

Research Article



Anti-Inflammatory Activity of Simple Ascidian, *Phallusia nigra* Sav

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ABSTRACT

The methanolic extract of the simple ascidian *Phallusia nigra* Sav. was screened for acute and chronic anti-inflammatory activity (carrageenan induced paw edema, cotton pellet granuloma). The reduction in paw volume was highly significant in Group V treated with 150 mg/kg bw followed by Group IV (100 mg/kg bw), indicating a dose dependent activity compared to control. A highly significant inhibition in the antiproliferative activity of the granulomatous tissue was observed with 150 mg/kg bw in chronic inflammatory studies. Studies on biochemical parameters of the serum, liver and exudates indicated lower lipid peroxide content and γ -glutamyl transpeptidase activity. The crude extracts were observed to decrease the increased acid and alkaline phosphatase activity and decrease the serum albumin content during chronic inflammation. The suppression of transudative, exudative and proliferative components of chronic inflammation may be due to the presence of bioactive compounds in this marine ascidian.

Keywords: Anti-inflammatory, simple ascidian, *Phallusia nigra*, methanolic extract.

INTRODUCTION

The untapped rich faunal diversity of the ocean has gained attention as a topic of research for evaluation of pharmacological properties and synthesis of novel new compounds. In this context the recent focus has been on the marine sedentary organisms called ascidians belonging to the Subphylum: Urochordata and Class: Ascidiacea. *Phallusia nigra* Sav. is a simple ascidian belonging to the family Ascidiidae occurring as the major component of fouling community on the hull of ships, piers, pilings, harbour installations and materials used for aquaculture operations in the Tuticorin Port Area¹. Since the report of *Phallusia nigra* from Tuticorin coast of India, studies on the ecology, distribution, seasonal variation in the occurrence, breeding biology, recruitment and succession in the fouling community, role as bioindicators², taxonomy^{3,4}, food value⁵, antibacterial activity to human pathogens⁶⁻⁹ and larvicidal potency¹⁰ chemical investigation¹¹⁻¹³ and pharmacology¹⁴⁻¹⁶ have been attempted. A review of literature reveals that a systematic pharmacologic screening of the anti-inflammatory activity of methanolic extract of *Phallusia nigra* is lacking. Hence the present study has been performed to assess the acute and chronic anti-inflammatory potential.

MATERIALS AND METHODS

Collection and identification

Phallusia nigra (Figure 1) was collected from Green Gate area (8°48'N and 78°11'E) of Tuticorin Port, Tamil Nadu by SCUBA diving and identified using Key to identification of Indian ascidians². A voucher specimen (AS 2083) was deposited in the Museum of the Department of Zoology, A.P.C. Mahalaxmi College for Women, Tuticorin-628002, Tamilnadu, India.



Figure 1: *Phallusia nigra* Sav.

Cleaning and extraction

Epibionts adhering to the test of *Phallusia nigra* were carefully removed, washed several times with sterile sea water, dried under shade and powdered. 100 g of *Phallusia nigra* was exhaustively extracted with methanol in a soxhlet apparatus, concentrated in a rotary vacuum evaporator and 15 g of a brown sticky mass was obtained.

Experimental animal

Mature adult Wistar albino rats of either sex weighing about 180 - 200 g were maintained in a well ventilated animal house at 25±2°C and humidity 60± 5% with constant 12 h of darkness and 12 h of light schedule. Clean boiled water and standard pellet diet (Hindustan Lever Ltd., India) were given 'ad libitum'. All the animals were acclimatised to laboratory conditions prior to experiments. 2 ml of 1% Vanillin was used as a flavouring agent to enhance the acceptability of the extract.

Acute oral toxicity studies

To determine the minimum lethal dose, acute oral toxicity studies were performed as per OECD guidelines¹⁷. Adult albino rats of either sex weighing 180 - 200 g were used.

The animals were divided into six groups of six each. Group I was given 2 ml of 1% saline and Group II received 2 ml of 1% vanillin both acted as control. The other four groups were administered 50, 100, 200 and 500 mg/kg bw of the methanolic extract with 2 ml of 1% vanillin orally using Intra Gastric Catheter respectively. All the experimental rats were fasted overnight. They were observed continuously for any gross behavioural changes and toxic manifestations like hyperactivity, grooming, convulsions, sedation, hypothermia and mortality during the first three hours. Thereafter the animals were continuously monitored at regular intervals for 7 days. No adverse effect or mortality was detected in this study up to 500 mg/kg bw dose. Hence sub-lethal doses of 50, 100 and 150 mg/kg bw doses of the extract were selected for the following experiments.

Carrageenan induced hind paw edema

Acute anti-inflammatory activity studies were performed following the Carrageenan induced hind paw edema suggested¹⁸ by Winter *et al.*, The animals were divided into five groups of six animals each. Group I acted as control and was given 1% saline. Group II received 10 mg/kg bw of standard reference drug – Indomethacin. Group III, IV and V were administered 50, 100 and 150 mg/kg bw of the methanol extract with 2ml of 1% vanillin respectively. 0.1 ml of 1% solution of Carrageenan was injected intradermally to the rats into the plantar surface of the right hind limb to induce paw edema. The paw volume was measured plethysmographically before induction (0 h) and after, at one hour intervals for four hours. The paw volume in Group II, III and IV were compared with that of the control. Percentage inhibition was calculated using the formula,

$$\text{Percentage inhibition} = \frac{V_c - V_t}{V_c} \times 100$$

V_c = Paw volume in control group

V_t = Paw volume in drug treated group

Statistical analysis

Values for anti inflammatory activities are expressed as "mean increase or decrease in paw volume \pm SEM". The level of significance was determined by students 't' – test values with * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, considered moderately significant, significant and highly significant¹⁹.

Cotton pellet granuloma

Wistar albino rats (180 – 200 g) of either sex were divided into five groups of six animals each. Cotton pellets weighing 30 ± 1 mg were autoclaved and implanted subcutaneously on both sides of the groin region of each rat as per²⁰ D'Arcy *et al.*, Group I served as control and received 1% saline. Group II was administered 10 mg of standard drug Indomethacin. Group III to V received 50, 100, and 150 mg/kg bw of the methanolic extract of *Phallusia nigra* respectively. The experiment was carried

out for a period of seven days and the extracts were administered orally. On the eighth day the animals were sacrificed and the pellets together with the granuloma tissues were carefully removed, dried in an oven at 60°C. The percentage of reduction in the wet and dry weight of granuloma in Group II, III, IV and V were compared with that of the control.

Biochemical parameters of liver, exudates and serum

For the analysis of biochemical parameters, the animals were sacrificed on the eighth day by cervical dislocation. Blood was collected and centrifuged to get the serum. The total protein in the serum and exudate was estimated colorimetrically²¹. Serum albumin was determined colorimetrically as per the procedure given²² by Doumas *et al.*, The liver was perfused with 0.86% cold saline to completely remove all the red blood cells, cut into small pieces, suspended in 10% (w/v) ice cold 0.1 M phosphate buffer (pH 7.4) and homogenised between 0 and 4°C. The lipid peroxide content of liver and exudates of granuloma was studied by the procedure²³ of Desai *et al.*, Acid phosphatase (EC 3.1.3.2) in the serum and exudate was assayed colorimetrically following the procedure suggested²⁴ by Fishman and Lerner. Colorimetric kinetic method suggested²⁵ by Persijn and Van der Slik was used to estimate the activity of γ -glutamyl transpeptidase (EC 2.3.2.2) GGTP in the exudate of granuloma. Alkaline phosphatase (EC 3.1.3.1) in the serum was determined²⁶ as per Bessey *et al.*, The results of enzyme activity were expressed as units of enzyme/mg of protein present in the serum and exudates.

RESULTS AND DISCUSSION

The results obtained for acute anti-inflammatory studies are presented in Table 1, Figures 2a,b,c. Group I (Control) showed a gradual increase in paw volume during the 4h of observation. Indomethacin 10 mg/kg bw (Group II) was highly significant after 3h and 4h of administration (50.85% to 61.08%). Group III which received 50 mg/kg bw of the methanolic extract was not significant but the mean increase in paw volume was less when compared to the control after 4 h. A highly significant mean decrease in paw volume after 3h and 4h (54.01% and 59.92%) was exhibited by Group IV treated with 100 mg/kg bw of the extract. The inhibitory activity of the methanolic extract at 150 mg/kg bw (Group V) was significant as early as the second hour (41.10%) and the percentage inhibition was very high after four hours (62.11%).

In general, a dose dependent anti-inflammatory activity was observed in all the three groups treated with the methanolic extract of *Phallusia nigra*. A comparison of the reduction in paw volume of the extract treated groups with the standard drug, Indomethacin (61.08%) indicated a highly significant anti-inflammatory activity (62.11%) in Group V after 4 hours.

Table 2, Figures 3a,3b,3c shows the results of chronic anti-inflammatory activity of the methanolic extracts of *Phallusia nigra*. Cotton pellet induced inflammation is due



to the formation of granuloma tissue indicating a proliferative phase. In Group I (control) there is an increase in the wet weight of cotton pellet indicative of the absorption of fluid by the pellet. Group II treated with Indomethacin showed significant reduction in granuloma with a percentage inhibition of 37.13. Group III, IV and V which received 50, 100 and 150 mg/kg bw of the extract showed 17.29%, 24.98% and 38.04% inhibition of inflammation. The percentage inhibition exhibited by 150 mg/kg bw (38.04%) was even greater than that observed for the standard drug, Indomethacin (37.13%). According to Swingle and Shideman²⁷, the wet weight of the granuloma is influenced by the fluid absorbed by the pellet and the dry weight by the excessive amount of granuloma tissue formed. The percentage reduction of the wet and dry weight granuloma showed a dose dependent increasing activity with a maximum of 38.04% and 49.83% respectively. A comparison of the results with that observed for the standard drug, Indomethacin (37.13% and 45.50%) showed that 150 mg/kg bw of the methanolic extract of *Phallusia nigra* has a higher inhibitory effect. The results are indicative of the antitransudative, exudative and antiproliferative properties of the methanolic extract of *Phallusia nigra*. The level of serum albumin in the present study was found to be almost equal to that of the reference drug (4.89gm/dl) with a dose dependent increase (Table 2).

Lipid peroxidation occurs during experimentally induced inflammation in rats and human. Bonta *et al.*, suggested²⁸ that lipid peroxides may be pro-inflammatory and can cause damage to tissues. In the present study a significant reduction in the percentage of lipid peroxide in the exudate (61.55%) and liver tissue (56.44%) of Group V was observed. The values were comparatively more significant than Group II (53.54%) and (48.16%) which received the reference drug. The lower percentage observed in Group III and IV may be due to a lag phase during which the process of lipid peroxidation is initiated and polyunsaturated fatty acids react with reactive oxidation species.

Nishikaze *et al.*, reported²⁹ marked increase in the activity of lysosomal enzyme *ie.*, acid phosphatase and alkaline phosphatase in the liver during inflammation. The level of serum acid phosphatase was increased in the control (2.94 U/mg protein/10⁴) during inflammation (Table 2) and this was normalised (1.83 U/mg protein/10⁴) with 100 mg/kg bw of the extract. The value was more significant compared to the standard drug (1.63 U/mg protein/10⁴).

Becker and Henson has reported³⁰ that the level of acid phosphatase in the exudates has got significance over its level in the serum as far as cotton pellet granuloma is considered. During inflammation there is an increase in the level of acid phosphatase (63.51%). The increase was controlled in all the three groups treated with the methanolic extract suggesting that *Phallusia nigra* has a curative effect during chronic inflammatory conditions.

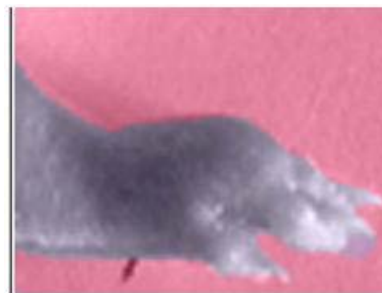


Figure 2a: Paw volume of rat showing inflammation induced by carrageenan



Figure 2b: Paw volume of the standard drug (Indomethacin) treated rat after 4 hours



Figure 2c: Paw volume of the rat treated with 150 mg/kg body weight of methanolic extract of *Phallusia nigra* after 4 hours



Figure 3a: Inflammation induced by cotton pellet before treatment



Figure 3b: Control rat treated with saline after 7 days



Figure 3c: Rat treated with 150 mg/kg bw of the methanolic extract of *Phallusia nigra*

Table 1: Effect of methanolic extract of *Phallusia nigra* and Indomethacin on carrageenan induced paw edema in rats

Group	Dose mg/kg bw	Paw volume in ml ± SEM and percentage inhibition				
		0 hour	+1 hour	+2 hour	+3 hour	+4 hour
Group I	Control	0.422±0.03	0.616±0.01	0.673±0.03	0.698±0.02	0.776± 0.04
Group II	Indomethacin	0.488±0.02	0.515±0.02* (16.39)	0.406±0.03** (39.96)	0.343±0.02*** (50.85)	0.302±0.01*** (61.08)
Group III	50	0.472±0.01	0.582±0.04 ns (05.51)	0.659±0.03ns (03.41)	0.673±0.01 (05.13)	0.612±0.02* (21.13)
Group IV	100	0.459±0.04	0.503±0.02* (18.34)	0.458±0.03** (31.94)	0.321±0.03*** (54.01)	0.311±0.02*** (59.92)
Group V	150	0.452±0.03	0.460±0.03* (25.32)	0.397±0.04** (41.10)	0.306±0.04*** (56.65)	0.294±0.03*** (62.11)

Data expressed in mean ± SEM; n = 6, Level of significance: *P < 0.05, **P < 0.01, ***P < 0.001 Control vs. Treated. Percentage inhibition is indicated in paranthesis.

Table 2: Effect of methanolic extract of *Phallusia nigra* on various biochemical parameters on cotton pellet induced granuloma

Group & Dose (mg/kg bw)	Granuloma wet wt	% of Reduction	Granuloma dry wt	% of Reduction	Albumin (g/dl)	Lipid peroxide		Acid phosphatase		Exudates GGTP (U/mg protein x10 ⁴)	Serum ALP (U/mg Protein x10 ⁴)
						Exudate (%)	Liver (%)	Serum (U/mg protein/10 ⁴)	Exudate (U/mg protein/10 ⁴)		
Normal-	-	-	-	-	5.08 ±0.23	-	63.13 ±3.97	1.98 ±0.021	-	-	0.939 ±0.053
G - I Control	36.59 ±2.19	-	21.23 ±1.43	-	4.12 ±0.43 ^a	100.00 ± 0.00	100.0 ± 0.0	2.94 ±0.11	63.51 ±3.63	16.13 ±0.66	1.863 ±0.034 ^a
G – II 10	21.89 ±2.44***	37.13	11.57 ±1.08**	45.50	4.89 ±0.16**	53.54 ± 2.88**	48.16 ±1.91*	1.63 ±0.17**	41.33 ± 2.16*	6.04 ±0.31*	1.586 ±0.012*
G – III 50	30.26 ±1.98*	17.29	14.68 ±1.22*	30.85	4.39 ±0.21	21.33 ±1.21	32.14 ± 1.46	2.31 ±0.16	36.34 ±1.93	9.14 ±0.14	1.621 ±0.054
G – IV 100	27.45 ±2.05**	24.98	12.59 ± 1.03**	40.69	4.63 ±0.11*	28.91 ±1.36	46.56 ±1.84*	1.83 ±0.14**	40.09 ±1.84*	7.36 ±0.24	1.514 ±0.041*
G - v 150	22.67 ±2.56***	38.04	10.65 ±1.11***	49.83	4.98 ±0.62**	61.55 ± 2.67**	56.44 ±1.54**	1.21 ±0.12*	48.11 ± 0.39**	5.11 ±0.56**	1.431 ±0.074*

*P < 0.05, **P < 0.01, ***P < 0.001 Control vs. Treated.

The level of γ -glutamyl transpeptidase (GGTP) showed a dose dependent reduction compared to the treated groups with control. The values observed for Group IV and V (7.36 U/mg protein x10⁴ and 5.11 U/mg protein x10⁴) showed a reduction similar to that of the standard drug. This may indicate the presence of specific phytochemicals in the methanolic extract of *Phallusia nigra*.

Serum alkaline phosphatase level is elevated during chronic inflammations as indicated in Table 2. On treatment with different doses of the methanolic extract there was a dose dependent decrease. Group V treated with 150 mg/kg bw showed a more significant decrease when compared to the reference drug indicating the efficacy of the extract in controlling chronic inflammation.

An evaluation of the anti-inflammatory activity of different doses of methanolic extract of *Phallusia nigra* revealed a highly significant reduction in paw volume in Group V (150 mg/kg bw) compared to that of the control and equal in potency to the standard drug, Indomethacin (Group II). Anti-inflammatory activity had been reported from many marine organisms like cyanobacterium³¹,

bacteria³², green algae³³, sponges^{34,35}, gorgonids³⁶ and ascidians³⁷.

The biochemical process leading to inflammation on carrageenan injection is induced by the activation of kinins, accumulation of neutrophils, release of mediators like prostanoids and cytokines³⁸. During the three stages in the induction of paw edema in albino rats initially there is a release of histamine and serotonin (0-2 h), secondly kinins (3 h) and lastly an elevated production of prostaglandins (4 h)³⁹. Phospholipase is the enzyme which is responsible for the release of arachidonic acid, the key molecule in the biochemical processes leading to the synthesis of prostaglandins during inflammatory response. Many marine organisms including ascidian^{40,41} have inhibitors of phospholipase A₂.

In the present study Group V, treated with 150 mg/kg bw of the extract showed significant inhibitory response to inflammation and the mean reduction in paw volume was highly significant after two hours. The results are suggestive of the presence of a potent anti-inflammatory principle in the methanolic extract of *Phallusia nigra*. It is suggested that the active compound in the extract may

play an inhibitory role either by drastically reducing superoxide production by neutrophils or by inhibiting phospholipase, COX 1 and 2 enzymes thus blocking the release of arachidonic acid and prostaglandin biosynthetic pathway.

The cotton pellet granuloma method is a standard widely used procedure to evaluate the inflammatory granuloma consisting of the transudative, exudative and proliferative component of chronic inflammation. It has been observed from the results that 150 mg/kg bw of the extract has equally significant anti-inflammatory activity compared to Indomethacin which is an indication of the antiproliferative activity of the extract resulting in the formation of less granulomatous tissue. The various biochemical parameters studied also supports the presence of some compounds which might have a role in controlling inflammatory condition.

CONCLUSION

Thus it can be concluded that the methanolic extract of *Phallusia nigra* shows anti-inflammatory potential in both acute and chronic phases of inflammation. A preliminary chemical screening of the methanolic extract of *Phallusia nigra* showed the presence of alkaloids, terpenoids, flavonoids, glycosides, phenolic compounds, tannins etc., which may act as antioxidants. Further studies on the isolation and structure determination of the active principle and its mode of action are suggested for the development of a new drug candidate in the treatment of inflammatory diseases.

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