Antinociceptive and Antiinflammatory Activity of Methanolic Extract of Leaves of *Shorea Robusta*

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Summary

A methanol extract of the dried leaves of *Shorea robusta* was investigated for anti-inflammatory and antinociceptive activities. The extract (200 and 400 mg/kg, p.o) produced a dose dependent inhibition of carrageenan – induced paw edema in rats. At the same doses, antinociceptive effect was also observed with hotplate device maintained at 55^0C, Aetic acid induced writhing, formaline induced paw licking, Tail clip and Tail flick models in mice. The results of the present study confirm the use of Shorea robusta traditionally for the treatment of painful inflammatory conditions.

Keywords: *Shorea robusta*, anti-inflammatory, antinociceptive, writhing,

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Introduction

Shorea robusta (family: Dipterocarpaceae) is a large deciduous tree 18-30m in height with smooth 'or' longitudinally fissured reddish brown 'or' grey bark[1]. *Shorea robusta* is a tropical hardwood found and developed in Southeast Asia. It prospers most commonly in Indonesia but can also be seen in Malaysia, the Philippines and certain parts of Northern India. The leaves are used to treat wounds, ulcers, itching, leprosy, gonorrhoea, cough, earache and headache. The oleoresin exuded from the cut bark has astringent and detergent properties. The bark is also used to treat diarrhoea, dysentery, wounds, ulcers, itching and vaginal discharges. In Unani medicine the resin is used for treating menorrhagia, enlargement of the spleen and for relieving eye irritations. . In Ayurveda the leaves are used as anthelmintic and alexiteric. The powdered stem bark 'or' bark paste is applied to stop bleeding and promote healing of cuts among the tribal inhabitants of southern Bihar& the Kondhs of southwestern Orissa [1]**.**

Materials and Methods

Plant Material

The leaves of *S.robusta* were collected near Kakatiya University campus, Warangal, Andhra Pradesh. It was authenticated by Prof V.S.Raju, Dept of Botany, Kakatiya University, Warangal. A voucher specimen of this plant material with No.KU/UCPSc/21/2006 has been retained in the Dept of Pharmacognosy and Ethnopharmacology, University College of Pharmaceutical Sciences, Warangal.

Preparation of Extracts:

The leaves were dried under shade, made into coarse powder and subjected to maceration process at room temperature in methanol for seven days with occasional shaking. The methanolic extract of *Shorea robusta* (MESR) collected was concentrated under reduced pressure at 50^0 – 55^0 C and stored in a vacuum desiccator. The suspension of the extract prepared in 2% gum acacia was used in the entire experimental studies.

Animals:

Wistar rats (150-250g) and albino mice (20-25g) of either sex were maintained under standard husbandry conditions and had free access to food and water *ad libitum* and were acclimatized to the laboratory environment for a period of one week before the commencement of experiment. All the animals were divided into different groups each consists of six animals were fasted overnight prior to the experiments. All the experiments were performed after obtaining prior permission from Institutional Animal Ethical Committee.

Drugs and Chemicals:

The drugs and chemicals used were Carrageenan (Sd.Fine ChemicalsLimited, Mumbai), Formaldehyde solution (37-41% w/v) (Universal Lab. Ltd., Mumbai), Gum acacia (M/s Hi-media, Mumbai), Methanol (BDH, Mumbai), Diclofenac sodium (Dr Reddys Labs, Hyderabad), Pentazocine(Pure Pharma Ltd., Mumbai), Indomethacin(M/s Jagsonpaul Pharmaceuticals Ltd., Faridabad).

Phytochemical screening:
The methanolic extract was screened for various phytoconstituents like steroids, alkaloids, tannins, flavanoids and glycosides by employing standard phytochemical tests [2].

Acute toxicity study:
Acute oral toxicity was performed in mice by following Organization for Economic Co-operation and Development (OECD) guidelines AOT No 425 [3].

Anti-nociceptive Activity
Hot Plate Method:
The hot plate test was used to measure analgesic activity described by Eddy and Leimbark (1953) [4] with minor modifications. Four groups of six animals each were selected for the present study. Group one served as control and received the vehicle (2% gum acacia) and group two receives the standard drug pentozocine 10mg/kg b.w, intraperitonially. The extract MESR at the concentrations of 200 and 400 mg/kg b.w was administered orally to group third and fourth respectively. 30min after administration of vehicle/extract/standard drug animals were placed individually on an aluminum hot plate kept at a temperature of 50 ± 0.5^0 C for a maximum period of 20 sec. The reaction time was recorded when the animals licked their fore-and hind paws and jumped.

$$\text{Percentage Variation} = \frac{\text{Drug latency} - \text{Base line latency}}{\text{Base line latency}} \times 100$$

Acetic acid – induced writhing test in mice:
The peripheral analgesic activity of methanolic extract of *S.robusta* leaves was determined by inhibitory effect of extract in the acetic acid induced writhing method [5]. The MESR at the different doses (200 and 400 mg/kg b.w) and standard drug diclofenac sodium (20 mg/kg) were administered orally, 1h prior to the injection of acetic acid, vehicle control group received 2% gum acacia administered orally. After 40min of administration of the test dose vehicle/extract/standard each of the mice was injected intraperitonially with acetic acid (0.7%) at a dose of 0.1 mL/10g to create pain sensation. The inhibition of writhing by methanolic extract of *S.robusta* leaves in mice was compared against inhibitory effect of writhing by standard diclofenec sodium given. The number of writhings was counted during 30 min test period, beginning 3 min after the injection of acetic acid. The percentage of pain protection was calculated.

$$\text{Percentage of Inhibition} = \frac{\text{Control mean} - \text{Treated mean}}{\text{Control mean}} \times 100$$

Formalin induced nociception in mice:
Albino mice of either sex were divided into five groups. Group I considered as normal control received orally with the vehicle 2% gum acacia, group II with standard indomethacin (5 mg/kg, i.p), group III and IV were given orally with different doses i.e., 200mg/kg and 400 mg/kg of methanolic extract of *S.robusta* leaves. One hour after the above treatment 20µl of 2.5% formalin was injected into the sub plantar region of each animal. Duration of paw lickings was monitored for 0-5 min (first phase) and at 20-25 min (second phase) after formalin challenge.

$$\text{Percentage of Inhibition} = \frac{\text{Control mean} - \text{Treated mean}}{\text{Control mean}} \times 100$$

Haffner's Tail clip method:

The method was described as early as 1929 by Haffner who observed the raised tail (straub phenomenon) in mice treated with opioid drugs and found the tail to be less sensitive to noxious stimuli after drug treatment. Mice (25-30 g) of either sex were divided into four groups with six animals in each group. The animals were administered the standard, control and extracts (200 and 400 mg/kg) by the oral route of administration 30min prior to testing. A bulldog clamp was applied to the root of the tail of the mice to induce pain. The animal quickly responded to this noxious stimulus by biting the clip 'or' the tail near the location of the clip. The time between the stimulation onset and the response was measured using a stopwatch [6].

Tail flick method: (Radiant heat method) :

Mice were randomly assigned to four groups of six animals each. A control group received 2% gum acacia. Pentazocine 10 mg/kg (acted as the standard drug) and the extracts at 200 and 400 mg/kg were administered orally. The tail flick latency was assessed by the analgesiometer. The strength of the current passing through the naked nichrome wire was kept constant at 6amps. 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 [7].

Anti inflammatory Activity:

Carrageenan – induced rat paw edema

The normal paw volumes of all the rats were measured initially and were divided into four groups (Groups I-IV) each comprises of six animals treated orally with the vehicle as control (2% gum acacia), standard diclofenac sodium 20 mg/kg and methanolic extract of *S.robusta* leaves 200 and 400 mg/kg respectively. The vehicle, drug and extract were administered 30 min prior to the injection of 0.1 ml of freshly prepared suspension of carrageenan in sub plantar region of the right hind paw of each rat. The paw volumes of all the rats were recorded at 1, 2, 3, 4 and 5h after carrageenan treatment by using plethysmometer[8]. A significant reduction in the paw volume compared to vehicle-treated control animals were considered as inflammatory response. The percentage inhibition of edema was calculated as follows:

$$\text{Percentage of Inhibition} = \frac{(V_T\text{-}V_O)\ \text{Control} - (V_T\text{-}V_O)\ \text{Treated group}}{(V_T - V_O)\ \text{Control}} \times 100$$

Vo= paw volume of the rat before administration of carrageenan .

Vt= paw volume of the rat after the administration of carrageenan at different time intervals.

Percentage inhibition of paw edema is proportional to anti-inflammatory activity.

Statistical analysis:

All the results were expressed as mean $\pm$ SEM and analysed by one way analysis of variance (ANOVA) followed by Dunnet's t–test and $P < 0.05$, 0.01, 0.001 were considered as significant. All statistical manipulations were carried out using statistical software Graph Pad Prism 3.0 (U S A).

Results

Preliminary Phytochemical Screening:
Preliminary phytochemical screening of the methanol extract of *S.robusta* leaves revealed the presence of alkaloids, triterpenes, phenols, anthraquinones and cardiac glycosides.

Acute toxicity test:
In the acute toxicity study even with the dose tested 2 gm/kg no mortality was observed during the 24 h period and the animals showed no stereotypical symptoms associated with toxicity such as convulsions, ataxia, diarrhoea or increased diuresis.

Hot Plate Test:
Table 1 shows the results of analgesic activity recorded with the two doses (200 & 400 mg/kg) of leaf extract of *S.robusta* with the hot plate (thermal stimulus) model. The two doses of the extracts had increased the reaction time in dose dependent manner. Higher dose i.e., 400 mg/ kg of methanolic extract of *S,robusta* had exhibited the highest anti-nociceptive effect to the thermal stimulus (164.6%) at 90 min which is comparable to the effect of standard pentazocine.

Acetic Acid – Induced Writhing Test:
Dose dependent antinociceptive effect was noted with the methanolic extract at the tested dose levels 200 and 400mg/kg (table no.2).In the acetic acid induced writhing model methanolic extract of *S.robusta* leaves with 400 mg/kg dose has exhibited a maximum of 93.66% inhibition of writhing and the activity was shown more than standard drug diclofenac sodium (74.87%) while lower dose 200 mg/kg have shown 66.29% reduction.

Formalin induced nociception in mice:
Methanolic extract of leaves of *S.robusta* has significantly reduced formalin induced paw nociception and paw licking in mice. In first phase the standard and the extract with two doses have significantly reduced the number of paw lickings as 60.9%, 43.9% and 54.5% respectively. Higher dose of the extract 400mg/kg has exhibited antinociceptive activity more than the standard indomethacin. In second phase the standard and the test doses of the extract as mentioned above reduced number of paw lickings to 69.7%, 75.9% and 82.5% respectively. The results were shown in table.3.

Tail clip method:
The methanolic extract of *S.robusta* (200 and 400mg/kg) produced significant ($p<0.01$) increase in the mean latency of biting of the tail-clip after 30min and was dose dependent (177.7% and 247.2%). The statistical data also shows that the extracts were more efficacious and comparable to that of standard pentazocin (100.5%).

Tail flick method:
The results shown in table.5 revealed that the methanolic extract of *S.robusta* leaves significantly increased reaction time after 30 min.The test extracts produced a dose dependent increase in the reaction time at various time intervals of observation i.e. 60, 90 and 120min. The increase in reaction time was more with both the test doses of the extracts during the latency period interval 60 min & 120 min as compared to standard drug pentazocine (10 mg/kg). The test extract shows more significant activity at120 min i.e. 162.9% and 185.6% than the standard (56.6%).

Carrageenan-induced paw edema test:
The anti inflammatory effect of the extract and the reference drug in Carrageenan induced paw edema model in rats have been shown in table.6. After Carrageenan administration paw edema in rats reached to a peak value at 5h and various doses of methanolic extract of *S.robusta* leaves have produced a significant inhibition in the edema volume at the end of 3h. Maximum percent inhibition of edema exhibited with 200 and 400 mg/kg of methanolic extract at 5h was 79.8%, 85.5% and the effect was comparable to that of the standard drug (86.46%).

Table. 1 Analgesic effect of methanolic extract of ***Shorea robusta*** leaves with Hot plate method

S.No	Group	Dose (mg/kg)	Reaction time after administration of control/ standard / extract in sec.				
			0	60	90	120	150
1.	Control		1.83 ± 0.17	1.5 ± 0.22	1.5 ± 0.22	1.5 ± 0.22	1.83 ± 0.16
2.	Pentazocine	10	3.5 ± 0.34**	7.00 ± 0.45**	7.33 ± 0.21**	5.83 ± 0.8**	2.6± 0.42
3.	*S.robusta*	200	3.66 ± 0.33**	8.66± 1.02**	10 ± 0.73**	10.5 ± 0.43**	4.83 ± 0.31**
4.	*S.robusta*	400	4.66 ± 0.21**	11.3 ± 0.49**	11.83 ± 0.31**	12.33 ± 0.21**	5.16 ± 0.31**

Values are expressed as mean ± S.E.M; n=6; significance at P < 0.05*, 0.01** and 0.001** as compared to the control

Table. 2 Analgesic effect of methanolic extract of ***Shorea robusta*** leaves on Writhing reaction induced by Acetic acid in mice

S.No	Group	Dose (mg/ kg)	No of writhings (mean ± S.E.M)	% Inhibition
1.	Control		81.6 ± 2.95	---
2.	Diclofenec sodium	20	20.5 ± 1.6**	74.87
3.	*S.robusta*	200	27.5 ± 4.95**	66.29
4.	*S.robusta*	400	5.2 ± 0.87**	93.66

Values are expressed as mean ±S.E.M; n=6; significance at P< 0.05*, 0.01** and 0.001*** as compared to the control

Table. 3 Analgesic effect of methanolic extract of ***Shorea robusta*** leaves with Formalin induced pain in mice

S.No	Group	Dose (mg/kg)	Licking time (sec)		% Inhibition	
			First Phase	Second Phase	First Phase	Second Phase
1.	Control		42.5 ± 3.82	38 + 4.04	---	---
2.	Indomethacin	5	16.66 ± 0.5***	11.5 ± 0.72***	60.94	69.73
3.	*S.robusta*	200	23.83 ± 1.7***	9.16 ± 0.7***	43.92	75.9
4.	*S.robusta*	400	19.33 ± 1.6***	6.66 ± 0.89***	54.51	82.47

Values are expressed as mean ±S.E.M; n=6; significance at P< 0.05*, 0.01** and 0.001*** as compared to the control

Table. 4. Analgesic effect of methanolic extract of ***Shorea robusta*** leaves with Tail Clip method in mice

S.No	Treatment	Dose (mg/kg)	Latency Period				
			0 min	30 min	60 min	90 min	120 min
1.	Control	----	2.16 ± 0.11	2.16 ± 0.11	2.33 ± 0.21	2.16 ± 0.16	2.33 ± 0.21
2.	Pentazocine	10	2.16 ± 0.16	4.33 ± 0.33** (100.46%)	3.66 ± 0.21** (57.08%)	3.16 ± 0.3* (46.29%)	2.5 ± 0.22 (7.3%)
3.	*S.robusta*	200	2.0 ± 0.0	6.0 ± 0.37** (177.77%)	5.16 ± 0.30** (121.45%)	4.0 ± 0.26** (85.18%)	2.16 ± 0.16 (-7.3%)
4.	*S.robusta*	400	2.83 ± 0.21	3.58 ± 0.24** (247.2%)	6.33 ± 0.16** (214.6%)	6.5 ± 0.13** (115.14%)	8.01 ± 0.3 (-7.3%)

Values are expressed as mean ±S.E.M; n=6; significance at P< 0.05*, 0.01** and 0.001*** as compared to the control

Table. 5. Analgesic effect of methanolic extract of ***Shorea robusta*** leaves with Tail Flick reaction time in mice

S.No	Treatment	Dose (mg/kg)	Latency Period					
			0 min	30 min	60 min	90 min	120 min	150 min
1.	Control	---	2.16 ± 0.11	2.16 ± 0.11	2.25 ± 0.11	2.16 ± 0.11	2.16 ± 0.11	2.16 ± 0.11
2.	Pentazocine	10	2.5 ± 0.18	2.75 ± 0.22** (10%)	3.83 ± 0.21** (53.2%)	3.92 ± 0.24** (56.64%)	3.5 ± 0.34** (40%)	2.92 ± 0.08** (16.64%)
3.	*S.robusta*	200	2.25 + 0.17	2.66 ±0.31 (18.22%)	4.0 ± 0.13** (77.7%)	5.09 ± 0.15** (125.7%)	5.92 ± 0.24** (162.9%)	2.66 ± 0.16 (18.2%)
4.	*S.robusta*	400	2.83 + 0.21	3.58 ± 0.24** (26.5%)	6.33 ± 0.16** (123.7%)	6.5 ± 0.13** (129.7%)	8.01 ± 0.3** (185.6%)	2.83 ± 0.16** (67%)

Values are expressed as mean ±S.E.M; n=6; significance at P< 0.05*, 0.01** and 0.001*** as compared to the control

Table. 6. Analgesic effect of methanolic extract of ***Shorea robusta*** leaves on Carrageenan- induced edema in rats

S.No	Treatment	Drug (mg/kg)	Paw edema volume					
			0 h mean + S.E.M (%PEI)	1 h mean + S.E.M (%PEI)	2 h mean +S.E.M (%PEI)	3 h mean + S.E.M (%PEI)	4 h mean + S.E.M (% PEI)	5 h mean + S.E.M (% PEI)
1.	Control	---	0.22 + 0.01	0.9 + 0.07	1.4 + 0.02	1.6 + 0.02	1.7 + 0.02	1.6 + 0.01
2.	Diclofenec	20	0.20 + 0.01	0.24 + 0.01** (73.2)	0.33 + 0.01** (76.5)	0.33 + 0.02** (78.7)	0.31 + 0.01** (82)	0.22 + 0.02** (86.5)
3.	S.robusta	200	0.12 + 0.004	0.23 + 0.01** (67.1)	0.44 + 0.02** (68.6)	0.42 + 0.02** (73.3)	0.42 + 0.02** (75.2)	0.32 + 0.01** (79.8)
4.	S.robusta	400	0.19 + 0.01	0.25 + 0.01** (71.9)	0.33 + 0.01** (75.9)	0.34 + 0.01** (78.4)	0.34 + 0.01** (79.9)	0.23 + 0.01** (85.5)

Values are expressed as mean $\pm$S.E.M; n=6; significance at P< 0.05*, 0.01** and 0.001*** as compared to the control

% PEI = Percentage of Edema Inhibition

Discussion and Conclusion

Pain and inflammation are associated with pathology of various clinical conditions like arthritis, cancer and vascular diseases [9-11]. In various traditional medical systems a number of natural products are used to relieve the symptoms of pain and inflammation. The methanolic extract of *S.robusta* leaves with two different dose levels exhibited a significant anti-nociceptive activity in different animal models of pain. In hot plate test, nociceptive reaction towards thermal stimuli in mice is a well validated model for detection of opiate like analgesic drugs where in pain response is from spinal origin [12]. Acetic acid – induced writhing has been used as a model of chemonociceptive induced pain, which peripherally increase PG-E2 and PG-F2 [13]. In both hot plate and acetic acid induced nociceptive models *S.robusta* leaves extract exhibited anti-nociceptive activity which indicates both central and peripherally mediated anti-nociceptive properties. The formalin induced pain test can be used to clarify the possible mechanism of antinociceptive effect of a proposed analgesic. Centrally acting drugs such as opioids inhibit both phases equally whereas peripherally acting drugs inhibit only the late phase. In formalin – induced pain experiment, the extracts at the test doses were found to inhibit the inflammatory pain better than the neurogenic induced pain. The maximal effect of *S.robusta* leaves shows in the late phase suggests that their activity may be resulting from their peripheral action, when compared with standard. The central analgesic property of the extracts was corroborated by the first phase of formalin- induced pain, hot plate, tail- clip and tail- flick results.

Carrageenan-induced paw edema is the standard experimental model of acute inflammation and carrageenan is the phlogistic agent of choice for testing anti inflammatory drugs as it is not known to be antigenic and devoid of apparent systemic effects. Moreover, the experimental model exhibits a high degree of reproducibility [14] Carragenan-induced edema is a biphasic response, the first phase is mediated through the release of histamine, serotonin and kinins where as the second phase is related to the release of prostaglandins and mediated by bradykinin, leucotrienes, polymorphonuclear cells and prostaglandins produced by tissue macrophages[15].The antiinflammatory activity shown by the fruit extract of *S.robusta*(200 and 400mg/kg) in carrageenan –induced paw inflammation over a period of 4h was quite similar to that exhibited by the group treated with standard diclofenac sodium.These results indicate that the extract acts on both initial and later phases of inflammation. Later phase activity might probably involve with arachidonic acid metabolites which produce an edematous response by mobilization of the neutrophils [16]. Based on the results obtained it can be concluded that the methanolic extract of fruits of *S.robusta* posses potential anti-nociceptive and anti inflammatory activities.

Based on the investigations results, it can be concluded that and *S.robusta* was endowed with peripheral and centrally acting analgesic properties as well as anti-inflammatory activity on acute inflammatory process.

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