Year:**2013**, Volume: **1**, Issue:**2**
First page: (**35**) Last page: (**43**)
ISSN: **2321-2187**
Online Available at **www.florajournal.com**

International Journal of Herbal Medicine

In vitro Anti-Oxidant, Anti-Nociceptive and Anti-Inflammatory Properties of *Pongamia Pinnata Stem Bark* in Experimental Animal Models

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This study aims to evaluate the anti-inflammatory and anti-nociceptivepotential of the ethanolic extract of *pongamia pinnata* stem bark (PSBE) in different experimental animal models.The antioxidant activity of ethanolic extract of*pongamia pinnata*and compared with ascorbic acid (Standard) and theanalgesic and anti-inflammatory activities in animal models. The extract has an anti-inflammatory effect demonstrated by its inhibitory effects on Carrageenan induced paw edema.PSBE (200, 500 and 1000 mg/kg) exhibited significant anti-inflammatory activity in acute (carrageenan induced hind paw edema) and chronic (cotton pellet granuloma) models of inflammationPSBE did not show any sign of toxicity and mortality up to a dose level of 10.125 g/kg, p.o. in mice. Both acute as well as chronic administration PSBE (200, 500 and 1000 mg/kg, p.o.) did not produce any gastric lesion in rats.The analgesic activity was tested by acetic acid-induced writhing response in albino mice and tail flick method in albino rats.Its ethanolic extract shows the most effective anti-inflammatory activityat doses of 200, 500 and 1000 mg/kg significantly throughout the observation period.In the tail flick model, the PSBE in the above doses increased the pain threshold significantly after 30 min., 1, 2, and 4 hr. of administration. *pongamia pinnata*showed dose-dependent action in all experimental animal models.

Keyword: *Pongamia pinnata, In iitro* Anti-oxidantActivity, Analgesic, Anti-inflammatory, acute toxicity.

1. Introduction

Inflammatory diseases including different types of rheumatic diseases are very common throughout the world. Although rheumatism is one of the oldest Known diseases of mankind affecting the majority of population, no substantial progress has been made in achieving a permanent cure. The greatest disadvantage in presently available potent synthetic drugs lies in their toxicity and reappearance of symptoms after discontinuation. Therefore, the screening and development of drugs for their anti-inflammatory activity is the need of hour and there are many efforts for finding anti-inflammatory drugs from indigenous medicinal plants[1].

Pongamia pinnata (Leguminosae), popularly known as 'Karanj' in Hindi, is a medium sized glabrous tree, found throughout India and further distributed eastwards, mainly in the littoral regions of South Eastern Asia and Australia[2].The leaves are hot, digestive, laxative, anthelmintic and cure piles, wounds and other inflammations[3].A hot infusion of leaves is used as a medicated bath for relieving rheumatic pains and for cleaning ulcers in gonorrhea and scroflilous enlargement[2,4]. While different extracts of

roots and seeds (ethanol, petroleum ether, benzene extracts and others) of *Pongamia pinnata* have been reported to have anti-inflammatory activity[1,5,6],ethanolic extract of the leaves of *P. pinnata* has significant antibacterial activity against the tested bacteria *Vibrio* sp., *Pseudomonas* sp., and *Streptococcus* species[7].

2. Materials and Method

2.1 Animals

For anti-inflammatory activity Male Wistar rats (200–300 g), kept in the Animal House at the College of pharmacy, GIS IPS, Dehradun (U.K.) was used. After procuring the animals were acclimatized for 10 days under standard husbandry conditions, room temperature ($27^0 \pm 3^0$C), relative humidity (65 ± 10 %) and 12 hours light / dark cycle. They were allowed free access to standard dry pellet diet and water *ad libitum* under strict hygienic conditions. All the described procedure were reviewed and approved by the Institutional Animal Ethical Committee (Reg. No: 1145/A/2007/CPCSEA).

2.2 Chemicals

Nitro blue tetrazolium, Riboflavin, Meta phosphoric acid and all the solvents used in the study were of analytical grade and were procured from S D Fine Chemicals Limited, Mumbai, India. 1, 1 diphenyl-2-picrylhydrazyl, 2, 2'-azino-bis- (3-ethylbenzothiazoline-6-sulfonic acid), Vitamin C, thiobarbituric acid, malondialdehyde and other chemicals were obtained from Sigma Chemical Company (St. Lousis, MO U.S.A). Carrageenan and Indomethacin were purchased from Hi-Media Laboratories, Mumbai, India. Water represents the double distilled water; standard orogastric cannula was used for the oral administration.

2.3 Plant Material

Fresh stem bark of *Pongamia pinnata* were collected from their natural habitats in and around Bareilly (Uttar Pradesh). The plant was authenticated by comparison with the herbarium and voucher specimen was lodged in the departmental herbarium of Botanical Research survey of India Dehradun.A voucher specimen has also been deposited in the herbarium of the institute for future references.Stem bark of *Pongamia pinnata* (500gm) were air dried at room temperature andpowdered coarsely. Hundred gram of the pulverized plant was extracted with ethanol using a soxlet apparatus. Theextract was filtered, pooledandconcentratedonrotavapour. The yield was 8.2% in powder extract. The extract of stem bark of *Pongamia pinnata* (PSBE)was administered as a suspension in 2% Gum acacia to the animals.Preliminary phytochemical screening method was carried out on the standard screening method[8].

2.4 Acute Toxicity Study (oral)

For acute toxicity, mice were divided into groups of eight animals each. One group served as a control and received (2 ml/kg, p.o.) alone. While the remaining groups were treated with increasing doses of the ethanolic extract: of 3.0, 4.5, 6.75 and 10.125 g/kg respectively. All treated animals were closely observed for any abnormal or toxic manifestations and for mortality up to the end of 24 h in each group to calculate LD_{50} described by Weil (1952)[9].Based on the results obtained from the preliminary toxicity study, the doses for further pharmacological studies were fixed to be 200, 500 and 1000 mg/kg, p.o.

2.5 Anti-inflammatory study

2.5.1 Carrageenin-induced Hind Paw Edema in rats –

In present study anti- inflammatory activity was determined in albino rats of either sex

according to the method [10]. Acute inflammation was produced by subplantar injection of 0.1 ml of 1% suspension of carrageenan with 2% gam acacia in normal saline, in the right hind paw of the rats, one hour after oral administration of the drugs. The paw volume was measured plethysmometrically (UgoBasile) at '0' and '3' hours after the carrageenan injection. Aspirin 100 mg/kg, p.o. suspended in 2% gum acacia was used as the standard drug.The inhibitory activity was calculated according to the following formula

$$\text{Percentage inhibition} = \frac{(C_t - C_0)_{\text{control}} - (C_t - C_0)_{\text{treated}}}{(C_t - C_0)_{\text{control}}} \times 100$$

Where C_t = paw circumference at time t, C_0 = paw circumference before carrageenin injection

2.5.2 Cotton pellet granuloma in rats –

The effect of PSBE on chronic or proliferative phase of inflammation was assessed in cotton pellet granuloma rat model[11].Autoclaved cotton pellets weighing 35±1 mg each were implanted subcutaneously through small incision made along the axilla or flank region of the rats anesthetized with ether. The different groups of rats were administered the PSBE (200, 500 and 1000 mg/kg, p.o.) and ASA (300 mg/kg, p.o.) once daily for seven consecutive days from the day of cotton pellet insertion. The control group received normal saline alone. On the eighth day, all the rats were sacrificed and the cotton pellets covered by the granulomatous tissue were excised and dried in hot air oven at 60 °C till a constant weight was achieved. Granuloma weight was obtained by subtracting the weight of cotton pellet on 0 day (before start of experiment) from the weight of the cotton pellet on eighth day (at the end of experiment).

2.5.3 Acute ulcerogenic activity –

The ulcerogenic potential of PSBE at three different doses (200, 500 and 1000mg/kg, p.o.) was tested in overnight fasted male rats. The control group was administered vehicle (1 ml/kg, p.o.), while the other group received standard drug, ASA (300 mg/kg, p.o.), respectively. All the animals were killed with anesthetic ether 5 h after the administration of test compounds. The stomachs were dissected out, incised along the greater curvature, and then put in diluted formaldehyde solution (2.5%). A few minutes later, mucosa of the stomach was observed for petechial hemorrhages and ulcers.

2.5.4 Chronic ulcerogenic activity–

The experiment was carried out using male Wistar rats with free access to feed and drinking water throughout the period of experiment. The rats were administered vehicle (1 ml/kg, p.o.), PSBE (200, 500 and 1000 mg/kg, p.o.) and ASA (300 mg/kg. p.o.), once daily for 14 consecutive days. All the animals were sacrificed 24 h after the administration of the last dose of the drug and the stomachs were removed and examined as in the acute experiment[6, 12].

2.6 Analgesic Activity

2.6.1 Acetic acid-induced writhing test –

The prescreened animals were divided into groups as shown in Table 2. Aspirin in doses suspended in 2% gam acacia was used as the standard drug. The drugs were autoclaved at 121 ^{O}C for 30 min and administered subcutaneously. Writhing was induced 30 min later by intraperitoneal injection of 10 ml/kg of 0.6% acetic acid in distilled water[13].The number of writhes was counted for 30 min immediately after the acetic acid injection.

2.6.2 Tail Flick Method–

The prescreened animals (reaction time: 3-4 sec) were divided into groups as shown in

Table 3. Pethidine 5 mg/kg acted as the standard drug. The drugs were administered intraperitoneally. The strength of the current passing through the naked nicrome wire was kept constant at 6 Amps. The distance between the heat source and the tail skin was 1.5 cm. The site of application of the radiant heat in the tail was maintained at 2.5 cm, measured from the root of the tail. The cut-off reaction time was fixed at 10 sec to avoid tissue damage[14].Acetylsalicylic acid is a well-known peripheral analgesic drug and was used as a positive control in the present investigation. The analgesic activity was calculated using the following formula:-

$$\% \text{ potential} = \frac{\text{Drug latancy (Test) - Base line lantency (Control)}}{\text{Base line latency (Control)}} \text{X } 10$$

2.7 *In vitro* Antioxidant Activity

2.7.1 DPPH Method

DPPH scavenging activity was measured by the spectrophotometric method of Facino RM[15]. Stock solution of DPPH (1.5 mg/ml in ethanol) was prepared such that 75 µl of it in 3 ml of ethanol gave an initial absorbance of 0.973. Decrease in the absorbance in presence of sample extract at different concentration (10-125 µg/ml) was noted after 15 min. IC_{50} was calculated from % inhibition.

2.7.2 Protocol for DPPH Free Radical Scavenging Activity

Preparation of stock solution of test sample: 100 mg of extract was dissolved in 100 ml of ethanol to get 1000 µg/ml solution.

- Dilution of test solution: 100, 200, 300, 400 and 500 µg/ml solution of test were prepared from stock solution.
- Preparation of DPPH solution: 15 mg of DPPH was dissolved in 10 ml of ethanol. The resulting solution was covered with aluminum foil to protect from light.
- Estimation of DPPH scavenging activity: 75 µl of DPPH solution was taken and the final volume was adjusted to 3 ml with ethanol, absorbance was taken immediately at 517 nm for control reading. 75 µl of DPPH and 50 µl of the test sample of different concentration were put in a series of volumetric flasks and final volume was adjusted to 3 ml with ethanol.
- Absorbance at zero time was taken in UV-Visible at 517 nm for each concentration.Final decrease in absorbance of DPPH with sample of different concentration was measured after 15 minute at 517 nm.

Percentage inhibitions of DPPH radical by test compound were determined by the following formula.

$$\% \text{ Reduction} = \frac{\text{Control absorbance- Test absorbance}}{\text{Control absorbance}} \text{ x } 100$$

Calculation of IC_{50} value using graphical method.

2.8 Reducing Power

The reducing power of the ethanolic extract of *Pongamia pinnata*was determined according to the method of Oyaizu. Accurately weighed 10 mg of the extract in 1 ml of distilled water were mixed in to the mixture of 2.5 ml of 0.2M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferri-cyanide. The mixture was then incubated at 50oC for 20 min. Following incubation, 2.5 ml of 10% tri chloro acetic acid was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1 %) and the absorbance was measured at 517 nm. Increased absorbance of the reaction mixture indicated the increased reducing power.

2.8.1 Assay for Nitric Oxide Scavenging Activity[16]

The method was followed for the experiment, sodium nitroprusside (10 mM) in phosphate buffered saline was mixed with different concentrations of the extract dissolved in ethanol and incubated at room temperature for 2½ h. Thesame reaction without the sample but equivalent amount of ethanol served as control. After incubation period, 0.5 ml of Griess reagent was added. Absorbance of the chromophore formed was measured at 546 nm. Ascorbic acid was used as positive control.

The procedure is based on the principle that, Sodium nitro prusside solution spontaneously generates nitric oxides, which reacts with oxygen to produce nitrite ions that can be estimated using Greiss reagent. Scavengers of nitric oxides compete with oxygen leading to reduceproduction of nitrite ions.

2.8.2 Assay for Superoxide Radical Scavenging Activity[17]

The assay for superoxide radical scavenging activity was performed as per standard procedure. The reion mixture contained 50 mM phosphate buffer (pH 7.6), 20 µg riboflavin, 12 mM EDTA and 0.1 mg/ml of NBT (nitro blue tetrazolium), all added in chronological sequence. Reaction was started by illuminating the reaction mixture containing different concentrations of the sample extract for 90 sec and then measuring the absorbance at 590nm. Ascorbic acid was taken as the positive standard.

2.8.3 Statistical analysis

Results are expressed as mean ± S.E.M. statistical evaluations were made using ANOVA followed by t-test (Prism 3.0) and P values less than 0.05 were considered significant. Data are represented as mean ± S.E.M.

3. Result and Discussion

On all examination, all animals given PSBE at the doses of 3, 4.5, 6.75 and 10.125 g/kg, p.o. were devoid of toxic symptoms and mortality. The results of the present study suggest that the PSBEat doses of 200, 500 and 1000 mg/kg significantly suppressed carrageenan-induced paw edema in rats (Table 1). The results were found to be highly significant ($P<0.001$) in comparison to the control. The study of PSBE on proliferative phase of inflammation indicated that PSBE (200, 500 and 1000 mg/kg, p.o.) reduced the granuloma formation with percentage inhibition 8.48%, 16.47% and 21.23% as compared with ASA (300 mg/kg, p.o.), which showed inhibition on granuloma formation with the percent inhibition 43.95%.Significant analgesic activity in acetic acid-induced writhing (Table 3) and tail flick models (Table 4). The PSBE(200, 500 and 1000 mg/kg, s.c.) suppressed the acetic acid-induced writhing response significantly in a dose-dependent manner (Υ =0.99).

The anti-inflammatory effects of the extract on acute inflammatory process such as carrageenan-induced edema in rats paw was dose dependent[18]. At 500 mg/kg, the extract showed at least 50% inhibitory activity throughout the measurement intervals was comparable to 1000 mg/kg of the extract. It is well known that non-steroidal anti-inflammatory and analgesic drugs mitigate the inflammatory pain by inhibiting the formation of pain mediators at the peripheral target sites where prostaglandins and bradykinin are proposed to play a significant role in the pain process[17]. Phytochemical screening of the ethanolic extract shows the presence of flavonoids and saponins. Flavonoids act as an anti-inflammatory response in the same way as the non-steroidal anti-inflammatory drugs, i.e. by inhibiting the enzymes that cause the synthesis of prostaglandins[15].

Further studies may reveal the mechanisms of action responsible for the analgesic and anti-inflammatory activities of *pongamia pinnata*. The free radical scavenging activity of the extract was examined in various in vitro models like, DPPH, NO, OH and lipid peroxidation was compared with the standards/ ascorbic acid. It was observed that ethanolic extract of *Pongamia pinnata*stem bark had higher activity than that of leaves. At a concentration of 0.5 mg/ml, the scavenging activity of ethanol extract of *Pongamia pinnata*stem bark reached 54.8%. Though the DPPH radical scavenging abilities of the extract were less than those of ascorbic acid (98%) and Nitric Oxide and Super Oxide shows % Inhibition maximum at 1000 µg/ml, the study showed that the extracts have the proton donating ability and could serve as free radical inhibitors or scavenging, acting possibly as primary antioxidants (Table 5 and 6).

4. Conclusion

The present study indicates the *pongamia pinnata* stem bark (PSBE) contained compounds, flavonoids and saponins. Its ethanolic extract shows the most effective anti-inflammatory activityat doses of 100, 200 and 1000 mg/kg significantly throughout the observation period. The results shows in the present study provide evidence that the ethanolic extract of *pongamia pinnata* stem bark possesses anti-inflammatory activity.

The antioxidant activity and anti-inflammatory activity was comparable with standard ascorbic acid, Indomethacin and aspirin. These findings provide scientific evidence to support traditional medicinal uses and indicate a promising potential for the development of an analgesic, antioxidant and anti-inflammatory agent from *Pongamia pinnata*plant. This medicinal plant by *in vitro* results appear as interesting and promising and may be effective as potential sources of novel analgesic, antioxidant and anti-inflammatory drugs.

Table 1: Anti-inflammatory effect of PSBE extracts on carrageenan-induced paw edema in rats

Groups	Dose orally (mg/kg, p.o.)	Change In Mean Paw Volume (ml)		Inhibition (%)	
		3h	4h	3h	4h
Control	---	0.88±0.20	0.91±0.24	----	-----
PSBE	200	0.54±0.03	0.42±0.02*	38.6%	53.8%
	500	0.42±0.12**	0.37±0.03**	52.3%	59.3%
	1000	0.36±0.02**	0.32±0.03**	60%	64.8%
ASA	300	0.21±0.01***	0.23±0.01**	76%	74.7%

*n=6 in each group;Values are mean ± SEM. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ significant from the control values.

Table 2: Anti-inflammatory effect of PSBE on cotton pellet granuloma in rats

cotton pellet granuloma in rats			
Group	Dose (mg/kg, s.c.)	Weight of cotton pellet granuloma(mg)	% of protection
Control	----	109.67±2.21	–
PSBE	200	100.37±1.62	8.48%

	500	91.17±2.08*	16.87%
	1000	86.50±1.91**	21.23%
ASA	300	61.46±1.67***	43.95%

n=6 in each group;Values are mean ± SEM. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ significant from the control values.

Table 3: Analgesic activity of PSBE on Acetic acid-induced writhing response in mice

Acetic acid-induced writhing response in mice			
Group	**Dose (mg/kg, s.c.)**	**No. of writhing movements**	**% of protection**
Control	----	56.8 ± 4.2	–
PSBE	200	34.5 ± 3.1**	33.39%
	500	32.5± 3.4**	37.25%
	1000	28.5 ± 2.8***	44.98%
ASA	100	20.2 ± 2.2***	61%

n=6 in each group;Values are mean ± SEM. $**p < 0.01$ significant from the control values, $***p < 0.001$ significant from the control values.

Table 4: Analgesic activity of PSBE on tail flick response in rats

Group	**Drug dose mg/kg, p.o.**	**Dose Reaction time in seconds at time (h)**			
		30min	**1h**	**2h**	**3h**
Control	---	9.5±0.42	10.5±0.25	11.2±0.48	12.5±0.42
PSBE	200	11.6±0.49	10.9±0.38	11.4±0.61	12.7±0.66
	500	10.7±0.91	11.5±0.35	12.2±0.79	13.2±0.54
	1000	11.2±0.65	11.8±0.33	15.3±0.66**	18.8±0.54**
Pethidine	5	11.9±0.40*	12.2±0.35**	16.2±0.87**	20.2±0.70**

n= 6 in each group, each value is the mean ± S.E.M. $*P< 0.05$ compared to control, $**P< 0.01$ compared to control

Table 5: *In vitro* antioxidant activity of *Pongamia pinnata* via DPPH Method –

S N.	**Conc. of Sample (g/ml)**	**Absorbance at 517nm**	**%Inhibition Anti-oxidant Activity**
1	0.1	0.5438	30.0%
2	0.2	0.8543	47.0%
3	0.3	0.8967	49.4%
4	0.4	0.9247	50.9%
5	0.5	0.9947	54.8%

DPPH Absorbance (control): 1.8144

Table 6: *In vitro* antioxidant activity of *Pongamia pinnata*Stem Bark–

Group	Conc (µg/ml)	% Inhibition	
		Nitric oxide	Super oxide
Ethanolic extract of *pongamia pinnata*	25	18.65 ± 0.23**	28.24±.1.70**
	50	37.10 ± 0.42**	32.92±1.37**
	75	68.95 ± 0.50**	39.33±1.04**
	100	80.37 ± 0.64**	46.04±0.44**
Ascorbic acid	10	42.74±0.41	45.48±0.84
	15	55.91±0.35	53.52±1.42
	20	79.24±0.21	68.30±0.65
	25	92.84±0.35	90.04±0.35

Result expressed as mean ± SEM from six observation **P<0.01, each values is the mean ± S.E.M and Data were analyzed by one way ANOVA.

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