ANTI-INFLAMMATORY STUDIES OF THE ALCOHOLIC EXTRACT OF *ZORNIA GIBBOSA*

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Summary

The present study was performed to evaluate the anti-inflammatory activity of the alcoholic extract of *Zornia gibbosa* by using different models viz. lipoxygenase, interleukin 6 bioassay and carrageenan induced rat paw edema. In carrageenan induced rat paw edema, the alcoholic extract of Zornia *gibbosa* was given in the dose level of 500 and 750 mg/kg body weight and % inhibition of paw volume (at 3 hr) was found to be 51.42 and 66.66 respectively ($P < 0.05$). Lipoxygenase and interleukin-6 bioassay of the extract showed significant and dose dependent anti-inflammatory activity. This finding shows that of *Z. gibbosa* can be a potential source of anti-inflammatory activity.

Keywords: *Zornia gibbosa,* Anti-inflammatory activity, IL-6 inhibition activity, lipoxygenase activity

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Introduction

Inflammation is a local response of living mammalian tissues to the injury. It is a body defense reaction in order to eliminate or limit the spread of injurious agents from the body. There are various components to an inflammatory reaction that can contribute to the associated symptoms and tissue injury.

Drugs which are in use presently for the management of pain and inflammatory conditions are either narcotics (e.g. opioids) or non-narcotics (e.g. salicylates) and corticosteroids (e.g. hydrocortisone). All of these drugs possess well known side and toxic effects. Moreover, synthetic drugs are very expensive to develop and whose cost of development ranges from 0.5 to 5 million dollars. On the contrary, many medicines of plant origin had been used since long time without any adverse effects. Exploring the healing power of plants is an ancient concept. For centuries people have been trying to alleviate and treat disease with different plant extracts and formulations (1). It is therefore essential that efforts should be made to introduce new medicinal plants to develop cheaper drugs. Plants represent still a large untapped source of structurally novel compounds that might serve as lead for the development of novel drugs (2).

Zornia gibbosa belonging to family Papilionaceae, is a diffuse herb. Leaves are digitately 2-foliolate; leaflets up to 2.5 cm long, lanceolate, dotted with black glands. Flowers are yellow, enclosed in leafy bracts four in spikes. Pods are with three to six joints, densely prickly. It is distributed throughout Kanyakumari, Ramanathapuram, South Arcot, Tiruneveli of Tamil Nadu State and also in tropical region of India. Traditionally, herb is used in inflammation, dysentery and roots as a soporific given to children (3). But till now, *Zornia gibbosa* has not been screened for its anti-inflammatory activity. Therefore the present aim of this study is to carry out a pharmacological evaluation of *Zornia gibbosa* for its anti-inflammatory activity.

Material and Methods

Plant Material: The fresh aerial parts of *Z. gibbosa* were collected from Manipal, Udupi district, Karnataka, India in November and was authenticated by Dr. Gopalakrishna Bhat, Professor, Department of Botany, Poorna Prajna College, Udupi, Karnataka, India. A voucher specimen has been deposited at the Department of Pharmacognosy, Manipal College of Pharmaceutical Sciences, Manipal, India.

Preparation of extract: About 500g of plant material was air dried, powdered and was taken in soxhlet extractor and extracted with ethanol (95%) as solvent. The extract was concentrated under vacuum and dried in desiccator. The percentage yield was found to be 4.0 w/w.

Animals: Swiss albino male rats and Swiss albino mice were procured from Central Animal House, Manipal University, Manipal. The animals were acclimatized to the experimental standard environmental condition (room having temperature 23 ± 2ºC, controlled humidity conditions, and 12:12 hour light and dark cycle) with standard laboratory diet and water *ad libitum*. Study was conducted after obtaining ethical committee clearance from the Institutional Animal Ethics Committee of KMC, Manipal. No. IAEC/KMC/07/2007-2008.

Chemicals: Lipoxidase, indomethacin, phorbol myristate acetate (PMA), penicillin and streptomycin and carrageenan were purchased from Sigma, USA. Boric acid, Sodium hydroxide was purchased from Rankem, India. Linoleic acid was purchased from Himedia. All other chemicals and solvents used in the study were of analytical grade.

Acute toxicity study: Acute toxicity studies were conducted to determine the safe dose using Swiss albino mice. Alcoholic extract was administered orally (200 to 2000 mg/kg body weight) to overnight fasted animals. After administration the animals were observed continuously for one hour, frequently for the next four hours and then after 24 hr of the treatment. The toxicological effects were observed in terms of mortality expressed, as LD_{50}. The number of animals dying during a period was noted (4). The dose selected for the extract was about $1/10^{th}$ of the maximum tolerated safe dose found from acute toxicity studies. They were administered once daily by oral route.

Anti-inflammatory activity: Anti-inflammatory activity was determined by lipoxygenase assay, Interleukin 6 bioassay and Carrageenan induced rat paw edema model.

Lipoxygenases (LO) Assay: Anti-lipoxygenase assay was studied using linoleic acid as substrate and lipoxidase as enzyme (5). Test solution/positive control of various concentrations was dissolved in 140µl of 0.2 M Borate buffer, pH 9.0 and added 60µl of lipoxidase enzyme solution (18667.5 U/ml). The tubes are mixed and incubated at 25°C for 5 min, after which, 2.0 ml of linoleic acid solution (166.6 g/ml) is added, mixed well and incubated at 25°C for 4 min. Following incubation the absorbance is measured at 234 nm. Indomethacin was used as reference standard. The % inhibition was calculated as follows:

$$\% \text{ inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of test})}{\text{Absorbance of control}} \times 100$$

IC_{50} was calculated using log-probit analysis.

Interleukin 6: Cell-Based Assay for THP-1 Cytokine-Release Assay. The human monocytic cell line THP-1 (American Type Culture Collection, Manassas, Va.) was maintained in RPMI supplemented with 2 mM L-glutamine, 100 U of penicillin per ml, 100 mg of streptomycin per ml with 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), and 10% fetal bovine serum (FBS). Induction of cell differentiation was obtained with 100 nM PMA (phorbol 12-myristate 13-acetate) for 24 hr. After incubation, nonadherent cells were removed by aspiration, and the adherent cells were washed with RPMI three times. For cell stimulation, the cells were further incubated with or without LPS for 24 hr in fresh complete medium with 10% fetal bovine serum. After cell plating, the test compounds or vehicle (0.5% DMSO) were added to each well, and the plate was incubated for 30 min at 37°C. Finally, 20 µl (10 µg/ml) of LPS per well was added, for a final concentration of 1 µg/ml (6).Plates were incubated at 37°C for 24 hr supernatants were harvested and assayed for IL6 by ELISA as described by the manufacturer (BD Biosciences). Percent inhibition of cytokine release compared to the control was calculated. IC50 value was calculated by nonlinear regression method.

Carrageenan induced rat paw edema: The anti-inflammatory activity of the extract was evaluated using the 'carrageenan' induced hind paw edema' method developed by Winter *et al* (7). Young male rats were taken for the experiment, divided into five groups, each containing six rats. The rats were kept on fasting overnight. Acute inflammation was produced by injecting 0.1 ml of a 2 % w/v homogenous suspension of carrageenan in distilled water. The test extract (500 mg/kg and 750 mg/kg, orally) and diclofenac sodium (13.5 mg/kg, orally) as reference agent were administered 60 min. before carrageenan injection. The volume of injected paws measured immediately 0 hr and 3 hr after injecting carrageenan using plethysmometer. The amount of paw swelling was determined time to time and expressed as percent edema relative to initial (0 min) hind paw volume. Percentage inhibition of edema produced by treated group was calculated against the respective control group using the following formula (8).

$$\% \text{ inhibition} = \{(\text{control} - \text{test})/\text{control}\} \times 100$$

Statistical analysis: The data was analyzed using One way Anova followed by Post Hoc Schiff's Test using SPSS computer software version 7.5. Level of significance was fixed at 0.05.

Results

Lipoxygenase bioassay: The ethanolic extract of *Zornia gibbosa* was tested for *in-vitro* lipoxygenase bioassay at the concentration ranging from 200 to 400 µg/ml. and indomethacin (40 to 80 µg/ml) as standard. The highest inhibition for the extract was found to 35.65 % at the concentration 400 µg/ml and for the indomethacin it was found to be 91.54% at 80 µg/ml. The results obtained for the lipoxygenase bioassay is summarized in the table 1.

Table 1: IC_{50} data of *Zornia Gibbosa* in lipoxygenase inhibition assay

Sample	Concentration (µg/ml)	% inhibition	IC_{50} (µg/ml)
Indomethacin	40	11.27	57.38 (54.61-60.15)
	60	50.68	
	80	91.54	
Zornia Gibbosa Extract	200	0.00	
	250	0.00	
	300	11.53	
	350	24.95	
	400	35.65	

For *Zornia Gibbosa* extract IC_{50} value could not be calculated, since sample did not show 50% inhibition at the highest concentration tested.

Interleukin 6 bioassay: The ethanolic extract of *Zornia gibbosa* was tested for *in-vitro* Interleukin 6 bioassay at the concentration ranging from 0.03 to 100 µg/ml. The highest inhibition for the extract was found to 51.35 % at the concentration 100 µg/ml. The IC_{50} value was found to be 97.37 µg/ml. The results obtained for the interleukin 6 bioassay is summarizes in the table 2.

Table 2: IC_{50} data of *Zornia gibbosa* in interleukin 6 inhibition assay

Conc. (µg/ml)	% Inhibition	IC_{50} (µg/ml)
0.03	0.24	97.37
0.1	0.60	
3	2.41	
10	8.03	
30	24.9	
100	51.35	

Carrageenan induced rat paw edema: The test extract at dosage of 500 mg/kg and 750 mg/kg as well as diclofenac Sodium (13.5 mg/kg), showed significant ($p < 0.05$) inhibition edema in dose dependent manner, 3 hr after carrageenan induced inflammation when compared with control (Table 3).

Table 3: Effect of ethanolic extract of *Zornia gibbosa* on carrageenan induced rat paw edema

Treatment	Dose (mg/kg)	Paw volume at 3 hr	% Inhibition of paw edema inflammation at 3 hr
Control	0.00	4.20±0.26	0.00
Alcoholic	500	2.04±0.21	51.42*
Alcoholic	750	1.4±0.78	66.66*
Diclofenac	13.5	0.98±0.36	76.66*

All values are express in Mean ± SE, n=5, * $p < 0.05$ significant

Discussion

Lipoxygenases (LO) are members of a class of non-heme iron-containing dioxygenases that catalyze the addition of molecular oxygen to fatty acids containing a cis-1, 4-pentadiene system to give an unsaturated fatty acid hydroperoxide. In mammals, lipoxygenases carry out the first step in the arachidonic acid cascade (9, 10). 5- and 15-LOs lead to the biologically active lipoxins, whereas 5-LO leads to 5, 6-epoxy-leukotrienes which are involved in a variety of inflammatory responses, including neutrophil chemotaxis, vascular permeability, and smooth muscle contraction (11). There is a good correlation between inhibitory activity towards the mammalian 5- lipoxygenase and soyabean lipoxygenase (15-lipoxygenase) (12, 13). In the present assay linoleic acid is used as the substrate. The conversion of linoleic acid to 13-hydroxyperoxylinoleic acid by soyabean lipoxygenase was followed spectrophotometrically by the appearance of a conjugated diene at 234 nm (14). The ethanolic extract of *Zornia gibbosa* has shown good inhibition of lipoxygenase enzyme.

Interleukin-6 (IL-6) is a cytokine originally indentified as T-cell –derived factor regulating B-cell growth and differentiation (15). Human IL-6 is an important component of the inflammatory cascade. In particular, the deregulation of IL-6 production has been implicated in a variety of inflammatory/autoimmune diseases including rheumatoid arthritis, cardiac myxoma, Castleman's diseases and mesangial proliferative glomerulonephritis (16). The proinflammatory cytokines IL-1 and tumor necrosis factor-α (TNF- α) markedly stimulate IL-6 production (17).

The present study establishes the anti-inflammatory activity of ethanolic extract of *Zornia gibbosa.* It is evident that caragneenan is a sulphated polysaccharides obtained from sea weed (Rhodophyceae) and commonly used to induced acute inflammation and is believed to be biphasic. The first phase is due to release of histamine and serotonin. The second phase is caused by the release of bradykinin, protease, prostaglandins and lysosome (18). It has been reported that second phase of edema is sensitive to most clinically most effective anti-inflammatory drugs, which has been frequently used to assess the antioedematous effect of natural products (19,20). Prostaglandins play a major role in the development of second phase of reaction that is measured at 3 hr. These mediators take part in the inflammatory response and are able to stimulate nociceptors and thus induced pain (21). Carrageenan induced for edema model in rat in known to be sensitive to cyclooxygenase inhibitors. Based on these reports it can be inferred that inhibitory effect of *Zornia gibbosa* extracts on carrageenan induced inflammation in rats could be due to inhibition of the enzyme cyclooxygenase leading to inhibition of prostaglandin synthesis.

Z. gibbosa, a traditional Indian herb, exert significant anti-inflammatory activity, which in part seems to be connected with lipoxygenase, interleukin and cyclooxygenase pathway. Further investigation is required to find active component of the extract related to anti-inflammatory activity.

Conclusion

Z. gibbosa showed good anti-inflammatory activity compared to those of standard non-steroidal anti-inflammatory activity. It is also suggested that the mechanism of action might be associated with inhibition lipoxygenase,

interleukin and cyclooxygenase pathway. However further studies are needed to isolate and characterize the mechanistic anti-inflammatory chemical constituent.

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