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Hepatoprotective Activity of Pod Extract of *Plumeria rubra* against Carbontetrachloride-Induced Hepatic Injury in Rats (Wistar)

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ABSTRACT

The present work has designed to study the protective effects of the alcoholic extract of Pod *Plumeria rubra* on CCl_4 induced hepatic injury in male albino rats (Wistar strain) was investigated. For acute and massive invasion of hepatopathy, CCl_4 (S.C) injection of CCl_4+Olive Oil in 1:1 ratio; 2ml/kg) was used and the insidious intoxication was evidenced by significant turmoil of various biochemical parameters followed by significant ($p<0.001$) weight loss in toxic control group. The administration of alcoholic pod extract (200mg/kg and 100mg/kg of body weight) for 7 days, elicited protective action since the elevated levels of marker enzymes (SGOT, SGPT, ALP) of liver functions were found to be decreasing progressively in a dose dependent manner. The final body weight significantly ($p<0.001$) increased when compared with the toxic control group. The total serum protein and the serum albumin were also approaching normal values. The results found in alcoholic extract 200mg/kg treated rats were quite promising and were comparable with a standard drug Silymarin. In the alcoholic extract 200mg/kg treated rat group, all the marker enzymes were analyzed to be decreasing significantly. The statistically processed results support the conclusion, that the alcoholic Pod extract of *Plumeria rubra* (200mg/kg and 100mg/kg) possesses dose dependent, significant protective activity against CCl_4 induced hepatotoxicity.

INTRODUCTION

The entire world population is turning towards natural drugs because of the widespread belief that ―green medicines are healthier and safer than synthetic ones (Trivedi, 2004). It is also gaining greater acceptance from the public and the medical profession due to greater advances in understanding the mechanism of action by which herbs can positively influence health and quality of life (Fugh-Berman, 2000). Natural products have also been the starting point for the discovery of many important modern drugs. This fact has led to chemical and pharmacological investigations and general biological screening programs for natural products all over the world (Farvin, *et. al.*, 2006). Liver is the important vital organ, regulating various physiological processes such as, metabolism, secretion, storage and detoxification of toxic substances. Therefore, damage to the liver inflicted by hepatotoxic agents is of great concern (Shanmugasundaram & Venkantaraman, 2006). The hepatotoxicity is mainly caused by toxic chemicals such as carbon tetra chloride (CCl_4), alcohol, drugs such as paracetamol, antidiabetic drugs. Most of the chemicals damage the liver cell mainly by inducing lipid peroxidation and other oxidative damages. The reactive oxygen species such as superoxide anion radical (O_2), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH) have been implicated in the pathophysiology of various clinical disorders including ischemia, atherosclerosis, acute hypertension, liver diseases and diabetes mellitus. With the shifting of attention from synthetic drugs to natural plant products (Manonmani, 2005), the use of plant extracts for the treatment for liver diseases are now on the increase. Plants that were once considered of no value are now being investigated, evaluated and developed into drugs with no side effects. One such potential plant is *Plumeria rubra* L. (Hindi: Lalchampa; English: True Frangipani) is a laticiferous tree and shrub, belonging to the Apocynaceae family. The decoction of bark and roots of *Plumeria rubra* plant is traditionally used to treat asthma, ease constipation, promote menstruation, reduce fever and the latex is used to soothe irritation (Wiart, 2002). In India, however, its fruit is used as an abortifacient (Zaheer, 2010). The decoction of the flowers of *Plumeria rubra* is reported to be used for control of diabetes mellitus in Mexico (Bobbarala, *et. al.*, 2009). The leaves of *Plumeria rubra* are used in ulcers, leprosy, inflammations, rheumatism, bronchitis, cholera, cold and cough and as rubefacient, antibacterial, antipyretic, antifungal, stimulant etc. (Kirtikar & Basu, 1991). The selection of the plant *Plumeria rubra* Linn. was made on the basis of its easy of availability, therapeutic value and degree of research work which is not done. Therefore, the present work was undertaken to validate scientifically the therapeutic role of *Plumeria rubra* pods.

MATERIALS AND METHODS

Collection of plant material:

The plant *Plumeria rubra* was collected during the flowering period of August to October from Melghat region (20° 51′ to 21° 46′ N and to 76° 38′ to 77° 33′ E) of Amravati district of Maharashtra state of India. It was identified and authenticated by experts from Botanical Survey of India, Pune (Accession No. DD- 1).

Preparation of the extract:

The pods of *Plumeria rubra* were collected, shade dried, powdered and subjected to Soxhlet extraction with ethanol for 48 hrs. and filtered. The extract was evaporated to near dryness on a water bath, weighed and kept at 4°C in refrigerator until used for experimental evaluation.

Phytochemical screening:

The presence of various plant constituents in the plant extract was determined by preliminary phytochemical screening as per Thimmaiah (2004).

Animals:

Inbred colony of adult Wistar albino rats (170 –200 g) of either sex were used for the pharmacological activities. They were kept in polypropylene cages at 25 ± 2° C, with relative humidity 45-55% under 12h light and dark cycles. All the animals were acclimatized to the laboratory conditions for a week before use. They were fed with standard animal feed (Treemurti feeds Nagpur, India.) and water ad libitum. The test extracts and the standard drugs were administered in the form of a suspension in water using 1% carboxymethylcellulose (CMC) as suspending agent. The protocol of present study was approved by Institutional Animal Ethical Committee constituted as per CPCSEA guidelines (1060/ac/07/CPCSEA/Dec2009).

Acute toxicity study:

Healthy female albino rats were starved for 3- 4 hours and subjected to acute toxicity studies as per Organization of Economic Co-operation and Development (OECD) guidelines No: 423 and a highest dose (200mg/kg) was selected for treatment. The rats were observed continuously for 2 hrs. for behavioral, neurological and autonomic profile and next 24 to 72 hrs. for any lethality or death (OECD, 2001).

Drugs and dosing schedule:

The animals were divided into five groups group I (control), group II (CCl_4 treated), group III (CCl_4 + Silymarin treated), group IV and V (CCl_4 + extract). Animals of groups II, III, IV and V were administered 50% (v/v) CCl_4 in olive oil in a single dose of 2ml/Kg of body weight per day for 4 days via the S.C. (Subcutaneous) route. Simultaneously but at different hours of the day, animals of group III treated with Silymarin suspension (10 mg/kg body weight, I.P. (Intra-peritoneal) I.P. for 4 days respectively. Animals of group IV and V were fed with alcoholic extract in doses of 100 mg/kg and 200 mg/kg body weight respectively, Animals of group I, were given distilled water in a volume of 10 ml/kg body weight.

Preparation of samples for Biochemical studies:

On the day 5, after the treatment period all of the subject animals were anaesthetized and sacrificed and blood was withdrawn from heart and their serum was separated by centrifugation at 3000 rpm at 30^0C for 15 min. This was subsequently analyzed for various biochemical parameters including serum transaminases viz. SGOT (Rietman & Frankel, 1957), SGPT (Rietman & Frankel, 1957), total protein (Gornall, 1949), alkaline phosphatase (Kind & King, 1954), bilirubin (Malloy & Evelyn, 1937), and total albumin (Lowry, 1951).

Histopathological evaluation of liver:

The formalin fixed liver tissues were embedded in paraffin wax and microtome sections of 5-6 µm were made from them. These thin sections were stained with haematoxylin and eosin for Light microscopy. Photomicrographs were subsequently made from these sections.

Statistical analysis:

The data obtained were analyzed using student's t-test and results expressed as Mean ± standard error of mean. Statistical differences between means were determined by ANOVA. Values of $p<0.05$ was considered significant.

RESULTS

TABLE I: Phytochemical analysis of *Plumeria rubra* ethanolic pod extract:

Test for Phytoconstituents	Alkaloids	Glycosides	Anthraquinones	Saponins	Flavonoids	Steroids	Tanins
Results	+	+	-	+	+	+	+

Positive = +, Negative= -

Preliminary phytochemical screening of the pod extract *Plumeria rubra* revealed the presence of alkaloids, glycosides, saponins, flavonoids, steroids and tannins whereas anthraquinones was not detected in the test.

TABLE NO. II: Effect of *Plumeria rubra* ethanolic pod extract and silymarin on biochemical parameters during CCl_4 induced acute liver damage in albino rats (n=6).

Sr. No.	S.G.O.T	S.G.P.T	A.L.P	Billirubin	CHL	ALB
Control (Normal)	58.50±1.51	28.3± 2.6	25.48±0.6	0.17±0.2	100.03±2.40	6.98±0.14
CCl_4treated	111.50±40.02	102.00±11.10	89.82±7.36	0.52±0.34	212.8±2.7	2.06± 0.28
Std. Silymarin	60.74±5.01***	38.50±2.108***	36.50± 1.34***	0.25±0.34***	105.10±6.9*	5.35± 0.27**
Extract 100 mg/kg	85.62±6.60**	65.67±4.63**	63.43±2.04**	0.32±0.4	109.65±10.48*	3.05±3.36
Extract 200 mg/kg	66.30± 5.47**	45.67±2.94**	40.15± 9.80**	0.28±0.40**	113.30±2.50**	3.99±0.40**

Values in Mean±S.E.M, N=6. *Significant reduction compared to Carbon tetrachloride (P<0.05) ** Significant increase compared to Carbon tetrachloride (P<0.05)

Phytochemical investigation:

Phytochemicals are known to work many general and specific functions in plants and may exhibit different biochemical and pharmacological actions in different species of animals when ingested. Their actions range from cell toxicity to cell protective effect (Reitman & Frankel, 1957). The preliminary phytochemical screening of the pod extract of *Plumeria rubra* revealed the presence of alkaloids, flavonoids, steroids, tannins and saponins whereas anthraquinone was not found. Similar finding was reported by Uboh, *et al.*, (2010) in aqueous extract of *Psidium guajava* leaves in rats.

Clinical toxicity symptoms such as salivation, respiratory distress, change in appearance of hair, weight loss as well as mortality were not observed at any period of experiment. Similarly no mortality and change in behavioral, neurological and autonomic profile were seen in treated groups of the rats up to highest dose of 2000 mg/kg body weight.

Serum Analysis:

The administration of CCl_4 induced acute liver damage which is well indicated (Table II) by increased SGOT (Serum glutamate oxalate transaminase), SGPT (Serum glutamate pyruvate transaminase), ALP (Alkaline phosphatase), TPTN (Total protein), CHL (Cholesterol) and TBL (Total bilirubin) when compared with normal control group. The highly significant ($p<0.0001$) reduction was observed in all the parameters in the ethanolic extract group in comparison with positive control treated group. Among these, ethanolic extracts show maximum percentage reduction in SGOT, SGPT, ALP and TBL after administration of CCl_4. Though 200mg/kg alcoholic extract group also show significant results but less pronounced when compared with the 100mg/Kg ethanolic extract group. These results suggest the possibility of the 200mg/Kg ethanolic extract to give very good protection against liver injury upon CCl_4 induction within 72 hrs.

Histochemical Studies:

Histopathological observations also provided supportive evidence for the biochemical analysis. Normal control (Figure 1) group showed a normal liver architecture, hepatocytes very well

arranged, central and portal veins without alterations. The livers of rats treated with CCl_4 (Figure 2) for 5 days showed total loss of hepatic architecture with extensive accumulation of connective tissue resulting in formation of continuous fibrotic septa, nodules of regeneration, fatty changes, noticeable alterations in the central vein, hepatic necrosis, vacuolization, congestion of sinusoids, Kupffer cell hyperplasia, crowding of central vein, pronounced inflammation from portal to portal tract bridges and apoptosis compared to the normal control. Oral administration of Silymarin (Figure 3) shows recovery of hepatic architecture with uniform central vein dilation and precentral hepatitis with lymphomonocytes surrounding the vein. Some little foci were observed in portal triaditis and only central zone hepatitis as Silymarin treated. There are thick inflammatory bridges between portal tracts with parenchymal collapse. Inflammation around portal tracts with some loss of lamina limitans, among these plant extract, treatment with ethanolic extract (Figure 4 & 5) returned the injured liver to quite normal. Less pronounced loss of the liver architecture without fibrosis and minimal inflammation. Only peripheral zonal fatty changes were observed as in extract treated groups.

DISCUSSION

Reactive oxygen species (ROS) are causative factors of degenerative diseases, including some hepatopathies. Liver is an important organ actively involved in metabolic functions and is a frequent target of number of toxicants. Carbon tetrachloride has been widely used for inducing experimental hepatic damage due to free radical formation during its metabolism by hepatic microsome, leading to lipid peroxidation, and consequently, liver damage. The resulting hepatic injury is characterized by leakage of cellular enzymes into blood stream and by necrosis and fibrosis (Adeneye, 2009).

Carbon tetrachloride is selected as hepatotoxicant in inducing injury to the liver as it is known to cause hepatotoxicity in man and experimental animals when given in overdose. Popularity of herbal remedies is increasing globally and at least one quarter of patients with liver diseases use ethnobotanicals (Sharma & Sharma, 2010). Many formulations containing herbal extract are sold in the Indian market for liver disorders (Saleem, *et.al.* 2010). Therefore the present study was aimed at evaluating the scientific basis for the traditional use of alcoholic extract of *Plumeria rubra* pod using *in vivo* experimental model. Several studies shown that α-tocopherol (Vitamin E) and Silymarin are potent antioxidant that could protect the liver against CCl_4 hepatotoxicity

indicating that oxidative stress could play a pivotal role in CCl_4 hepatic injury (Yoshikawa, *et.al.*, 1982). To confirm the effects of the herbal medicinal plants in this study, biochemical and histological parameters were used. Assessment of liver damage can be made by estimating the activities of serum ALT, AST, ALP, TB which are enzymes and proteins originally present in higher concentration in cytoplasm. The elevated levels of these entire marker enzymes observed in the CCl_4 treated group II rats in the present study corresponded to the extensive liver damage induced by toxins, the tendency of these marker enzymes to return towards a near normalcy in group 3 (Silymarin), 4 and group 5 (ethanolic extract) treated rats was a clear manifestation of hepatoprotective effect of alcoholic Pod extract of *Plumeria rubra*. The protective effect of *Plumeria rubra* pod is also proved by histopathological examination of livers of rats treated with ethanolic extracts prior to CCl_4 administration which were almost normal in structure with slight changes. Although the exact mechanisms behind this protection are uncertain, many theories have been proposed. In the last decades, special attention has been paid towards edible plants, especially those that are rich in secondary metabolites and nowadays, there is an increasing interest in the antioxidant activity of such phytochemicals present in diet. However we can assume that the protective factor in this study is due to presence of alkaloids, flavonoids, saponins, tannins and terpenoids. The probable mechanism is mediated by their higher quantity of flavonoids or terpenoids in ethanolic extract or by their combination via antioxidant and free radicals scavenging activities (Kaur, *et.al.* 2006). Flavonoids, phenolic acids and some terpenoids have been reported to possess antioxidant activities by different mechanisms (Narayana, *et.al.* 2001). The second probable mechanism is due to effective blocking of oxidative stress and cytokines production, ethanolic extract protected against lipopolysaccaride induced liver damage through decreasing the level of TNF- and IL6 and prevented cytotoxic effect of oxygen free radicals and cytokines (Rice-evans, *et.al.*, 1997). In conclusion the present study demonstrated that ethanolic extract of *Plumera rubra* pods in comparison with silymarin groups has better hepatoprotective effect in CCl_4 induced liver damage. However, it is necessary to determine other parameters such as oxidative stress markers and molecular biology assays to confirm our findings. However, further studies will be required on molecular level and isolation of active constituents to substantiate this effect.

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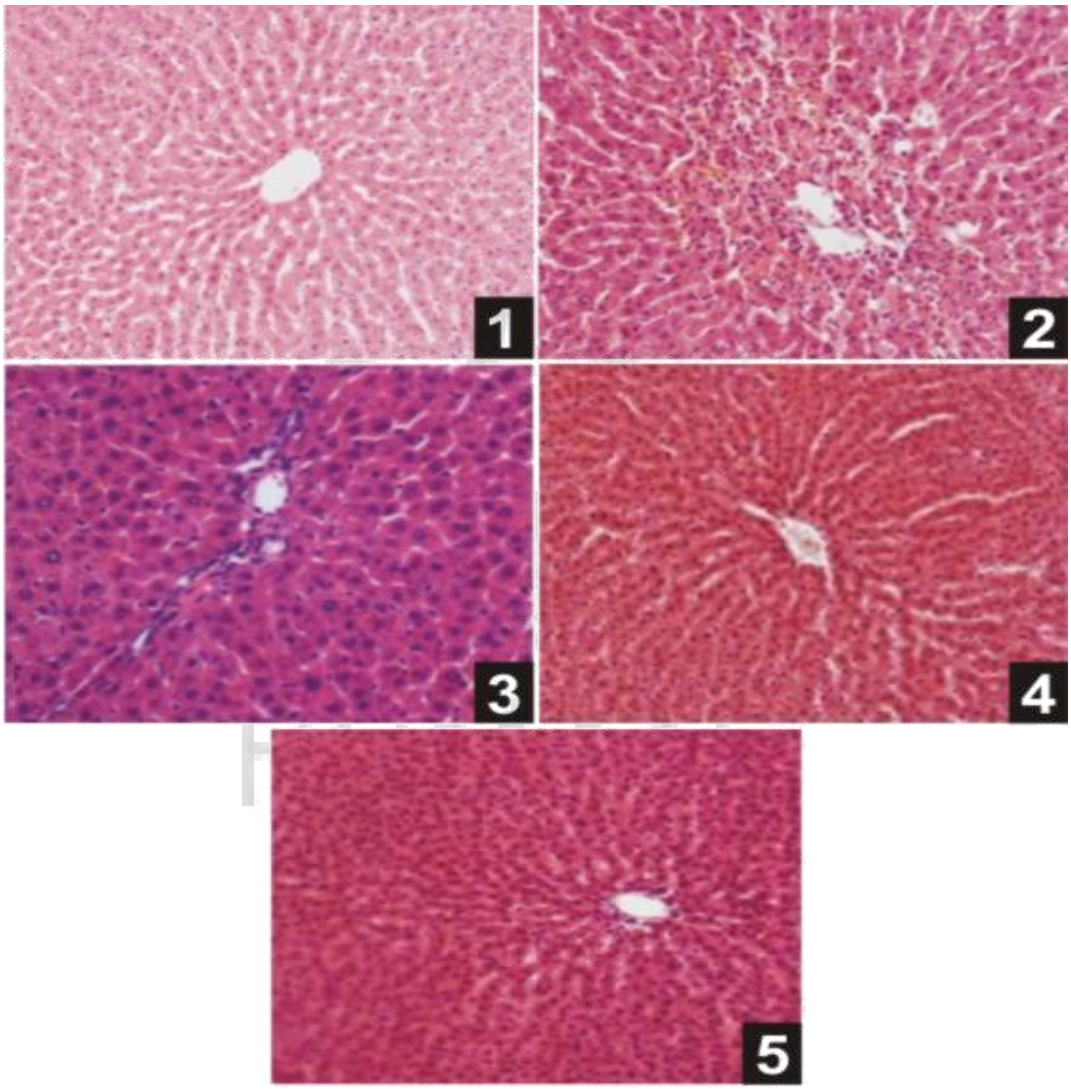

Figure. 1-5 Transverse section showing histology of different groups. Fig. 1: Control. Fig. 2: CCl_4. Fig. 3: CCl_4 + Silymarin. Fig. 4: CCl4 + 100 mg/Kg. Fig. 5: CCl4 + 200 mg/Kg