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Evaluation of hepatoprotective and antioxidant activity of methanolic extract of *Pongamia pinnata* leaves

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ABSTRACT

The aim of present work was to conduct experiment to check traditional claim of methanolic extract of *Pongamia pinnata*(PP) leaves as hepatoprotective and antioxidant. The hepatotoxicity was induced in rats using paracetamol 3 gm/kg on 8th day of treatment whereas *In vitro* estimation of antioxidant activity was carried out using two different methods i.e.by hydroxyl radical scavenging activity and total reduction capability. The different doses (100, 200, 400 mg/kg) of the methanolic extract of PP were screened for hepatoprotective and antioxidant activity. The (aspartate amino transferase (AST), serum alanine amino transferase (ALT), serum alkaline phosphatase (SALP) values recorded 24 hours after paracetamol administration were significantly reduced in pretreatment with methanolic extract of *Pongamia pinnata* group. It was also found that the methanolic extract of *Pongamia pinnata* showed dose dependant hydroxyl radical scavenging activity and total reduction capacity.

Keywords: *Pongamia pinnata*, hepatoprotective, antioxidant

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INTRODUCTION

The rate of hepatotoxicity has been reported to be much higher in developing countries like India (8% - 30%) compared to that in advanced countries (2%-3%) with a similar dose schedule [1]. Paracetamol (acetaminophen) is a commonly and widely used NSAID for the management of variable degree of pain and inflammation. Long term administration of large doses of paracetamol deplete the normal levels of hepatic glutathione, an enzyme that protects hepatocytes by combining with the reactive metabolite of paracetamol thus preventing their covalent binding to liver proteins [2]. Reactive oxygen species occur in tissues participating in potentially deleterious reactions controlled by a system of enzymatic and non- enzymatic antioxidants which eliminate pro-oxidants and scavenge free radicals, [3]. The main characteristic of an antioxidant is its ability to trap free radicals. Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxy and thus inhibit the oxidative mechanisms that lead to degenerative diseases.

Use of herbal drugs as medicines for the treatment of a range of diseases can be traced back since ancient time's i.e. during the Vedic period in India [4]. Being the outcome of therapeutic experiences of generations of practicing physicians of indigenous systems of medicine for over hundreds of years medicinal plants have played a key role in world health [5, 6]. Hence, in spite of the great advances observed in modern medicine in recent decades, herbal medicines still make an important contribution to health care [7]. Literature survey of *Pongamia pinnata* revealed its potential being like hepatoprotective and antioxidant [8]. However it remains to be scientifically validated. Hence present study titled "Evaluation of hepatoprotective and antioxidant activity of methanolic extract of *Pongamia pinnata* leaves" has been conducted.

MATERIALS AND METHODS

Plant material

Leaves of *Pongamia pinnata* (PP) were purchased from local vendors and were identified and authenticated from National institute of science communication and information sources (NISCAIR). Certification No: NISCAIR/RHMD/Consult/08-09/1052/83/06.

Chemicals and Drugs

Paracetamol, Phosphate buffer, Hydrogen peroxide and Vitamine C.

Preparation of Extract

Pongamia pinnata (PP) leaves were dried and charged to extractor along with methanol. The mass was heated for 5-6 hours in a closed system by re-pumping the extract to herb bed. This procedure was repeated. The extracts were combined, filtered and concentrated under



vacuum. This was subjected to spray drying to separate extract in the powder form. This powder was further subjected to multimill to obtain fine mesh size powder. It was sieved by a sifter and mixed in the blender to obtain a uniform particle sized powder [9].

Storage of extracts

Methanolic extract of PP was stored in tightly closed glass bottles in refrigerator at 2-8 °C.

Preparation of extract solutions

Test solutions (T.S) of Methanolic extract of PP was prepared in distilled water in order to make concentration 100 mg/ml.

Animals

Wistar albino mice (18- 22gm) and rats (120-150gm) were used. They were maintained at $25 \pm 2^\circ$ C and relative humidity of 45 to 55% and under standard environmental conditions (12 hour. light 12 hour. dark cycle). The animals had free access to food (Chakan Oil Mills, Pune, India) and water ad libitum. Local Institutional Animal Ethical Committee (IAEC) approved the protocol. All experiments were carried out between 12:00- 16:00 h.

Preparation of drug solution

Accurately weighed quantity of powdered extract of PP was dissolved in the distilled water to prepare the appropriate stock solution of the drug i.e. 10 mg/ml, 20 mg/ml and 40 mg/ml respectively. The doses were administered orally by selecting the appropriate concentration (10ml/kg) of the stock solution.

Route of administration

Methanolic extract of PP and Paracetamol were administered by oral route.

Acute toxicity study

Healthy adult male wistar albino mice (18- 22g) were subjected to acute toxicity studies as per guidelines (AOT 425) suggested by the organization for economic co-operation and development (OECD-2000). The mice were administered with the different doses of methanolic extract of PP or distilled water (10ml/kg). The dose progression or reduction was carried out as suggested by the AOT-425 guidelines.

Methods

The different doses (100, 200, 400 mg/kg) of the Methanolic extract of PP were

screened for following different pharmacological activities.

- Evaluation of hepatoprotective activity using paracetamol induced hepatotoxicity model.
- Evaluation of antioxidant activity.

Evaluation of Hepatoprotective activity using paracetamol model

Thirty pre-selected rats were randomly divided into five groups, each containing six rats. Wherein group I served as control and received vehicle (10ml/kg) for period of 08 days. Animals of Group II received vehicle for 07 days and Paracetamol 3 g/kg on the 08th day. Animals of Group III, IV and V received PP extract (100, 200 and 400 mg/kg/day) respectively for 07 days and Paracetamol 3 g/kg on the 08th day. 24 hours after paracetamol administration serum parameters (aspartate amino transferase (AST), serum alanine amino transferase (ALT), serum alkaline phosphatase (SALP) were estimated to assess hepatoprotective effect and thereafter all the animals were sacrificed and histopathological examination of cell necrosis, fatty change, hyaline degeneration, ballooning degeneration, and infiltration of kupffer cells and lymphocytes was carried out [10].

Assessment of liver functions

Blood samples of the rats were withdrawn from retrobulbar venous plexus with the help of a glass capillary under light anesthesia and were kept at room temperature for 02 hours, so that the coagulation process gets completed. The blood samples were centrifuged and serum thus separated was used for the estimation of AST, ALT, and SALP. Animals were then sacrificed and dissected. Their livers were taken out, washed with water, dried gently with filter paper and preserved in 10% formosaline. The activity of AST and ALT were measured and estimation of SALP was carried out [11].

Histopathological investigations

The liver samples fixed for 48 hours in 10% formosaline were dehydrated by passing successively in different mixtures of ethyl alcohol–water (50, 80, and 95%, and finally in absolute alcohol), cleared in xylene and embedded in paraffin. Sections (4–5mm thick) were prepared and then stained with hematoxylin and eosin dye for microscopic observation of cell necrosis, fatty change, hyaline degeneration, ballooning degeneration, and infiltration of kupffer cells and lymphocytes [12].

Evaluation of antioxidant potential by Hydroxyl Radical Scavenging Activity

The hydroxyl radicals scavenging activity was measured with Fenton reaction. The reaction mixture contained 60 µl of 1 mM ferric chloride (FeCl₃), 90 µl of 1mM 1, 10-phenanthroline, 2. 4 ml of 0.2 M phosphate buffer (pH 7.8), 150 µl of 0.17 M hydrogen peroxide (H₂O₂), and 1.5 ml of PP extracts at various concentrations. After the addition of H₂O₂, all the solutions were

incubated at room temperature for 05 minutes and the absorbance of the mixture was measured at 560 nm with a spectrophotometer. The hydroxyl radicals scavenging activity was calculated using the following equation.

$$\% \text{ Inhibition} = [(A_0 - A_1) / A_0 \times 100]$$

Where, A_0 was the absorbance of the control (blank) and A_1 was the absorbance in the presence of different concentrations of the extract [13].

Evaluation of antioxidant potential by Total Reduction Capability

Total reduction capability of PP (20 μ g/ml, 40 μ g/ml, 60 μ g/ml, 80 μ g/ml and 100 μ g/ml) and Vit C (20 μ g/ml, 40 μ g/ml, 60 μ g/ml, 80 μ g/ml and 100 μ g/ml) were estimated by using the method of Oyaizu. The different concentrations of PP and Vit C were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (1%, 2.5 ml). The mixture was incubated at 50 $^{\circ}$ C for 20 min. A portion of (2.5 ml) trichloroacetic acid (10 %) was added to the mixture. Then it was centrifuged for 10 min at 1000 g. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and Ferric chloride (0.5 ml, 0.1%) and the absorbance was measured at 700 nm by using spectrophotometer [14].

Statistical Analysis

The comparison was made against the vehicle treated control group and the data was expressed as mean \pm SEM. The data was analysed using suitable test with respect to individual models mentioned later [15].

RESULTS

Acute toxicity assessment

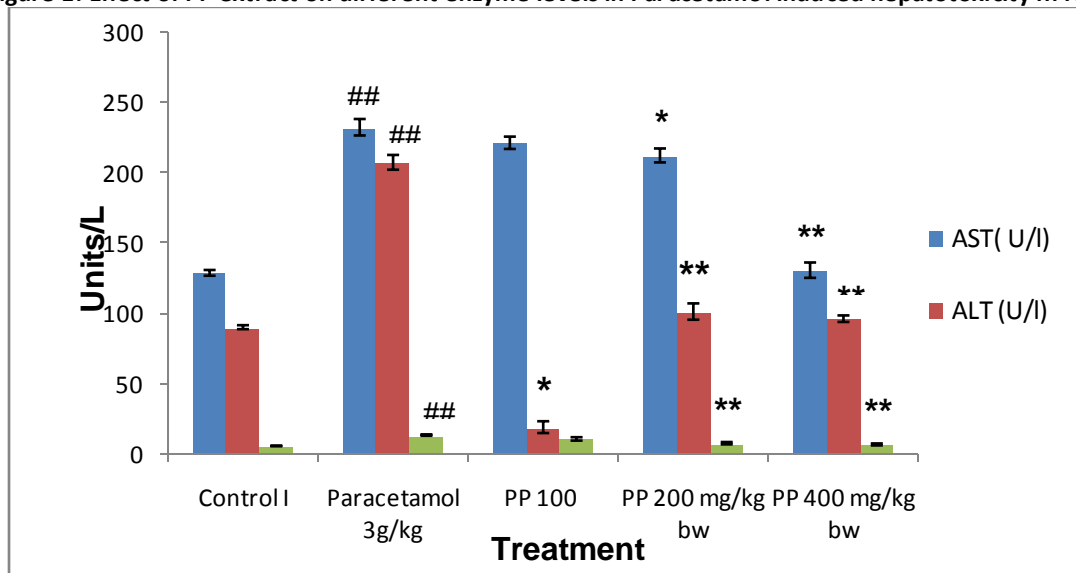
Oral administration of methanolic extract of *Pongamia pinnata* did not produce any toxic effect in mice. It was found to be safe and no mortality was observed up to dose 2000 mg/kg.

Evaluation of hepatoprotective activity using paracetamol model

As shown in Figure 1, the AST, Alt and SALP values recorded 24 hours after Paracetamol administration for vehicle treated control rats, Paracetamol treated rats and rats pretreated with PP extract 400, 200 and 100 mg/kg for 7 days. Paracetamol treated group exhibited a significant increase ($P < 0.01$) in the AST values as compared to vehicle treated control group. The pretreatment with PP extract 200mg/kg ($P < 0.05$) and 400 mg/kg ($P < 0.01$) significantly reduced the AST values.

In ALT, Paracetamol treated group exhibited a significant increase ($P < 0.01$) in the values. PP extract 200 and 400 mg/kg were equipotent and more significant ($P < 0.01$) than 100 mg/kg ($P < 0.05$) in reducing ALT.

Figure 1: Effect of PP extract on different enzyme levels in Paracetamol induced hepatotoxicity in rats



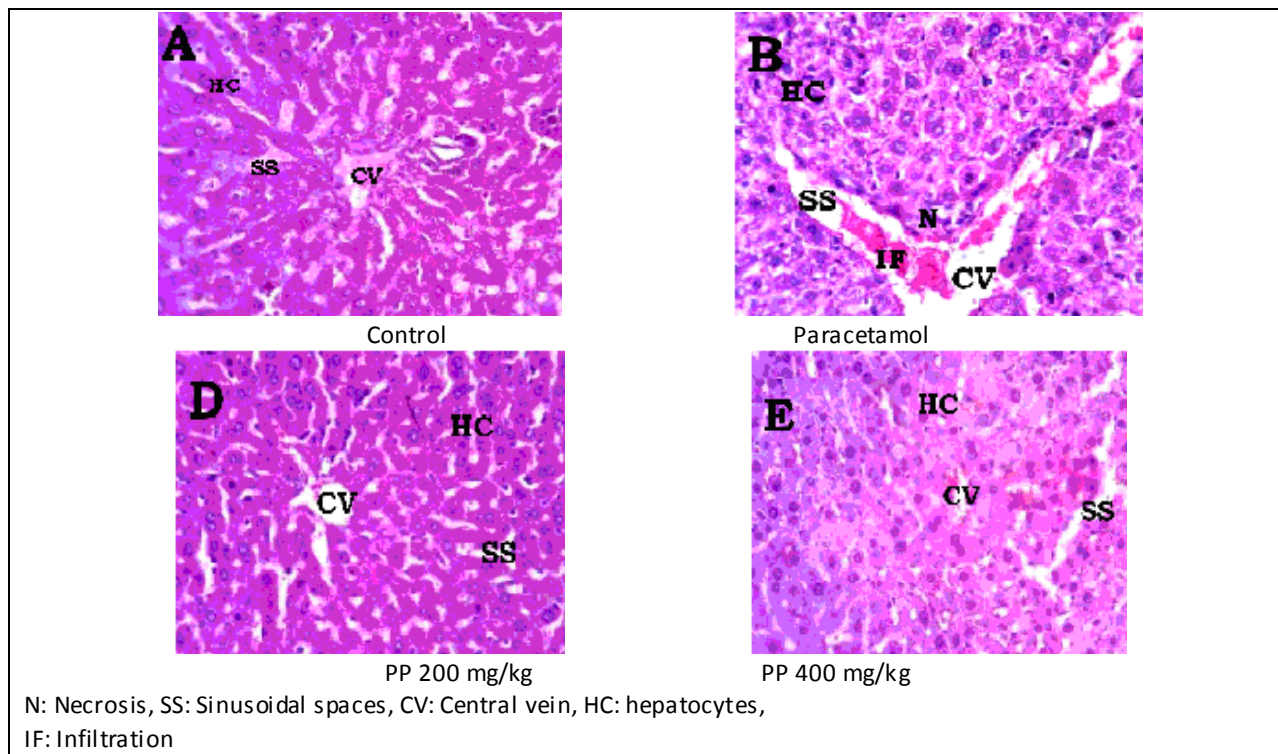
Results are expressed as mean \pm SEM (n = 6). Data was analysed by student t test and one way analysis of variance (ANOVA) followed by Dunnett's 't' test. ## $p < 0.01$ as compared to control, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ as compared to Paracetamol control.

The SALP values were significantly increased ($P < 0.01$) in Paracetamol treated group and were significantly reduced by the pretreatment with PP extract 200mg/kg and 400 mg/kg ($P < 0.01$) equally. PP 100 mg/kg was found to be insignificant in this regard.

As shown in Figure 2, the histopathological studies showed the normal texture and cell rearrangement in the vehicle treated control group. Paracetamol treated control group showed severe necrosis (N), disappearance of hepatocytes (HC) in areas of inflammation and increased sinusoidal spaces (SS) in the Paracetamol treated control group. These effects were significantly reduced in the group treated with PP 200 and 400 mg/kg.

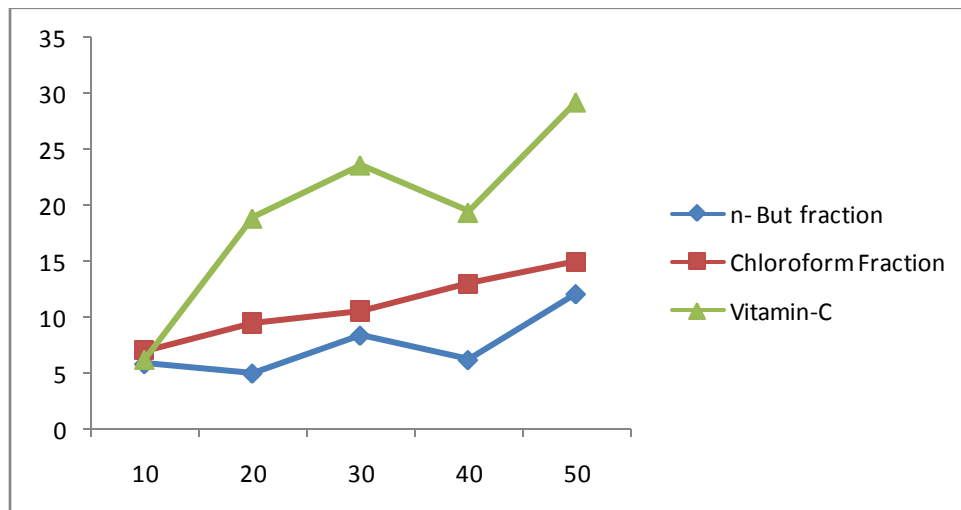
Histopathological investigations

Figure 2: Effects of PP extract, on histopathological changes in livers of control and experimental animals



Evaluation of antioxidant potential by Hydroxyl Radical Scavenging Activity:

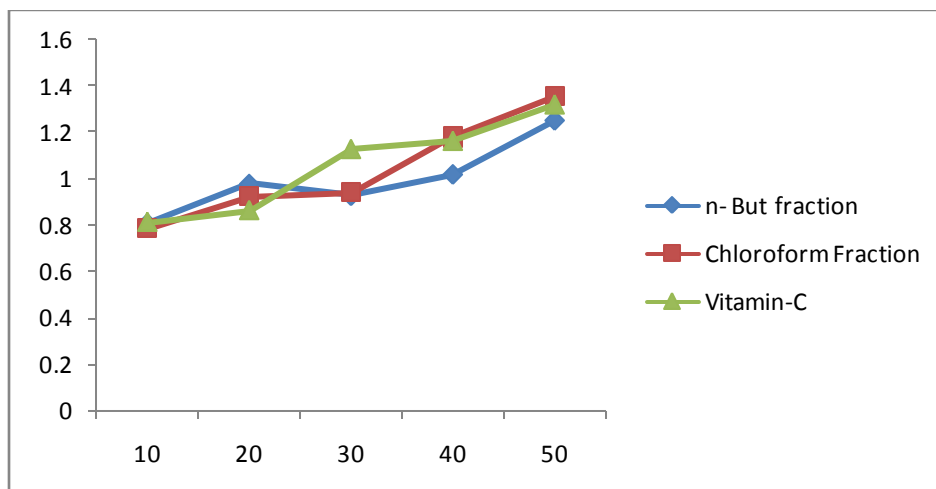
Figure 3: Hydroxyl radical scavenging activity of PPBF and PPCFat different concentrations



The PP extract showed dose dependant increase in percent inhibition i.e. hydroxyl radical scavenging activity. Detailed results are mentioned in Figure 3.

Evaluation of antioxidant potential by Total Reduction Capacity:

Figure 4: Reducing power assay of PPBF and PPCFat different concentrations



The PP extracts showed dose dependant total reduction capacity by dose dependent increase in absorbance. Detailed results are mentioned in Figure 4.

DISCUSSION

The use of natural products with therapeutic properties is as ancient as human civilisation and has had magical-religious significance and different points of view regarding the concepts of health and disease existed within each culture. For a long time, mineral, plant and animal products were the main sources of drugs [16]. The traditional claim, scientific observations and phytochemical presence have close relationship towards the actual therapeutic outcome.

In the present investigation, preliminary phytochemical analysis The earlier scientific studies have revealed that methanolic extract of *Pongamia pinnata* showed the prominent presence of steroids, triterpenoids, glycosides, saponins, flavonoids, proteins, tannins and carbohydrates are chiefly responsible for the pharmacological actions and thus thereby supported the traditional claim [17, 18]. This gives a green signal towards further exploration of this plant for the validation of traditional claims for various complaints for which there is either no or very limited satisfactory pharmacotherapy.

Next to this, toxicity is one of the most important parameters for any medication so it is necessary to evaluate the toxicity profile so as to confirm its safety prior subjected to any preclinical pharmacological screening. In light of this, the acute toxicity studies of methanolic extract of *Pongamia pinnata* was carried out. Our findings indicated that the extract was found

to be devoid of any toxic symptoms and no mortality was found up to 2000 mg/kg [19]. Thus it successfully completed the first step towards its label as a potential drug in the future. From this report three different doses i.e 100, 200 and 400 mg/kg of extract were selected for further study.

In this regard, PP extract at the dose of 200 and 400 mg/kg is found to be significant in reducing paracetamol induced hepatotoxicity. This effect was associated with the significant improvement in various hepatic enzymes like AST, ALT, and SALP which are well known diagnostic indicators of liver dysfunction [20]. The histopathological examination has also showed prevention of hepatic damage and regeneration of damaged hepatocytes which is perhaps the major objective of the therapy [21].

The plant exhibited its usefulness are prominently associated with oxidative stress which remains as one of the important cause for producing wide range of cellular damage. The reactive oxygen species (ROS) such as superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\bullet OH$) formed during oxidative stress. Clinical evidence suggests that oxidative stress and inflammatory processes linked to free radical over generation may be the key in the generation of diabetes, cardiovascular diseases, ischemia, reperfusion injury, atherosclerosis, acute hypertension, haemorrhagic shock, cancer and other chronic diseases [22]. It is well known thing that these free radicals and the reactive species derived from them cause damage through mechanisms of covalent binding and lipid peroxidation with subsequent tissue injury [23, 24]. The agents combating with these alterations produced by oxidative stress are termed as antioxidants which can prove to be useful in improving these complications. Antioxidant agents of natural origin have attracted special interest because they can protect human body from damage caused by free radicals. Numerous medicinal plants and their formulations are used for liver disorders in ethno medical practices as well as in traditional systems of medicine in India [25].

As the plant has shown its potential effectiveness in treating various disorders for which most common mechanism may be through its antioxidant potential. On the contrary, *Pongamia pinnata* has been traditionally claimed to possess antioxidant properties. So in order to assess its efficacy as a potent antioxidant agent the plant was investigated using two in vitro models namely hydroxyl radical scavenging activity and total reducing ability models.

Hydroxyl radical is the most reactive oxygen species among all reactive oxygen species owing to its strong ability to react with various biomolecules. Hydroxyl radical reacts with several biological materials oxidatively by hydrogen withdrawal, double-bond addition, electron transfer and radical formation, and initiates autoxidation, polymerization, and fragmentation. Hydroxyl radicals are highly reactive biological molecules and its scavenging may provide an important therapeutic approach against oxidative stress induced ailments [26]. The PP extract showed dose dependant increase in percent inhibition i.e. hydroxyl radical scavenging activity.



Total reducing ability is considered as the ability of Fe^{3+} - Fe^{2+} transformation. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity [27].

The PP extracts showed dose dependant total reduction capacity by dose dependent increase in absorbance indicating total reducing ability in turn potential antioxidant activity. These results indicate its usefulness in various disorders associated with oxidative stress and as hepatoprotective.

CONCLUSION

To conclude *Pongamia pinnata* (PP) leaves extract showed beneficial activity as hepatoprotective and antioxidant. It was also concluded that hepatoprotective activity of PP extract may be prominently due to its antioxidant potential.

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