

## Evaluation of Hepatoprotective and *In-vitro* Cytotoxic Activity of Leaves of *Premna serratifolia* Linn

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Received 14 August 2008, accepted in final revised form 20 November 2008

### Abstract

*Premna serratifolia* is used by the traditional practitioners as cardiotoxic, antibiotic, anti-coagulant, stomachic, carminative, hepatoprotective, antitumor etc. The present study aims in the evaluation of hepatoprotective and *in-vitro* cytotoxic activity of alcoholic extract of leaves of *Premna serratifolia* Linn. Hepatoprotective activity is studied by carbon tetrachloride induced hepato-toxicity in rats and the *in-vitro* cytotoxic activity is carried out by tryphane blue exclusion method using EAC cell lines. The degree of protection in hepatoprotective activity has been measured by using biochemical parameters such as serum glutamate oxalate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP), bilirubin and total protein. The results suggest that the alcoholic extract at the dose level of 250mg/kg has produced significant ( $p < 0.001$ ) hepatoprotection by decreasing the activity of serum enzymes, bilirubin, and lipid peroxidation which is comparable to that of standard drug silymarin. The alcoholic extract also does exhibit the  $IC_{50}$  value of 75 $\mu$ g/ml which indicates the significant *in-vitro* cytotoxic activity of the extract. It is concluded that alcoholic extract of leaves of *Premna serratifolia* Linn is not only an effective hepatoprotective agent, but also possesses significant antitumor activity.

**Keywords:** *Premna serratifolia* Linn; Alcoholic extract hepatoprotective; *In-vitro* cytotoxic activity.

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DOI: 10.3329/jsr.v1i1.1046

### 1. Introduction

Nature has provided an excellent storehouse of remedies to cure all the ailments of mankind. In ancient days, almost all the medicines used were from natural sources, particularly from plants. Plants continue to be an important source of new drugs even today. The importance of botanical, chemical and pharmacological evaluation of plant-derived agents used in the treatment of human ailments has been increasingly recognized in the last decades. Herbal remedies are widely used for the treatment and prevention of

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various diseases and often contain highly active multitude of chemical compounds. Modern research is now focusing greater attention on the generation of scientific validation of herbal drugs based on their folklore claim. In this modern era, a large Indian population still relies on the traditional system of medicine, which is mostly plant based.

Free radical initiating auto oxidation of cellular membrane lipids can lead to cellular necrosis and is now accepted to be important in connection with a variety of pathological conditions [1]. Liver is an aerobic organ which generates reactive oxygen species that induce oxidative tissue damage. These radicals react with cell membranes and induce lipid per oxidation or cause inflammation, which may result as important pathological mediators in many clinical disorders such as heart disease, diabetes, gout and cancer [2]. Reduction of these radicals by antioxidant molecules is crucial to the protection of cells against various disorders. Development of life threatening diseases like cancer and also liver disorders are linked to the availability of these antioxidants. Flavonoids are phenolic compounds widely distributed in plants, and have been reported to exert multiple biological effects, including antioxidant and free radical scavenging abilities [3].

*Premna serratifolia* Linn (Verbenaceae), otherwise known as “Agnimantha” in Ayurvedic system of medicine, is a small-sized tree or a large shrub, up to 9m in height [4] with a comparatively short trunk and numerous branches. It is widely used by the traditional practitioners as cardiogenic, antibiotic, anti-coagulant, stomachic, carminative, hepatoprotective, antitumor [5] etc. Preliminary phytochemical studies revealed the presence phytochemicals like flavanoids, alkaloids, triterpenoids in the alcoholic extract. The present study aims in the evaluation of hepatoprotective and *in-vitro* cytotoxic activity of alcoholic extract of leaves of *Premna serratifolia* Linn.

## **2. Materials and Methods**

### **2.1. Plant materials**

The leaves of *Premna serratifolia* Linn were collected in the month of March- April. It was authenticated by Prof. P. Jayaraman, Botanist and Director, PARC, Chennai. A voucher specimen was deposited in the department for future reference. The leaves were shade dried, coarsely powdered and subjected to successive extraction using n-hexane and alcohol in soxhlet apparatus. Both the extracts were then dried under reduced pressure and the percentage yields of the extracts were 8.2%, 20% respectively. Hepatoprotective and *in-vitro* cytotoxic activity were carried out by using alcoholic extract.

### **2.2. Experimental animals**

24 male Albino rats of Wister strain weighing 150-200 g were used for the study. They were maintained under standard environmental condition (temperature 25-28<sup>0</sup>C and 12h light/dark cycle) and they were allowed with standard laboratory feed and water *ad libitum*. The animals were given a week's time to get acclimatized with the laboratory

condition. Initial body weights of all animals were recorded. Ethical clearance for the use of animals was obtained from the committee constituted for the purpose.

### 2.3. Acute toxicity study

Acute toxicity study was carried out using Acute Toxic Class Method as described in OECD (Organization of Economic Co-operation and Development) Guidelines No. 423.

### 2.4. Hepatoprotective activity

#### *Experimental design* [6]

A total of 24 Albino rats were divided into 4 groups of 6 animals each:

- Group I:** Vehicle control and received orally with normal saline 5ml/kg body weight daily for 9 days.
- Group II:** Carbon tetra chloride control.
- Group III:** Served as test and received alcoholic extract of leaves of *Premna serratifolia* Linn (250 mg/kg p.o) daily for 9 days.
- Group IV:** Animals served as standard received silymarin (25mg/kg) per oral [7] for 9 days.

On day 7 and 9, Carbon tetra chloride 0.1 ml/kg/day [8] of body weight i.p was given to all rats except the rats in group I. After 24 hours of the second dose of CCl<sub>4</sub> blood was collected from all the animals by retro-orbital plexus and the serum was separated by centrifugation at 2000 rpm for 10 minutes, since many of the biochemical and histological changes are known to manifest after 24 hours of CCl<sub>4</sub> administration [9]. The Serum was used for the assay of hepatic marker enzymes, Serum Glutamate Oxaloacetate Transaminase (SGOT), Serum Glutamate Pyruate Transaminase (SGPT), Alkaline phosphatase (ALP), Bilirubin and Total Protein. After collection of blood samples the rats in different groups were sacrificed and their livers were excised immediately and washed in ice-cold normal saline.

### 2.5. Assessment of biochemical parameters

Estimation of SGOT and SGPT were based on the reference method described in International Federation of Clinical Chemistry [10]. The reagent supplied in the kits (Bayer Diagnostic Kits) were reconstituted, mixed with serum as directed. The SGOT and SGPT were measured at 340nm and expressed as IU/L. The serum alkaline phosphatase was estimated by mixing with the reagent (p-nitro phenyl phosphate, magnesium, buffers and stabilizers) with serum, estimated at 405 nm and expressed as IU/L. Biurete method [11] was followed for the estimation of total protein. Serum was mixed with Biuret

reagent and incubated for 10min at 37°C. The total protein was estimated at 555nm and expressed as g/dl. Total Bilirubin was estimated by Jandrossik and Grof [12] method measured at 546 nm and expressed as mg/dl.

## **2.6. Histopathological examination**

The blood was collected by retro-orbital plexus, the liver was removed, sliced and washed in saline. Liver pieces were preserved in 10% formalin (10% formaldehyde diluted using normal saline) for histopathological study. The pieces of liver were processed and embedded in paraffin wax. Sections were made about 4-6 µm in thickness, stained with haematoxylin and eosin. They were mounted and observed under light microscope for histological changes.

## **2.7. In-vitro cytotoxic activity [13]**

The *in-vitro* cytotoxic activity was carried out by using EAC cells lines by incubating  $1 \times 10^6$  EAC cells in 1 ml phosphate buffer saline with alcoholic extract of leaves of *Premna serratifolia* at 30°C for 3 hrs in CO<sub>2</sub> atmosphere. The viability of the cells was determined by trypan blue exclusion method.

## **2.8. Statistical analysis**

The data are expressed as mean  $\pm$  SEM. Statistical analysis was made by one way ANOVA followed by Tukey-Kramer multiple comparison test; *p* values <0.05 are considered as significant. Highest significant difference test has been performed with Graph pad instat software.

## **3. Results**

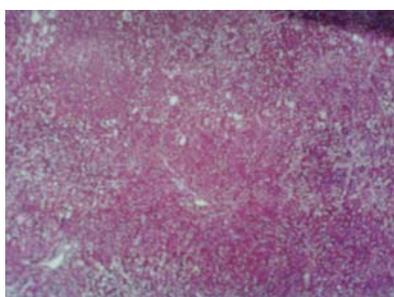
The effects of alcoholic extract on SGOT, SGPT, ALP, bilirubin and total protein levels in carbon tetrachloride induced liver damage in rats are summarized in Table 1. Administration of CCl<sub>4</sub> (0.1ml/kg b.wt) and 24 hrs of intoxication resulted in a significant elevation of serum marker enzymes SGPT, SGOT, ALP and Bilirubin and reduced protein content when compared with normal control. On administration of alcoholic extract of leaves of *Premna serratifolia* Linn and Silymarin (25mg/kg) the levels of the enzymes were found to be retrieving towards normality.

Histopathological observation of group I showed a normal hepatic architecture (Fig. 1 a) and in group II (CCl<sub>4</sub> treated) severe hepatotoxicity was evidenced by profound necrosis (Fig. 1 b). In group III and IV, the liver exhibited an almost normal architecture barring a little deformation of hepatocytes with pyknosis and clearing of cytoplasm (Fig. 1 c, d).

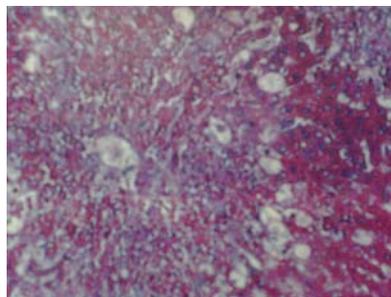
Table 1. Effect of hepatoprotective activity of the ethanolic extract of the leaves of *Premna serratifolia* Linn against CCl<sub>4</sub> induced hepatotoxicity in rats.

Treatment	SGOT (IU/L)	SGPT (IU/L)	Alkaline Phosphatase (IU/L)	Total Protein (g/dl)	Total Bilirubin (mg/dl)
Vehicle control	43 ± 1.29	130.25 ± 2.28	184.2 ± 1.75	9.4 ± 0.15	0.58 ± 0.025
CCl <sub>4</sub> Treated	180.75 ± 1.88 <sup>#</sup>	263.75 ± 1.49 <sup>#</sup>	475.2 ± 2.39 <sup>#</sup>	6.5 ± 0.21 <sup>#</sup>	1.65 ± 0.29*
Alcoholic extract (250mg/kg)	62.75 ± 1.50 <sup>#</sup>	185 ± 1.58 <sup>#</sup>	226 ± 4.30 <sup>#</sup>	8.58 ± 0.14*	0.67 ± 0.04
Silymarin (25mg/kg)	47 ± 1.780	114 ± 1.71 <sup>#</sup>	193.5 ± 1.55	8.83 ± 0.11	0.47 ± 0.02

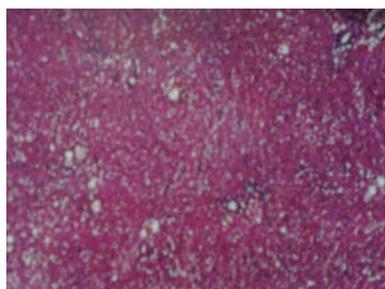
Values are expressed as mean ± SEM of 6 animals in each group. \**p*<0.01, <sup>#</sup>*p*<0.001 as compared to group I



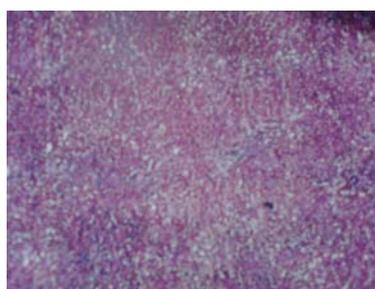
a. Group-I: Normal rat (normal cell).



b. Group-II: CCl<sub>4</sub> treated rat (severe cell necrosis).



c. Group-III: Methanolic Extract treated rat (almost normal architecture).



d. Group-IV: Silymarin treated rat (almost normal architecture).

Fig. 1. Histopathology of liver tissues.

The *in-vitro* cytotoxicity was assessed by tryphane blue exclusion method and the IC<sub>50</sub> of the extract was found to be 75µg/ml (see Table 2).

Table 2. *In-vitro* cytotoxic activity.

Conc. µg/ml	Viable cell count	Dead cell count	Total cell count	% cell viability	% cell death	IC <sub>50</sub> µg/ml
50	96	86	182	52.7	47.2	
100	105	260	315	28.8	69.2	
200	125	300	425	29.4	70.5	75
400	60	380	440	13.6	86.3	
800	42	430	472	8.9	91.1	
1000	09	480	489	1.8	98.1	

#### 4. Discussions

The liver damage in CCl<sub>4</sub> induced hepatotoxin is mainly assessed by determining the serum enzyme levels. Liver is considered to be highly sensitive to toxic agents. CCl<sub>4</sub> administration causes necrosis or membrane damage of liver thereby release of enzymes into the circulation which can be determined by using serum.

In the present study it was observed that the animals treated with CCl<sub>4</sub> resulted in the significant hepatic damage as shown by the elevated levels of marker enzymes. These changes in the marker level will reflect in hepatic structural integrity. The rise in the SGPT plays a vital role in the conversion of alanine to pyruvate and glutamate (amino acids to ketoacid) [14]. The pre-treatment with extract significantly attenuated the elevated levels of the serum markers. SGPT is more specific to liver, and is thus a better parameter for detecting the liver injury [15].

The normalization of the serum markers by the extract suggests that they are able to condition the hepatocytes so as to protect the membrane integrity against CCl<sub>4</sub> induced leakage of marker enzymes in the circulation. The above changes can be considered as an expression of the functional improvement of hepatocytes, which may be caused by an accelerated regeneration of parenchyma cells [16]. Serum ALP and bilirubin levels on the other hand are related to the function of hepatic cells. Increase in serum level of ALP is due to increased synthesis, in presence of increasing biliary pressure [17]. Our findings suggest that the treatment with extract caused significant inhibition of ALP and bilirubin levels.

Liver cell injury induced by CCl<sub>4</sub> involves the biotransformation of the toxin carbon tetrachloride by cytochrome P-450 to produce the trichloromethyl free radical, which causes peroxidative degradation in the adipose tissue resulting in fatty infiltration of the hepatocytes. Trichloromethyl free radicals elicit lipid peroxidation of membrane lipids in the presence of oxygen generated by metabolic leakage from mitochondria. All these events culminate in the loss of integrity of the cell membranes and damage of hepatic

tissue [18]. Treatment with the 250mg/kg ethanolic extract of the leaves significantly reduced the elevated liver enzymes and bilirubin level, indicating the hepatoprotective action.

Comparative histopathological study of the liver tissue from different groups of rats also confirmed the hepatotoxic levels of carbon tetrachloride. The study also confirms the preventive effect of *Premna serratifolia* which causes regeneration of hepatic cells.

Hepatotoxic effect of carbon tetrachloride is due to oxidative damage by free radical generation and antioxidant property is claimed to be one of the mechanisms of hepatoprotective drugs. The preliminary phytochemical reports revealed that the ethanolic extract of the leaves were found to contain higher concentration of flavonoids and alkaloids. It has been reported that the flavonoid possess antioxidant properties by free radical scavenging. Flavonoids are phenolic compounds widely distributed in plants, which have been reported to exert multiple biological effects, including antioxidant and free radical scavenging abilities. Over expression of fibrogenic cytokines as well as increased transcription and synthesis of collagen can be down regulated, at least in experimental models by the use of antioxidants. A study has demonstrated that natural phenolics inhibit stellate cell activation by perturbing signal transduction pathway and cell protein expression. The co-administration of hepatoprotective agents may induce the hepatocytes to resist the toxic effects of carbon tetrachloride. The results indicate that the ethanolic extract of the leaves of *Premna serratifolia* Linn has significant hepatoprotective activity.

The *in-vitro* cytotoxic activity was assessed by dye exclusion method. The alcoholic extract of *Premna serratifolia* Linn showed the IC 50 value of 75µg/ml indicating the cytotoxic activity of the extract. The activity may be due to the presence of flavanoids in the plant extract. Flavanoids have a chemopreventive role in cancer through their effects on signal transduction in cell proliferation [19] and angiogenesis [20].

## 5. Conclusion

The present investigation revealed that the alcoholic extract of leaves of *Premna serratifolia* Linn exerted significant protection against CCl<sub>4</sub> induced hepato toxicity by its ability to ameliorate the lipid per oxidation. The extract also showed good *in-vitro* cytotoxic activity. Our result indicated that the potent hepatoprotective and cytotoxic activity of the extract may be due to its antioxidant and free radical scavenging properties which may be due to presence of flavanoids in the plant. Further investigation is under progress to evaluate the *in vivo* anticancer activity and to determine the exact phytoconstituents that is responsible for its hepatoprotective and cytotoxic activity.

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