

Hepatoprotective and Antioxidant Effect of *Pisonia aculeata* L. against CCl₄- Induced Hepatic Damage in Rats

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Abstract

Ethanol extract of *Pisonia aculeata* (EPA) was evaluated for hepatoprotective and antioxidant activities in rats. The plant extract (250 and 500 mg/kg, p.o.) showed a remarkable hepatoprotective and antioxidant activity against carbon tetrachloride (CCl₄)-induced hepatotoxicity as judged from the serum marker enzymes and antioxidant levels in liver tissues. CCl₄-induced a significant rise in aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP), total bilirubin, gamma glutamate transpeptidase (GGTP), lipid peroxidase (LPO) with a reduction of total protein, superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and glutathione S-transferase (GST). Treatment of rats with different doses of plant extract (250 and 500 mg/kg) significantly ($P < 0.001$) altered serum marker enzymes and antioxidant levels to near normal against CCl₄-treated rats. The activity of the extract at dose of 500 mg/kg was comparable to the standard drug, silymarin (50 mg/kg, p.o.). Histopathological changes of liver sample

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were compared with respective control. Results indicate the hepatoprotective and antioxidant properties of *P. aculeata* against CCl₄-induced hepatotoxicity in rats.

Keywords

Pisonia aculeata • Carbon tetrachloride • Biochemical parameters • Antioxidants • Lipid peroxidation • Histopathology

Introduction

The liver is the key organ regulating homeostasis in the body. It is involved with almost all the biochemical pathways related to growth, fight against disease, nutrient supply, energy provision and reproduction [1]. The liver is expected not only to perform physiological functions but also to protect against the hazards of harmful drugs and chemicals. In spite of tremendous scientific advancement in the field of hepatology in recent years, liver problems are on the rise. Jaundice and hepatitis are two major hepatic disorders that account for a high death rate [2]. Presently, a few hepatoprotective drugs and that too from natural sources, are available for the treatment of liver disorders. Hence, people are looking at the traditional systems of medicine for remedies to hepatic disorders.

Pisonia aculeata L. (Nyctaginaceae) is a large scandent shrub distributed through out India. The leaves and bark are used by the tribes and native medical practitioners to treat various ailments including liver disorders, inflammation, swelling, cough and tumours. They are also used as a counter irritant for swelling and rheumatic pain [3]. However there are no reports regarding the pharmacological activities of this plant. The present study is aimed to evaluate the hepatoprotective and antioxidant activity of ethanol extract of the leaves of *Pisonia aculeata* against CCl₄-induced hepatotoxicity in rats.

Experimental

Chemicals

Silymarin was purchased from Micro Labs, India. 1-chloro-2,4-dinitrobenzoic

acid (CDNB), 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), Reduced glutathione (GSH) and glutathione were purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. Thiobarbituric acid was purchased from E-Merck, India. All other chemical used were of analytical grade.

Collection and extraction

The fresh leaves of *P. aculeata* were collected in and around Kolli hills in Salem district, Tamilnadu, India, in the month of September 2006 and authenticated by Dr. R. Gopalan, Botanical Survey of India, Coimbatore, Tamilnadu, India. A voucher specimen has been retained in our laboratory for future reference (P.Ch.002/2006).

The leaves were shade dried and pulverized. The powder was treated with petroleum ether for dewaxing and removal of chlorophyll. Later, it was packed (1 kg) in a Soxhlet apparatus and subjected to continuous hot percolation for 8 h using 2.5 l ethanol (95% v/v) as solvent. The extract was concentrated under vacuum and dried in a desiccator (yield 72 g, 7.2 % w/w). Without any purification, aliquot portions of the crude extract were suspended in 5% gum acacia for use on each day of our experiment.

Phytochemical screening

A preliminary phytochemical screening of *P. aculeata* was carried out. The phytochemical profile was performed as described by Wagner et al [4]. The presence of alkaloid (Dragendroff reagent and Mayer's reagent), flavonoids (Shinoda test), steroids (Lieberman Burchard test) and terpenes (Vanillin sulfuric acid reagent) were analyzed.

Animals

Swiss albino mice (20–25 g) and male Wistar rats (150–200 g) were procured from Venkatershwara Enterprises, Bangalore, Karnataka, India, and used throughout the study. They were housed in microlon boxes in a controlled environment (temperature 25 ± 2 °C and 12 h dark/light cycle) with standard

laboratory diet and water *ad libitum*. The study was conducted after obtaining Institutional Animal Ethical Committee clearance.

Acute toxicity studies

Acute oral toxicity (AOT) of *P. aculeata* was determined using Swiss albino mice. The animals were fasted for 12 h prior to the experiment and were administered with single dose of extracts dissolved in 5% gum acacia and observed for mortality up to 48 hour (short term toxicity). Based on the short-term toxicity, the dose of next animal was determined as per OECD guideline 420.

Hepatoprotective activity

Rats were divided into five groups, each group consisting of six animals.

- Group I: Controls received the vehicle of normal saline (2 ml/kg, p.o.).
- Group II: Received CCl₄ (2 ml/kg, s.c.) at every 72 h for 10 days [5].
- Group III: Received silymarin 50 mg/kg p.o. for 10 days and simultaneously administered CCl₄ (2 ml/kg, s.c.) at every 72 h.
- Group IV: Received alcohol extract of *P. aculeata* 250 mg/kg p.o. for 10 days and simultaneously administered CCl₄ (2 ml/kg, s.c.) at every 72 h.
- Group V: Received alcohol extract of *P. aculeata* 500 mg/kg p.o. for 10 days and simultaneously administered CCl₄ (2 ml/kg, s.c.) at every 72 h.

At the end of experimental period, all the animals were sacrificed by cervical decapitation. Blood samples were collected, allowed to clot. Serum was separated by centrifuging at 2500 rpm for 15 min and analyzed for various biochemical parameters.

Assessment of liver function

Biochemical parameters i.e., aspartate amino transferase (AST) [6], alanine amino transferase (ALT) [6], alkaline phosphatase (ALP) [7], γ-glutamate transpeptidase (GGTP) [8], total bilirubin [9] and total protein [10], were analyzed according to the reported methods. The liver was removed, morphological changes were observed. A 10% of liver homogenate was used for antioxidant studies such

as lipid peroxidation (LPO) [11], superoxide dismutase (SOD) [12], Catalase [13], glutathione peroxidase (GPx) [14], and glutathione S-transferase (GST) [15]. A portion of liver was fixed in 10% formalin for histopathological studies.

Histopathological studies

After draining the blood, liver samples were excised, washed with normal saline and processed separately for histopathological observation. Initially the materials were fixed in 10% buffered neutral formalin for 48 hour and then with bovine solution for 6 hour. Paraffin sections were taken at 5 mm thickness processed in alcohol-xylene series and was stained with alum hematoxylin and eosin. The sections were examined microscopically for histopathology changes.

Statistical analysis

The values were expressed as mean $\pm$ SEM. Statistical analysis was performed by one way analysis of variance (ANOVA) followed by Tukey multiple comparison tests. P values < 0.05 were considered as significant.

Results

Preliminary phytochemical studies revealed the presence of alkaloids, steroids, saponins, triterpenes, flavonoids and polyphenolic compounds. For the acute oral toxicity studies, the extract treated animals were observed for mortality up to 48 h. Based on the results the extract did not produce any mortality up to 2000 mg/kg body weight.

The effect of *P. aculeata* on serum marker enzymes is presented in Table 1. The levels of serum AST, ALT, ALP, total bilirubin, GGTP were markedly elevated and that of protein decreased in CCl₄ treated animals, indicating liver damage. Administration of *P. aculeata* extract at the doses of 250 and 500 mg/kg remarkably prevented CCl₄-induced hepatotoxicity in a dose dependent manner.

Analysis of LPO levels by thiobarbituric acid reaction showed a significant (P<0.001) increase in the CCl₄ treated rats. Treatment with *P. aculeata* (250 mg/kg and 500 mg/kg) significantly (P<0.001) prevented the increase in LPO level which

was brought to near normal. The effect of *P. aculeata* was comparable with that of standard drug silymarin (Table 2).

Tab. 1. Effect of *Pisonia aculeata* on biochemical parameters in CCl₄-induced hepatotoxicity in rats

Treatment	Dose (mg/kg)	AST U/L	ALT U/L	ALP U/L
Control	–	48.52 ± 2.26	95.25 ± 1.6	126.17 ± 1.80
CCl ₄	2 ml/kg	296.56 ± 1.45 ^a	481.21 ± 1.81 ^a	251.52 ± 1.82 ^a
Silymarin	50	87.75 ± 2.15 ^{a,d}	131.73 ± 1.62 ^{a,d}	138.8 ± 2.93 ^b
<i>P. aculeata</i>	250	116.21 ± 2.25 ^{a,d}	221.91 ± 1.8 ^{a,d}	166.8 ± 2.04 ^{a,d}
	500	89.1 ± 0.85 ^{a,d}	152.5 ± 3.1 ^{a,d}	143.83 ± 1.8 ^{a,d}
Treatment	Dose (mg/kg)	Total bilirubin mg%	Total Protein mg%	GGTP U/L
Control	–	0.98 ± 0.01	9.34 ± 1.20	48.15 ± 1.30
CCl ₄	2 ml/kg	3.22 ± 0.02 ^a	6.08 ± 0.45 ^c	72.1 ± 2.14 ^a
Silymarin	50	1.25 ± 0.02 ^{a,d}	8.62 ± 0.36	45.3 ± 1.76 ^d
<i>P. aculeata</i>	250	2.07 ± 0.05 ^{a,d}	7.62 ± 0.31	62.6 ± 1.08 ^{a,e}
	500	1.56 ± 0.01 ^{a,d}	8.22 ± 0.56	51.05 ± 3.26 ^d
N = 6; Values are expressed as mean ± SEM				
^a P < 0.001; ^b P < 0.01; ^c P < 0.05 Vs Control				
^d P < 0.001; ^e P < 0.05 Vs CCl ₄				
Data were analyzed by using one way ANOVA followed by Tukey multiple comparison test.				

Tab. 2. Effect of *Pisonia aculeata* on antioxidants level in CCl₄-induced hepatotoxicity in rats

Treatment	Dose (mg/kg)	LPO	SOD	Catalase
Control	–	11.42 ± 0.85	35.42 ± 2.10	62.12±1.67
CCl ₄	2 ml/kg	24.86 ± 1.14 ^a	21.05 ± 1.35 ^a	39.20 ± 1.35
Silymarin	50	13.01± 0.87 ^d	29.36 ± 1.48 ^f	49.18 ± 1.92 ^{a,f}
<i>P.aculeata</i>	250	15.54 ± 0.51 ^{b,d}	23.36±1.29 ^a	37.18 ± 2.14
	500	12.92±1.18 ^d	26.18±1.40 ^c	46.33 ± 2.10 ^a
Treatment	Dose (mg/kg)	GPx	GST	
Control	–	48.65 ± 2.36	0.48 ± 0.07	
CCl ₄	2 ml/kg	24.28 ± 2.10 ^a	0.12 ± 0.02 ^a	
Silymarin	50	38.14± 1.45 ^{c,d}	0.36 ± 0.04 ^{b,e}	
<i>P.aculeata</i>	250	29.84 ± 2.15 ^{a,e}	0.27±0.04	
	500	34.25± 1.75 ^a	0.32 ± 0.05 ^e	

N = 6; Values are expressed as mean ± SEM
^a P< 0.001; ^b P< 0.05; ^c P< 0.01 Vs Control
^d P< 0.001; ^e P< 0.05; ^f P< 0.01 Vs CCl₄
 Data were analyzed by using one way ANOVA followed by Tukey multiple comparison test.
 LPO = μ moles of MDA/ min/mg protein
 SOD = Units/min/mg protein
 CAT = μ mole of H₂O₂ consumed/ min/mg protein
 GPx = μ moles of GSH oxidized/min /mg protein
 GST = μ moles of CDNB conjugation formed/min /mg protein

CCl₄ treatment caused a significant ($P < 0.001$) decrease in the level of SOD, Catalase, GPx and GST in liver tissue when compared with control group (Table 2). The treatment of *P. aculeata* at the doses of 250 and 500 mg/kg resulted in a significant increase of SOD, Catalase, GPx and GST when compared to CCl₄ treated rats. The liver of silymarin treated animals also showed a significant increase in antioxidant enzymes levels compared to CCl₄ treated rats.

Morphological observations showed an increased size and enlargement of the liver in acetaminophen treated groups. These changes were reversed by treatment with silymarin and also *P. aculeata* at the doses tested.

Histopathological studies, showed acetaminophen to produce extensive vascular degenerative changes and centrilobular necrosis in hepatocytes. Treatment with different doses of *P. aculeata* extract produced mild degenerative changes and absence of centrilobular necrosis when compared with control. All these results indicate a hepatoprotective potential of the extract.

Discussion

Carbon tetrachloride is one of the most commonly used hepatotoxins in the experimental study of liver diseases. The hepatotoxic effects of CCl₄ are largely due to its active metabolite, trichloromethyl radical [16]. These activated radicals bind covalently to the macromolecules and induce peroxidative degradation of membrane lipids of endoplasmic reticulum rich in polyunsaturated fatty acids. This leads to the formation of lipid peroxides. This lipid peroxidative degradation of biomembranes is one of the principle causes of hepatotoxicity of CCl₄ [17]. This is evidenced by an elevation in the serum marker enzymes namely AST, ALT, ALP, total bilirubin, GGTP and decrease in protein.

In the assessment of liver damage by CCl₄ the determination of enzyme levels such as AST, ALT is largely used. Necrosis or membrane damage releases the enzyme into circulation and hence it can be measured in the serum. High levels of AST indicates liver damage, such as that caused by viral hepatitis as well as cardiac infarction and muscle injury, AST catalyses the conversion of alanine to

pyruvate and glutamate and is released in a similar manner. Therefore ALT is more specific to the liver, and is thus a better parameter for detecting liver injury. Elevated levels of serum enzymes are indicative of cellular leakage and loss of functional integrity of cell membrane in liver [18]. Serum ALP, bilirubin and total protein levels on other hand are related to the function of hepatic cell. Increase in serum level of ALP is due to increased synthesis, in presence of increasing biliary pressure [19].

Administration of CCl_4 caused a significant ($P < 0.001$) elevation of enzyme levels such as AST, ALT, ALP, GGTP, total Bilirubin and decrease in total protein when compared to control. There was a significant ($P < 0.001$) restoration of these enzyme levels on administration of the leaf extract in a dose dependent manner and also by silymarin at a dose of 50 mg/kg. The reversal of increased serum enzymes in CCl_4 -induced liver damage by the extract may be due to the prevention of the leakage of intracellular enzymes by its membrane stabilizing activity. This is in agreement with the commonly accepted view that serum levels of transaminases return to normal with the healing of hepatic parenchyma and the regeneration of hepatocytes [20]. Effective control of ALP, bilirubin and total protein levels points towards an early improvement in the secretory mechanism of the hepatic cells.

The efficacy of any hepatoprotective drug is dependent on its capacity of either reducing the harmful effect or restoring the normal hepatic physiology that has been distributed by a hepatotoxin. Both silymarin and the plant extract decreased CCl_4 induced elevated enzyme levels in tested groups, indicating the protection of structural integrity of hepatocytic cell membrane or regeneration of damaged liver cells.

The increase in LPO level in liver induced by CCl_4 suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanism to prevent formation of excessive free radicals. Treatment with *P. aculeata* significantly reverses these changes. Hence it is likely that the mechanism of hepatoprotection of *P. aculeata* is due to its antioxidant effect.

Decrease in enzyme activity of superoxide dismutase (SOD) is a sensitive index in hepatocellular damage and is the most sensitive enzymatic index in live injury. Curtis and Mortiz [21], SOD has been reported as one of the most important enzymes in the enzymatic antioxidant defense system. It scavenges the superoxide anion to form hydrogen peroxide and thus diminishing the toxic effect caused by this radical. In *P. aculeata* causes a significant increase in hepatic SOD activity and thus reduces reactive free radical induced oxidative damage to liver.

Catalase (CAT) is an enzymatic antioxidant widely distributed in all animal tissues, and the highest activity is found in the red cells and liver. CAT decomposes hydrogen peroxide and protects the tissues from highly reactive hydroxyl radicals [22]. Therefore reduction in the activity of CAT may result in a number of deleterious effects due to the assimilation of superoxide radical and hydrogen peroxide. A higher dose (500 mg/kg) increases the level of CAT as produced by silymarin, the standard hepatoprotective drug.

Glutathione is one of the most abundant tripeptide, non-enzymatic biological antioxidant present in the liver. It removes free radical species such as hydrogen peroxide, superoxide radicals and maintains membrane protein thiols. Also it is substrate for glutathione peroxidase (GPx) [23]. Decreased level of GSH is associated with an enhanced lipid peroxidation in CCl₄ treated rats. Administration of *P. aculeata* significantly ($P < 0.001$) increased the level of GPx and GST in a dose dependent manner.

Extensive vascular degenerative changes and centrilobular necrosis in hepatocytes was produced by CCl₄. Treatment with different doses of ethanolic extract of leaves of *P. aculeata* produced only mild degenerative changes and absence of centrilobular necrosis, indicating its hepatoprotective efficiency.

Free radical mediated process has been implicated in pathogenesis of most of the diseases. The protective effect of *P. aculeata* on CCl₄ induced hepatotoxicity in rats appears to be related to inhibition of lipid peroxidation and enhancement of antioxidant enzyme levels in addition to free radicals scavenging action. Preliminary phytochemical studies reveal the presence of flavanoids in methanolic extract of *P.*

aculeata. Flavanoids are hepatoprotectives [24, 25]. The observed antioxidant and hepatoprotective activity of *P. aculeata* may be due to the presence of flavanoids. Further studies to characterize the active principles and to elucidate the mechanism are in progress.

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