P. niruri*, it protected liver from CCl_4 induced oxidative stress and subsequent hepatotoxicity. Results suggested that the protein isolate might reduce oxidative stress by scavenging ROS and enhancing antioxidative defense mechanism. In addition, when post-treated, the protein isolate also cured liver from CCl_4 induced damages. However, in the time course studies, the effect was not significant for first three days. After 3 days, the recovery process was faster than the normal recovery process. Data indicate that the protein isolate has the ability to stimulate repair mech-

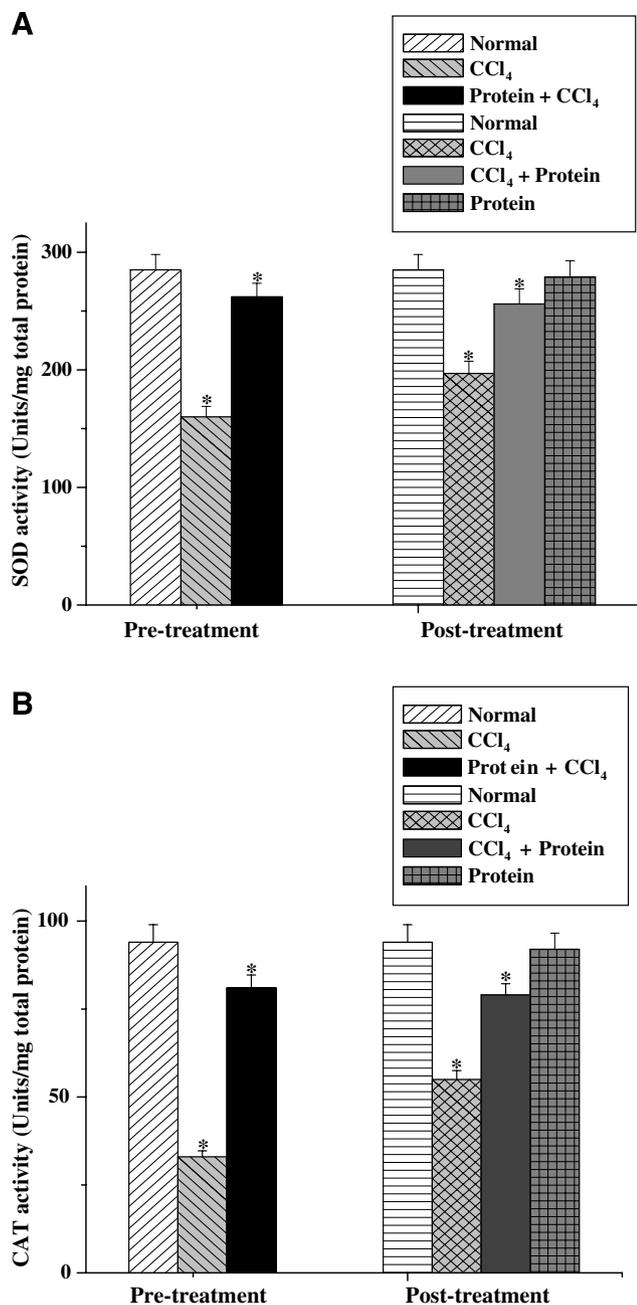


Fig. 7. A. Effect of *P. niruri* protein isolate on SOD activity – Legends of this figure is identical as that described in the legend of Fig. 3A. Each value represents mean \pm SD ($n = 6$) ($*P < 0.05$). B. Effect of *P. niruri* protein isolate on CAT activity – Legends of this figure is identical as that described in the legend of Fig. 3A. Each value represents mean \pm SD ($n = 6$) ($*P < 0.05$).

anism present in liver following some pathway(s), which is not clear at present.

In conclusion, we would like to mention that the protein isolate of *P. niruri* could be used as a protective agent against toxicity caused by various hepatotoxicants, like CCl₄. Further works are needed to fully characterize the protein(s) present in the herb and elucidate its possible mode of action and that is in progress.

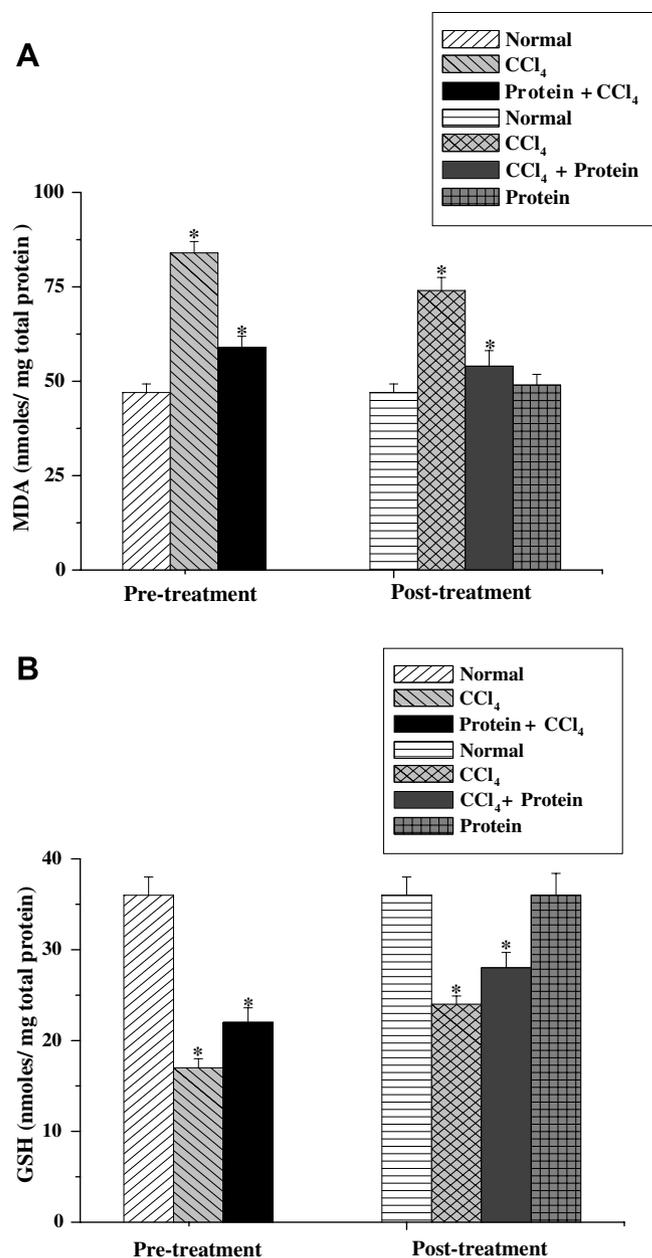


Fig. 8. A. Effect of *P. niruri* protein isolate on cellular lipid peroxidation – Legends of this figure is identical as that described in the legend of Fig. 3A. Each value represents mean \pm SD ($n = 6$) ($*P < 0.05$). B. Effect of *P. niruri* protein isolate on cellular GSH levels – Legends of this figure is identical as that described in the legend of Fig. 3A. Each value represents mean \pm SD ($n = 6$) ($*P < 0.05$).

Conflict of interest statement

There is no conflict of interest regarding financial, personal, or the relationship with other people or organizations.

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References

- Bei, W., Peng, W., Ma, Y., Xu, A., 2004. NaoXinQing, an anti-stroke herbal medicine, reduces hydrogen peroxide-induced injury in NG108-15 cells. *Neurosci. Lett.* 363, 262–265.
- Bonaventura, J., Schroeder, W.A., Fang, S., 1972. Human erythrocyte catalase: an improved method of isolation and a reevaluation of reported properties. *Arch. Biochem. Biophys.* 150, 606–617.
- 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.
- Chatterjee, M., Sarkar, K., Sil, P.C., 2006. Herbal (*Phyllanthus niruri*) protein isolate protects liver from nimesulide induced oxidative stress. *Pathophysiology* 13, 95–102.
- Chauhan, C.K., Nanivadekar, S.A., Billimoria, F.R., 1992. Effect of a herbal hepatoprotective product on drug metabolism in patients of cirrhosis and hepatic enzyme function in experimental liver damage. *Ind. J. Pharmacol.* 24, 107–110.
- Ellman, G.L., 1959. Tissue sulphhydryl group. *Arch. Biochem. Biophys.* 82, 70–77.
- Esterbauer, H., Cheeseman, K.H., 1990. Determination of aldehydic lipid peroxidation products: Malonaldehyde and 4-hydroxynonenal. *Meth. Enzymol.* 186, 407–421.
- Guerra, M.C., Speroni, E., Broccoli, M., Cangini, M., Pasini, P., Minghetti, A., et al., 2000. Comparison between chinese medical herb *Pueraria lobata* crude extract and its main isoflavone puerarin antioxidant properties and effects on rat liver CYP-catalysed drug metabolism. *Life Sci.* 67, 2997–3006.
- Gyamfi, M.A., Aniya, Y., 2002. Antioxidant properties of Thonningianin A, isolated from the African medicinal herb, *Thonningia sanguinea*. *Biochem. Pharmacol.* 63, 1725–1737.
- Halliwell, B., Gutteridge, J.M.C., 2000. Free Radicals in Biology and medicine. Oxford University Press, p. 148–149.
- Halliwell, B., Gutteridge, J.M., Cross, C.E., 1992. Free radicals, antioxidants, and human disease: where are we now? *J. Lab. Clin. Med.* 119, 598–620.
- Kadiiska, M.B., Gladen, B.C., Baird, D.D., Dikalova, A.E., Sohal, R.S., Hatch, G.E., et al., 2000. Biomarkers of oxidative stress study: are plasma antioxidants markers of CCl₄ poisoning? *Free. Radic. Biol. Med.* 28, 838–845.
- Kind, P.R.N., King, E.J., 1954. Estimation of plasma phosphatase by determination of hydrolyzed phenol with anti-pyrene. *J. Clin. Path.* 7, 322–326.
- Lin, T.J., Su, C.C., Lan, C.K., Jiang, D.D., Tsai, J.L., Tsai, M.S., 2003. Acute poisonings with *Breynia officinalis* – an outbreak of hepatotoxicity. *J. Toxicol. Clin. Toxicol.* 41, 591–594.
- Loomis, W.D., 1969. Removal of phenolic compounds during the isolation of plant enzymes. *Meth. Enzymol.* 13, 555–563.
- Myagmar, B.E., Shinno, E., Ichiba, T., Aniya, Y., 2004. Antioxidant activity of medicinal herb *Rhodococcum vitis-idaea* on galactosamine-induced liver injury in rats. *Phytomedicine* 11, 416–423.
- Naik, A.D., Juvekar, A.R., 2003. Effects of alkaloidal extract of *Phyllanthus niruri* on HIV replication. *Indian J. Med. Sci.* 57, 387–393.
- Noguchi, T., Fong, K.L., Lai, E.K., Alexander, S.S., King, M.M., Olson, L., Poyer, J.L., Mccay, P.B., 1982. Specificity of aphenobarbital-induced cytochrome P450 for metabolism of carbon tetrachloride to the trichloromethyl radical. *Biochem. Pharmacol.* 31, 615–624.
- Packer, J.E., Slater, T.F., Willson, R.L., 1978. Reactions of the carbon tetrachloride-related peroxy free radical with aminoacids: pulse radiolysis evidence. *Life Sci.* 23, 2617–2620.
- Padma, P., Setty, O.H., 1999. Protective effect of *Phyllanthus* against CCl₄-induced mitochondrial dysfunction. *Life Sci.* 64, 2411–2417.
- Paoletti, F., Aldinucci, D., Mocali, A., Caparrini, A., 1986. A sensitive spectrophotometric method for the determination of superoxide dismutase activity in tissue extracts. *Anal. Biochem.* 154, 536–541.
- Poyer, J.L., Mccay, P.B., Lai, E.K., Jenzen, E.G., Davis, E.R., 1980. Confirmation of assignment of the trichloromethyl radical spin adduct detected by spin trapping during ¹³C-carbon tetrachloride metabolism in vitro and in vivo. *Biochem. Biophys. Res. Commun.* 94, 1154–1160.
- Recknagel, R.O., Glendek Jr., E.A., Dolazk, J.A., Waller, R.L., 1989. Mechanism of carbon tetrachloride toxicity. *Pharmacol. Ther.* 43, 139–154.
- Reitman, S., Frankel, S., 1957. A colorimetric method for the determination of serum glutamic oxaloacetic and glutamic pyruvic transaminases. *Am. J. Clin. Path.* 28, 56–63.
- Sebastian, T., Setty, O.H., 1999. Protective effect of *Phyllanthus* against ethanol-induced mitochondrial dysfunction. *Alcohol* 17, 29–34.
- Stal, P., Olson, J., 2000. Ubiquinone, oxidative stress, and liver carcinogenesis. In: Kagan, Ve, Quinn, D.J. (Eds.), *Coenzyme Q: Molecular Mechanisms in Health and Disease*. CRC Press, Boca Raton, pp. 317–329.
- Syamasundar, K.V., Singh, B., Thakur, R.S., Husain, A., Kiso, Y., Hikino, H., 1985. Antihepatotoxic principles of *Phyllanthus niruri* herbs. *J. Ethnopharmacol.* 14, 41–44.
- Thyagarajan, S.P., Subramanian, S., Thirunalasundari, T., Venkateswaran, P.S., Blumberg, B.S., 1988. Effect of *Phyllanthus amarus* on chronic carriers of hepatitis B virus. *Lancet* 2 (8614), 764–766.
- Unander, D.W., Webster, G.L., Blumberg, B.S., 1995. Usage and bioassays in *Phyllanthus* (Euphorbiaceae). IV. Clustering of antiviral uses and other effects. *J. Ethnopharmacol.* 45, 1–18.
- Venkateswaran, P.S., Millman, I., Blumberg, B.S., 1987. Effects of an extract from *Phyllanthus niruri* on hepatitis B and woodchuck hepatitis viruses: in vitro and in vivo studies. *Proc. Natl. Acad. Sci. USA* 84, 274–278.
- Wei, Y.H., 1998. Oxidative stress and mitochondrial DNA mutations in human aging. *Proc. Soc. Exp. Boil. Med.* 217, 53–63.
- Yamamoto, Y., Yamashita, S., Fujisawa, A., Kokura, S., Yoshikawa, T., 1998. Oxidative stress in patients with hepatitis, cirrhosis and hepatoma, evaluated by plasma antioxidants. *Biochem. Biophys. Res. Commun.* 247, 166–170.
- Yamamoto, Y., Yamashita, S., 1999. Plasma ubiquinone to ubiquinol ratio in patients with hepatitis, cirrhosis, and hepatoma, and in patients treated with percutaneous transluminal coronary reperfusion. *Bio. Factors* 9, 241–245.