

The Protein Fraction of *Phyllanthus niruri* Plays a Protective Role against Acetaminophen Induced Hepatic Disorder via its Antioxidant Properties

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The aim of this study was to investigate the hepatoprotective action of the protein fraction of *Phyllanthus niruri* against acetaminophen (APAP) hepatotoxicity. The partially purified protein fraction of *P. niruri* was injected intraperitoneally in mice either prior to (preventive) or after the induction of toxicity (curative). Levels of different liver marker enzymes in serum and different antioxidant enzymes, as well as lipid peroxidation in total liver homogenates were measured in normal, control (toxicity induced) and *P. niruri* protein fraction-treated mice. *P. niruri* significantly reduced the elevated glutamate pyruvate transaminase (GPT) and alkaline phosphatase (ALP) levels in the sera of toxicity induced mice, compared with the control group. Lipid peroxidation levels were also reduced in mice treated with *P. niruri* protein fraction compared with the APAP treated control group. Among the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST) levels were restored to almost normal levels compared with the control group. *P. niruri* treatment also enhanced reduced hepatic glutathione (GSH) levels caused by APAP administration. The results demonstrated that the protein fraction of *P. niruri* protected liver tissues against oxidative stress in mice, probably acting by increasing antioxidative defense. Copyright © 2006 John Wiley & Sons, Ltd.

Keywords: acetaminophen; hepatotoxicity; oxidative stress; *Phyllanthus niruri*; antioxidant; hepatoprotection.

INTRODUCTION

Phyllanthus niruri is widely used all over Asia 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 *P. niruri* inhibited the replication of woodchuck hepatitis virus *in vivo* and decreased its pathological effect on woodchuck liver. Human clinical studies have been carried out with *P. niruri* (Thyagarajan *et al.*, 1988) and it has been shown that it has potent activity against various hepatic disorders such as viral hepatitis and toxicity caused by different drugs and environmental toxicants (Unander *et al.*, 1995; Padma and Setty, 1999). However, the mechanisms responsible for this hepatoprotective action of *P. niruri* are not yet well defined.

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 to produce highly reactive substances such as free radicals. In our laboratory, studies on the hepatoprotective action of *P. niruri* against acetaminophen (APAP) toxicity are

being carried out, and so APAP-intoxicated mice were injected with the soluble protein fraction of *P. niruri*, prior to and after the intoxication, for evaluation of preventive and curative properties, respectively. Thereafter, serum levels of the marker enzymes glutamate pyruvate transaminase (GPT) and alkaline phosphatase (ALP), which are related to hepatic integrity, were determined. To evaluate whether the protein fraction of *P. niruri* has any antioxidative properties, the levels of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST) and cellular glutathione (GSH) and lipid peroxidation in the liver were also examined.

MATERIAL AND METHODS

Materials. Fresh *P. niruri* was collected from a local market. Ammonium sulphate ((NH₄)₂SO₄), sodium dodecyl sulphate (SDS), ethylene diamine tetraacetic acid (EDTA), phenyl methyl sulphonyl fluoride (PMSF), di-sodium hydrogen phosphate (Na₂HPO₄), manganese dichloride (MnCl₂), 1-chloro-2,4-dinitrobenzene (CDNB), 5,5'-dithiobis-(2-nitro benzoic acid) (DTNB) (Ellman's reagent), 2-mercaptoethanol, reduced GSH, trichloro acetic acid (TCA), thiobarbituric acid (TBA), diethanol amine and triethanol amine were purchased from Sisco Research Laboratories (SRL). Bradford reagent, N,N,N',N'-tetramethylethylenediamine (TEMED) and APAP were purchased

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from Sigma Chemical Co., USA. Kits for the determination of SGPT and ALP were purchased from Span Diagnostics Ltd., India.

Extraction of proteins from the leaves. Fresh leaves were extracted with 50 mM phosphate buffer, pH 7.4 at 4 °C in a cyclomixer. The extract was filtered through cheesecloth and the homogenous filtrate centrifuged at 20 000 × g for 30 min at 4 °C. The resultant clear supernatant was brought to 60% ammonium sulphate saturation and kept overnight. It was then centrifuged at 20 000 × g for 30 min at 4 °C, and the pellet collected was reconstituted in 50 mM phosphate buffer, pH 7.4. All the experiments described here were carried out using this 60% ammonium sulphate saturated fraction.

Protein estimation. Different protein concentrations were measured according to the method of Bradford (1976).

Animal experiments. Swiss albino mice (male, body weight 25 ± 2 g) were acclimatized under laboratory conditions for 2 weeks before starting the experiments. They were provided with standard pellet diet and water *ad libitum*. The animals were divided into several groups of six in different models of experiments.

The first group served as controls; the second group received only APAP orally at a dose of 300 mg/kg of body weight for 2 days; and the third group was pretreated with the protein fraction for 3 days by intraperitoneal injection at a dose of 5 mg/kg body weight of mice, followed by APAP at the same dose as above. The concentration of the protein fraction to be used was determined by carrying out a dose response study with the protein fraction pretreated group, using doses in the range 1–40 mg/kg of body weight. A separate experiment was also conducted with the same doses of proteins to determine the effect of protein fraction on normal mice liver. The fourth group was post treated with the protein fraction for 3 days in the same way as above, after APAP treatment. With this group a time course study was also done by injecting *P. niruri* protein fraction for 1, 2, 3 and 4 days. Optimum results were obtained when the protein was administered for 3 days.

Evaluation of liver function. Blood samples collected from puncturing mice hearts were kept overnight to clot and then centrifuged at 3000 × g for 10 min. About 200 mg liver tissue was homogenized in 10 volumes of appropriate buffer and centrifuged at 10 000 × g for 20 min at 4 °C. The supernatant and blood serum were used to estimate the hepatotoxicity produced by APAP. Serum GPT was measured by the 2,4, dinitro phenyl hydrazine (DPNH) method of Reitman and Frankel (1957) and alkaline phosphatase was estimated by Kind and King's method (1954).

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).

Catalase assay. The activity of CAT was measured in liver homogenates by the method of Bonaventura *et al.* (1972).

Glutathione-S-transferase assay. GST activity was measured by the method of Habig and Jakoby (1980).

Superoxide dismutase assay. The principle of this method is based on the oxidation of nicotinamide adenine dinucleotide (NADH) mediated by super oxide radical (Paoletti *et al.*, 1986). The presence of SOD 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. The amount of SOD activity was calculated with respect to the blank value.

Measurement of hepatic glutathione level. Hepatic GSH level was determined by the method of Ellman (1959).

Statistical analysis. The data were expressed as mean ± SEM ($n = 6$), and n refers to the number of animals. Differences between the groups were analysed by one-way ANOVA, using the Epistat package.

RESULTS

Effect of *P. niruri* protein fraction on normal mice

To determine whether the *P. niruri* protein fraction had any adverse effect on the physiological condition of the normal mice, different doses of protein in the range 1–40 mg/kg of body weight were administered to the normal mice. As evident from the results, the protein fraction did not show any adverse effect on the SGPT level of normal mice (Fig. 1). The values of SGPT obtained using different concentrations of protein fraction are within the normal level.

Effect of *P. niruri* protein fraction against APAP induced hepatotoxicity: Preventive role

Dose response study. As described earlier, a dose response study was done by injecting different concentrations of protein prior to APAP administration. The doses used were in the range 1–40 mg/kg of body weight of protein. Serum GPT levels and liver SOD levels of all the mice were measured. The results are shown in Figs 2A and 2B. It is evident from the graphs that optimum results were obtained at a dose of 5 mg/kg body weight of protein, which was then used in all the experiments.

Serum GPT and ALP levels. GPT and ALP levels in the serum of the APAP treated mice were increased to 130 ± 1.6 units/mL of serum and 35 ± 1.9 KA units/mL of serum respectively indicating severe liver damage. Protein pretreatment for 3 days at a dose of 5 mg/kg body weight significantly reduced the levels of both the markers to 28 ± 0.5 units/mL of serum and 23 ± 1.4 KA units/mL of serum respectively for SGPT and ALP. Corresponding normal values were

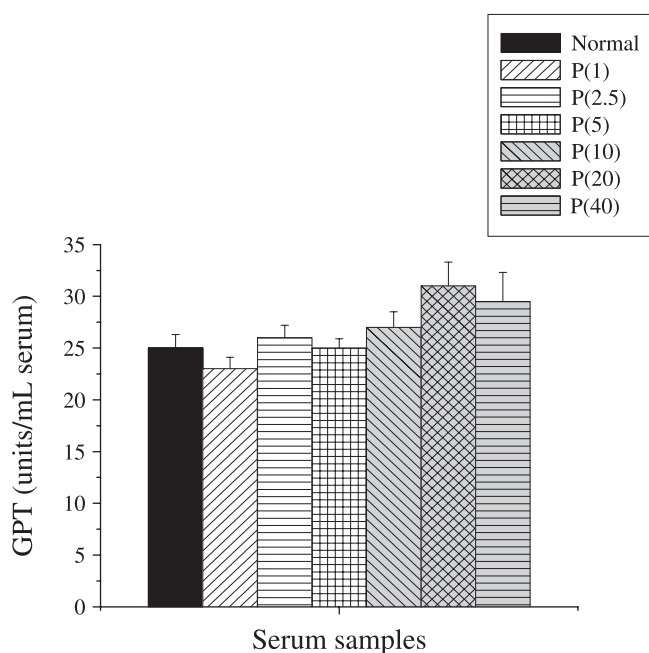


Figure 1. Effect of *P. niruri* protein fraction GPT levels in normal mice. Normal mice (control); P(1), protein treated at a dose of 1 mg/kg body weight; P(2.5), protein treated at a dose of 2.5 mg/kg body weight; P(5), protein treated at a dose of 5 mg/kg body weight; P(10), protein treated at a dose of 10 mg/kg body weight; P(20), protein treated at a dose of 20 mg/kg body weight; P(40), protein treated at a dose of 40 mg/kg body weight. Mice were treated with the protein fraction for 3 days. Each value represents mean ± SEM (*n* = 6). Note: The values of GPT obtained using different concentrations of protein fraction were within the normal range.

25 ± 0.7 units/mL of serum and 15 ± 1.1 KA units/mL of serum, respectively.

Cellular lipid peroxidation. Lipid peroxidation, measured as malonaldehyde (MDA) in experimental mice liver homogenates is shown in Fig. 3A. The MDA contents in liver homogenates of APAP treated mice were increased 2.1 fold (75 ± 1.7 nmol/g tissue) compared with the normal mice group (35 ± 2 nmol/g tissue). MDA level of protein treated group was almost lowered to normal levels (41 ± 2.1 nmol/g tissue).

CAT activity. The CAT activities in mice liver homogenates are shown in Fig. 3B. The CAT activity in liver homogenates of the APAP treated group (40 ± 0.5 units/mg total protein) was found to be significantly lower than the normal group (116 ± 4.5 units/mg total protein). Treatment of the mice with the protein prior to APAP treatment increased the CAT activity by 2.4 fold (91 ± 2.4 units/mg total protein) compared with the APAP treated mice.

GST activity. The GST activity in liver homogenates of different mice groups is shown in Fig. 3C. There was not much significant change in the GST level of the normal, APAP treated and protein pre-treated mice. The normal mice showed a GST level of 4 ± 0.37 units/mg total protein. These was reduced by 1.3 fold in APAP treated mice (3 ± 0.07 units/mg total protein), while the level increased by 1.2 fold to 3.7 ± 0.04 units/mg total protein in mice treated with the protein prior to APAP treatment.

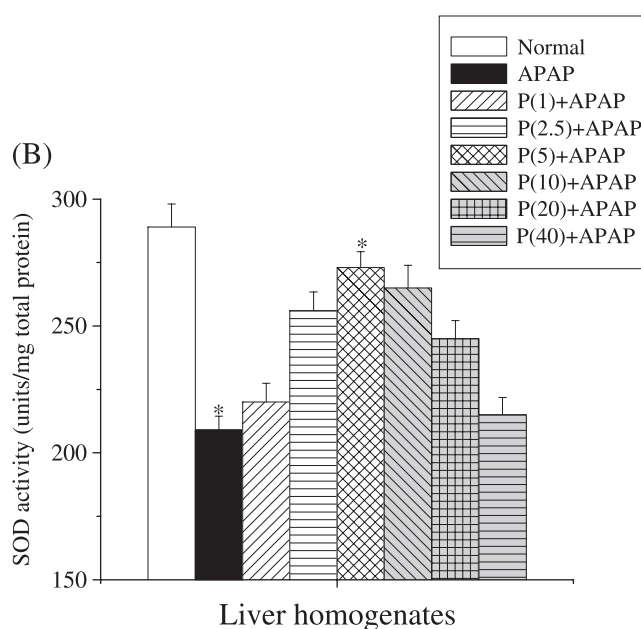
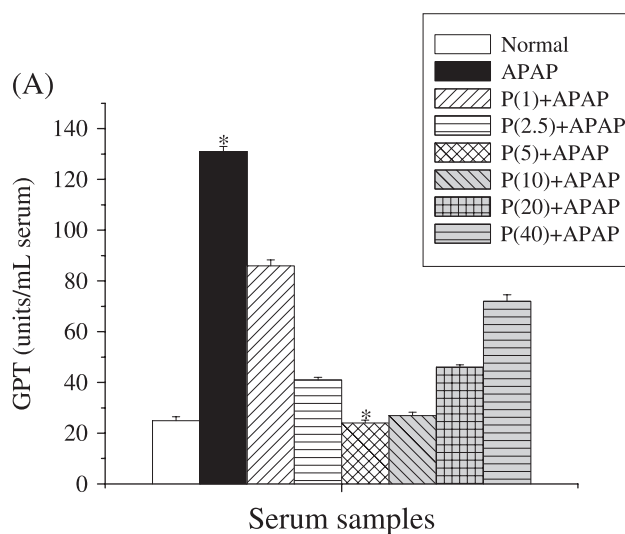
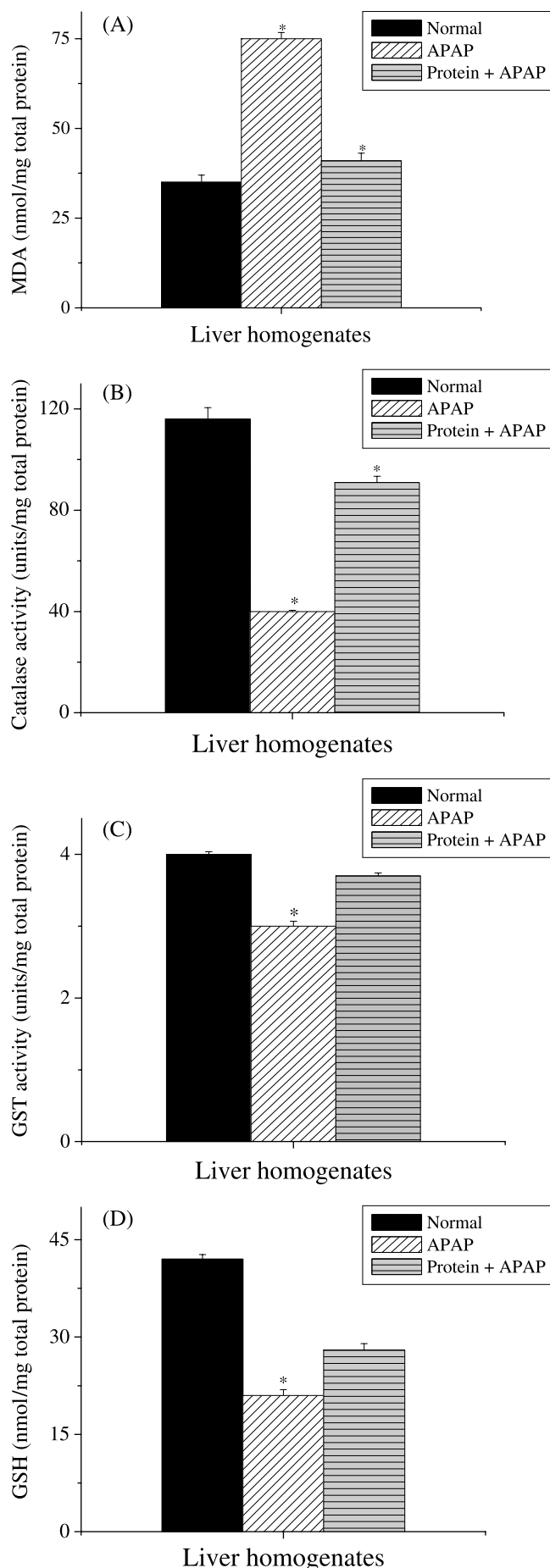


Figure 2. Dose response study of the effects of protein(s) of *P. niruri* against APAP toxicity. (A) GPT levels: Normal mice (control); APAP, APAP treated mice at a dose of 300 mg/kg body weight; P(2.5) + APAP, protein pretreated at a dose of 2.5 mg/kg body weight + APAP treated; P(5) + APAP, protein pretreated at a dose of 5 mg/kg body weight + APAP treated; P(10) + APAP, protein pretreated at a dose of 10 mg/kg body weight + APAP treated; P(20) + APAP, protein pretreated at a dose of 20 mg/kg body weight + APAP treated; P(40) + APAP, protein pretreated at a dose of 40 mg/kg body weight + APAP treated. Mice were pretreated with the protein fraction for 3 days as it gave optimum results. Each value represents mean ± SEM (*n* = 6). Protein (5 mg/mL dose) treated group was significantly different (*p* ≤ 10⁻⁶) from the APAP control group. (B) SOD levels: Legends of this figure are identical to that described in the legend of Fig. 2A. Each value represents mean ± SEM (*n* = 6). Protein (5 mg/mL dose) treated group was significantly different (*p* = 0.0000019) from the APAP control group.

Cellular GSH levels. Changes in GSH content of different liver homogenates are shown in Fig. 3D. GSH level was reduced by 2 fold (21 ± 0.9 nmol/mg total protein) in APAP treated mice compared with normal mice group (42 ± 0.7 nmol/mg total protein). GSH level was increased to 28 ± 1 nmol/mg total protein in protein pretreated mice.



Effect of *P. niruri* protein fraction against APAP induced hepatotoxicity: Curative role

Time dependent effect of *P. niruri* treatment. A time dependent study was done by injecting protein for 1, 2, 3 and 4 days after 2 days of APAP administration at a dose of 300 mg/kg body weight. Serum GPT levels and liver SOD levels of these mice were measured. The results are shown in Fig. 4A, 4B. From the results it is evident that protein injection for 3 days gave optimum results. So, further experiments were carried out by injecting the protein fraction for 3 days.

Serum GPT and ALP levels. GPT and ALP levels in the serum of the APAP treated mice were increased to 60 ± 1 units/mL of serum and 32 ± 1.2 KA units/mL of serum respectively, indicating liver damage. Protein pretreatment for 3 days at a dose of 5 mg/kg bodyweight reduced the levels of both GPT and ALP to 23 ± 0.8 units/mL of serum and 20 ± 0.9 KA units/mL of serum, respectively.

Cellular lipid peroxidation. Malonaldehyde levels in experimental mice liver homogenates are shown in Fig. 5A. MDA contents in liver homogenates of APAP treated mice was increased 1.5 fold (69 ± 2.1 nmol/g tissue) compared with the normal mice group (47 ± 2 nmol/g tissue). MDA level of protein treated group was lowered close to normal levels (54 ± 1.3 nmol/g tissue).

CAT activity. CAT activities in mice liver homogenates are shown in Fig. 5B. APAP treatment lowered the CAT activity (70 ± 5 units/mg total protein) by 1.3 fold than the normal mice (90 ± 4 units/mg total protein). Protein post treatment increased the CAT activity marginally to 78 ± 6.5 units/mg total protein.

GST activity. GST activity in liver homogenates of different mice groups is shown in Fig. 5C. Here also there was not much significant change in the GST level of the normal, APAP treated and protein post-treated mice. The normal mice showed a GST level of 4 ± 0.37 units/mg total protein. GST activity was reduced by 1.15 fold in APAP treated mice (3.5 ± 0.07 units/mg total protein), compared to normal mice (4 ± 0.12 units/mg total protein). While the level increased by 1.1 fold

Figure 3. (A) Effect of *P. niruri* protein fraction on cellular lipid peroxidation. Normal mice (control); APAP, APAP treated mice at a dose of 300 mg/kg body weight; Protein + APAP, protein pretreated at a dose of 5 mg/kg body weight for 3 days + APAP treated at a dose of 300 mg/kg body weight for 2 days. Each value represents mean \pm SEM ($n = 6$). Protein treated group was significantly different ($p \leq 10^{-6}$) from the APAP control group. (B) Effect of the *P. niruri* protein fraction on CAT activity. Legend of this figure is identical to that described in the legend of Fig. 3A. Each value represents mean \pm SEM ($n = 6$). Protein treated group was significantly different ($p \leq 10^{-6}$) from the APAP control group. (C) Effect of *P. niruri* protein fraction on GST activity. Legend of this figure is identical to that described in the legend of Fig. 3A. Each value represents mean \pm SEM ($n = 6$). Protein treated group was significantly different ($p = 0.000025$) from the APAP control group. (D) Effect of *P. niruri* protein fraction on cellular GSH levels. Legend of this figure is identical to that described in the legend of Fig. 3A. Each value represents mean \pm SEM ($n = 6$). Protein treated group was significantly different ($p \leq 10^{-6}$) from the APAP control group.

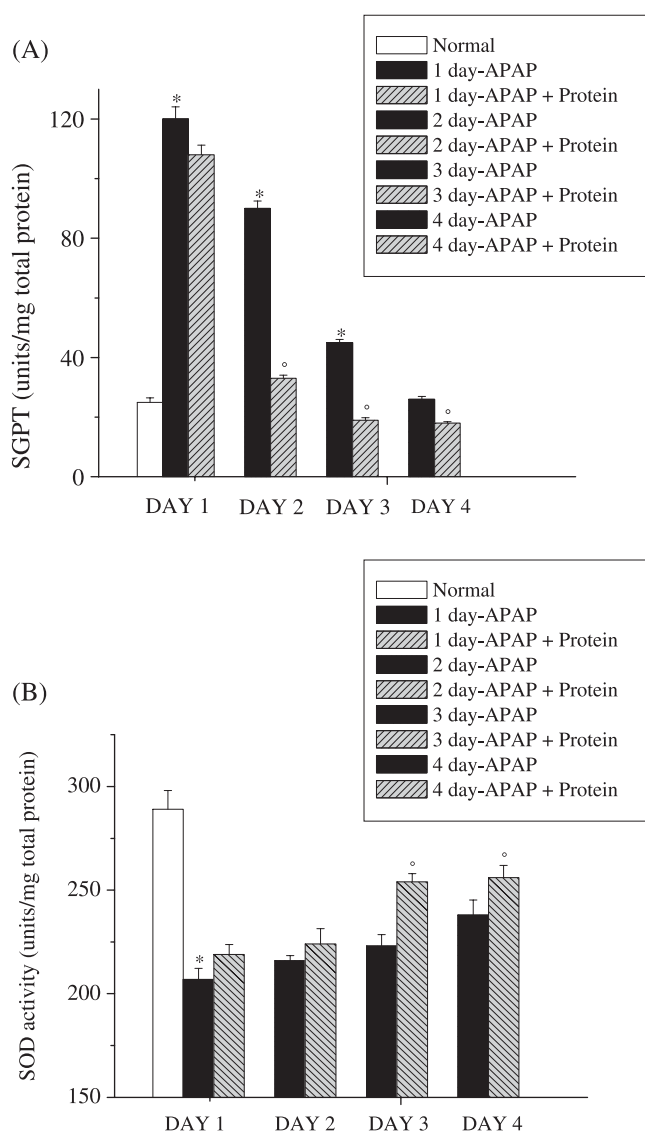


Figure 4. Time dependent study of the effect of protein(s) of *P. niruri* against APAP toxicity. (A) GPT levels: Normal mice (control); 1 day-APAP, APAP treated mice at a dose of 300 mg/kg body weight and killed after 1 day; 1 day APAP + Protein, APAP treated + protein post treated at a dose of 5 mg/kg body weight for 1 day; 2 day APAP, APAP treated mice at a dose of 300 mg/kg body weight and killed after 2 days; 2 day APAP + Protein, APAP treated + protein post-treated at a dose of 5 mg/kg body weight for 2 days; 3 day APAP, APAP treated mice at a dose of 300 mg/kg body weight and killed after 3 days; 3 day APAP + Protein, APAP treated + protein post-treated at a dose of 5 mg/kg body weight for 3 days; 4 day APAP, APAP treated mice at a dose of 300 mg/kg body weight and killed after 4 days; 4 day APAP + Protein, APAP treated + protein post-treated at a dose of 5 mg/kg body weight for 4 days. Each value represents mean \pm SEM ($n = 6$). *Significantly different ($p \leq 10^{-6}$) from the normal group. °Significantly different ($p \leq 10^{-6}$) from the APAP treated group. (B) SOD levels: Legend of this figure is identical to that described in the legend of Fig. 4A. *Significantly different ($p = 0.0000034$) from the normal group. °Significantly different ($p = 0.0000085$) from the APAP treated group.

to 3.8 ± 0.04 units/mg total protein in mice treated with the protein after APAP treatment.

Cellular GSH levels. The GSH contents in liver homogenates of APAP treated mice was decreased 1.4 fold (28.5 ± 0.9 nmol/mg total protein) compared to the normal mice group (40 ± 1 nmol/mg total protein). GSH level of protein fraction treated group was increased

to 32 ± 0.6 nmol/mg total protein. Result is shown in the Fig. 5D.

DISCUSSION

The liver is a major target organ for toxicity of xenobiotics and drugs, because most orally ingested drugs pass through the liver and are metabolized into toxic intermediates in the liver. APAP is the most commonly used analgesic and antipyretic and is responsible for more hospitalizations after overdose than any other common medication. APAP, when used in high doses, can cause potentially fatal hepatic necrosis (Proudfoot and Wright, 1970; Thomas, 1993) most probably via formation of NAPQI, a toxic quinone metabolite, which is produced by the oxidation of APAP by cytochrome P450 enzymes (Dahlin *et al.*, 1984). The risk of APAP hepatotoxicity is greater in alcoholics, where damage can occur even with therapeutic doses (Johnston and Pelletier, 1997). Under normal circumstances, a therapeutic dose of APAP results in the production of a small fraction (5%) of NAPQI after metabolism by cytochrome P450 enzymes (Johnston and Pelletier, 1997), with the majority of APAP excreted as conjugates of glucuronic acid or as the sulphate (Prescott, 1980). The small amount of NAPQI that is produced is rapidly detoxified in a reaction with GSH to form mercapturic acid or cysteine conjugates and is excreted, resulting in no hepatic damage (Zimmerman, 1998). Hepatic necrosis occurs only when the amount of NAPQI produced exceeds the binding capacity of the liver's store of GSH, resulting in increased utilization of GSH and depletion of GSH stores. The biotransformation and mechanism of hepatotoxicity of APAP have been reviewed recently (Nelson, 1995; Cohen and Khairallah, 1997). Excessive NAPQI combines covalently with hepatocyte membrane proteins (Zimmerman, 1998; Nelson, 1995), which results in the generation of reactive oxygen species (ROS) including the super-oxide anion O_2^- , H_2O_2 and the hydroxyl radical (Nelson, 1995; Michael *et al.*, 1999). ROS significantly affect the cellular membrane and induce peroxidation of lipids and also affect antioxidant defense mechanisms, reducing the intracellular concentration of GSH, and decreasing the activity of SOD and CAT. They also decrease the detoxification system produced by GST (Halliwell and Gutteridge, 2000).

The present study demonstrates that the protein fraction of the herb *P. niruri* has hepatoprotective properties against hepatic damage induced by a drug like APAP. In this study the mice were treated with APAP at a dose of 300 mg/kg body weight, which resulted in severe hepatic toxicity as evident from the increased levels of liver marker enzymes GPT and ALP. It also induced a significant depletion of the antioxidant enzymes SOD and CAT, whereas the change in GST activity was less marked. Moreover, APAP treatment enhanced lipid peroxidation significantly and reduced hepatic levels of GSH, which is required for the detoxification of NAPQI. In order to elucidate further the protective role of *P. niruri*, the effect of the protein fraction was examined by administering it prior to APAP in mice. *P. niruri* was able to reduce the elevated levels of the liver marker enzymes GPT and ALP significantly. The data also suggested that even at

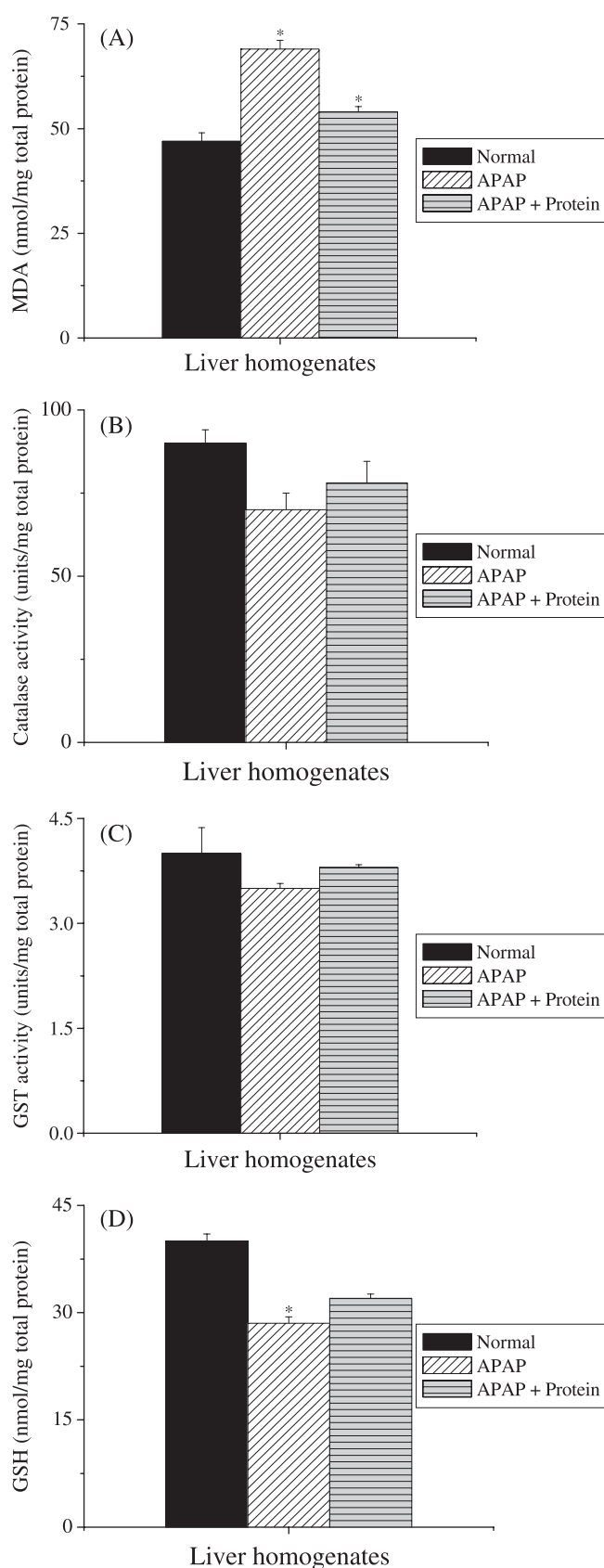


Figure 5. (A) Effect of *P. niruri* protein fraction on cellular lipid peroxidation. Normal mice (control); APAP, APAP treated mice at a dose of 300 mg/kg body weight; APAP + Protein treated, APAP treated at a dose of 300 mg/kg body weight for 2 days + protein post-treated at a dose of 5 mg/kg body weight for 3 days. Each value represents mean \pm SEM ($n = 6$). Protein treated group was significantly different ($p \leq 10^{-6}$) from the APAP control group. (B) Effect of the *P. niruri* protein fraction on CAT activity. Legend of this figure is identical to that described in the

high protein concentrations, there was no toxicity to the liver as is evidenced from Fig. 1, although in the presence of APAP it could not reduce the GPT level as expected at the higher concentration (40 mg/kg body weight). The reason for this effect is not yet clear; it may be due to the presence of other materials which at higher concentrations affect the normal physiology of the liver. The protein fraction of *P. niruri* also restored the levels of antioxidant enzymes such as SOD and CAT almost back to the normal levels. SOD plays an important role in the elimination of ROS derived from the peroxidative process in liver tissues (Halliwell *et al.*, 1992), and the observed increase in SOD activity suggests that the *P. niruri* protein fraction has an efficient protective mechanism in response to ROS. CAT is also a key component of antioxidative defense system. Here CAT activity was increased and then restored to normal levels on administration of *P. niruri* protein fraction. These findings indicate that *P. niruri* may be associated with decreasing oxidative stress and reducing free radical-mediated tissue damage, and may help in activating antioxidative defense mechanisms. It also reduced cellular lipid peroxidation and increased the depleted levels of cellular GSH, although this increase in the levels of cellular GSH and GPT was not as prominent as those obtained for CAT and SOD. These findings seem to indicate that *P. niruri* protects the liver through its preventive action on lipid peroxidation and scavenging ROS, but its effect is less pronounced on the detoxification system produced by GSH.

The results suggested the protein fraction of *P. niruri* acts at the cellular level, reducing oxidative stress and enhancing the antioxidative defense mechanism of the cells. It is not yet clear whether the protein fraction directly reduces oxidative stress by scavenging ROS or by boosting the antioxidant enzyme system; it is likely that a combination of both processes is involved. However, the effects were more pronounced when it was used as a preventive agent, i.e. when injected prior to APAP administration. It may, therefore, be assumed that the protein fraction of *P. niruri* holds more promise as a preventive agent than a curative one in this situation.

In conclusion, the protein fraction present in *P. niruri* may find a use as a protective agent against APAP toxicity. Further work is in progress to fully characterize the protein(s) present in the herb and elucidate the possible mode of action.

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legend of Fig. 5A. Each value represents mean \pm SEM ($n = 6$). Protein treated group was significantly different ($p \leq 10^{-6}$) from the APAP control group. (C) Effect of *P. niruri* protein fraction on GST activity. Legend of this figure is identical to that described in the legend of Fig. 5A. Each value represents mean \pm SEM ($n = 6$). Protein treated group was significantly different ($p = 0.00242$) from the APAP control group. (D) Effect of *P. niruri* protein fraction on cellular GSH levels. Legend of this figure is identical to that described in the legend of Fig. 5A. Each value represents mean \pm SEM ($n = 6$). Protein treated group was significantly different ($p = 0.0000845$) from the APAP control group.

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