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Wound healing activity of the methanolic extract of *Phallusia nigra* Sav.

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ABSTRACT

The methanolic extract of the simple ascidian *Phallusia nigra* Sav. was screened for wound healing activities (incision wound model, excision wound model and dead space model). In excision and incision wound models, the wound contraction was highly significant in Group V treated with 15% w/w of the methanolic extract followed by Group IV (10% w/w) indicating a dose dependent activity compared to that of the standard drug, framycetin sulphate (2% w/w). Epithelialization period was also found to be highly significant when compared with that of the standard, in both the models. In the dead space model, significant increase in the wet and dry weight of the granulation tissues, tensile strength and hydroxyproline content of the granulation tissue was observed in the animals treated with the methanolic extract of *Phallusia nigra*.

Key words: Wound healing, Simple ascidian, *Phallusia nigra,* Methanolic extract.

1. INTRODUCTION

A large proportion of natural compounds have been extracted from marine organisms, especially sponges, ascidians, bryozoans and molluscs and some of them are currently in clinical trials [1]. To date, almost all of the drugs derived from natural sources come from terrestrial organisms. But recently, systematic searches for new drugs have shown that marine animals produce more antibiotic, anti-cancer and anti-inflammatory substances than any group of terrestrial organisms. Particularly promising groups include sponges, tunicates, ascidians, bryozoans, octocorals, some molluscs, annelids and echinoderms [2,3]. Tunicates have been reported to be rich sources of biologically active compounds and ranked third for their overall activities, next to sponges and bryozoans [4]. Although research on bioactive compounds from ascidians have been recently initiated, it is significant that the first marine natural product, Didemnin B entering in to human clinical trial, is an ascidian metabolite. Most of the ascidians are utilized as food in various countries and they are known to produce bioactive metabolites which prevent bio-fouling and this can be considered as a kind of autogenic protection [5]. This mechanism has proved to be timely alternative natural medicine to human beings. *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 area. Though marine animals exhibit a lot of pharmacological properties, wound healing activity studies have not been performed in any of these groups especially ascidians. Hence in the present investigation wound healing activity has been studied on the simple ascidian, *Phallusia nigra* Sav.

2. MATERIALS AND METHODS

2.1. Collection and identification

Phallusia nigra Sav. (Fig.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 ascidians [6]. 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.

Fig. 1: *Phallusia nigra* Sav.

2.2. 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 Powder was exhaustively extracted with methanol in a Soxhlet apparatus, concentrated in a rotary vacuum evaporator when 15 g of a brown sticky mass was obtained. The methanolic extract was incorporated into a simple ointment base BP. Three formulations of the extract ointment 5% (w/w), 10% (w/w) and 15% (w/w) were prepared by incorporating 5 g, 10 g and 15 g of extract in 100 g of simple ointment base BP respectively for topical administration. The extracts were suspended in 1% carboxymethyl cellulose (CMC) and used for oral administration.

2.3. Experimental animal

Mature adult Wistar Albino rats of either sex weighing about 180-200 g were maintained in a well ventilated animal house at $25^0 \pm 2^0$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.

2.4. Acute oral toxicity studies

To determine the minimum lethal dose, acute oral toxicity studies were performed as per OECD guidelines. 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 mg/kg bw, 100mg/kg bw, 200 mg/kg bw and 500 mg/kg bw of the methanolic extract of *Phallusia nigra* 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 500mg/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.

2.5. Wound healing activity

Excision, incision and dead space wound models were used to evaluate the wound healing activity of the methanolic extract of *Phallusia nigra* Sav.

2.5.1. Excision wound model

The rats in these studies were inflicted with an excision wound as described by Morton and Malone [7], under light ether anaesthesia Superficially, a single circular wound of 500 mm^2 was made on a depilated ethanol-sterilized dorsal thoracic region of the rats. The animals were then randomized into five groups (n = 6/group); Rats in: Group 1 (ointment control) received a topical application of 50 mg of the Simple ointment BP; Group II (standard drug) were treated with a topical application of 50 mg of 2% framycetin sulphate cream (FSC); Group III, IV and V were treated with 5%, 10% and 15% of methanolic extract ointment respectively. The ointments were applied topically with a fine brush once daily till the wound was completely healed.

2.5.1.1. Determination of wound contraction

Wound contraction was monitored planimetrically by tracing the wound margin on a transparent paper every alternate day and retracing the wound margin on a millimetre scale graph paper. Wound contraction which contributes for wound closure or reduction in the wound area was expressed as percentage reduction of the original wound area (500 mm^2). The percentage wound contraction was determined using the following formula:-

$$\text{Percentage Wound Contraction} = \frac{\text{Healed area}}{\text{Total wound area}}$$

(Healed area = Original wound area – resent wound area). To apply this equation, the wound margins were traced and measured to calculate the non-healed area which was subtracted from the original wound area to obtain the healed area.

2.5.2. Incision wound model

In this model, 6 cm long paravertebral incisions were made through the full thickness of the skin on either side of the vertebral column of rats as described [8] by Ehrlich and Hunt. The wound was then closed with interrupted sutures 1 cm apart. The animals were then randomized into five groups (n = 6/group). Each treatment outlined here was utilized each day (for 8 consecutive days) after the wound infliction. Rats in: Group 1 (ointment control) received a topical application of 50 mg of the Simple ointment BP; Group II (standard drug) were treated with a topical application of 50 mg of 2% framycetin sulphate (FSC) Group III, IV and V were treated with 5%, 10% and 15% methanolic extract of *Phallusia nigra* respectively.

2.5.3. Dead space wound model

Dead-space wounds were created under light ether anesthesia by subcutaneous implantation of sterilized cylindrical grass piths (2.5 x 0.3 cm), one on either side of the dorsal paravertebral surface of the rat [9]. Animals were then randomized into five groups (n = 6/group). Each treatment outlined here was utilized each day (for 10 consecutive days) after the pith implantations. Rats in: Group 1 (ointment control) received a topical application of 50 mg of the Simple ointment BP; Group II (standard drug) were treated with a topical application of 50 mg of 2% framycetin sulphate cream (FSC); Group III, IV and V were treated with 5% w/w, 10% w/w and 15% w/w of methanolic extract of *Phallusia nigra* respectively. The tensile strength of the wound was measured as described by Lee [10].

2.5.4. Histological study

The granulation tissues were fixed in 10% neutral formalin solution for 24 hours and dehydrated with a sequence of ethanol-xylene series of solution. The tissues were embedded with paraffin at 40^0-60^0C. Microtome sections of 10 μ thickness were taken. The processed sections were stained with hematoxylin and eosin and observed under the light microscope.

2.6. Statistical analysis

Results obtained from all the wound models have been expressed as mean ± standard error. Treated groups have been compared with the corresponding control groups. P values have been calculated by Student's t-test by comparing with the control and standard.

3. RESULTS AND DISCUSSION

The effect of methanolic extract of *Phallusia nigra* on the contraction of excision wound is presented in Table 1. Topical application of the methanolic extract ointment of *Phallusia nigra* showed a significant and dose dependent effect on the healing process. It can be noted that the wound contracting ability of extract treated groups *viz* group IV (10% w/w) and group V (15% w/w) were found to be highly significant from 8^{th} to 14^{th} day when compared to that of the standard, framycetin sulphate (2% w/w). Epithelialization period was also found to be highly significant in group V (12.31±0.14) when compared to that of the standard (15.66±0.36).

The effect of methanolic extract of *Phallusia nigra* on the contraction of incision wound is presented in Table 2. In incision wound model, the extract treated groups even at very low dose produced significant increase in wound contraction when compared with the standard. Highly significant increase was noticed in group V (15% w/w). On day 16, group V (15% w/w) showed 99.98%, wound contraction which is higher than that of the standard (98.11%). Epithelialization period was also found to be highly significant in group V (11.34±0.23) when compared to that of the standard (12.34±1.06).

The effect of wound healing activity by dead space method was evaluated by determining the granuloma weight of the dead space wound of different groups *viz* control treated with simple ointment base B.P, standard group treated with framycetin sulphate (2%) ointment and the test group treated orally with the extracts at different concentration (5% w/w, 10% w/w and 15% w/w). The results are presented in Table 3. In the dead space model, significant increase in the wet and dry weight, tensile strength and hydroxyproline content of the granulation tissue were observed in the animals treated with the methanolic extract of *Phallusia nigra*. There is a significant increase with the granuloma wet and dry weights when we move from lower to higher dose. Group V (15% w/w) shows the highest activity. Hydroxyproline content was found to be maximum in group V (93.6 ± 3.40) compared to that of standard (90.1 ± 3.32).

Histological profiles of the granulation tissues of the control and the methanolic extract treated animals are presented in Fig.2. Fig. 2a. represents control showing less collagenation, more macrophages and lymphocytes. Fig. 2b. represents the standard, Framycetin sulphate (2% w/w) treated granuloma showing more amount of collagen formation, tissue infiltration with macrophages and lymphocytes. Fig. 2c. represents the 5% w/w extract treated granuloma tissue showing less collagen fibers and less infiltration of tissue. Fig. 2d. .represents the 10% w/w extract treated granulation tissue containing moderate collagen, fibroblasts, and blood capillaries. Fig. 2e. represents the 15% w/w extract treated granuloma showing more collagen and fibroblasts with absence of inflammatory cells. Increased collagen formation was observed in the methanolic extract treated animals when compared to that of control. The methanolic extract of *Phallusia nigra* at the dose of 15% w/w was more effective in promoting collagen formation.

Wound healing process begins with the restoration of a damaged tissue as closely as possible to its natural state and wound contraction is the course of shrinkage in wounded area. The healing primarily depends on the repairing ability of the tissue in addition to type and degree of damage and general health status of the tissue.

Table - 1: Effect of methanolic extract of *Phallusia nigra* on the contraction of excision wound

Groups	Epithelializa-tion Period (days)	Percentage of Wound contraction in post wounding days						
		2	4	6	8	10	12	14
Group I (Control)	19.41±0.32	15.59±0.31	29.66±0.53	36.27±0.62	43.44±0.31	69.44±0.93	88.64±0.56	93.56±0.55
Group II Standard (2%)	15.66±0.36*	22.38±0.45*	53.23±0.37*	68.77±0.35*	86.57±0.22*	90.21±0.66*	92.11±0.36*	94.11±0.38
Group III (5% w/w)	16.14±0.16*	20.16±1.21	45.36±0.96	58.11±0.75	70.11±0.86*	83.36±0.69*	91.27±0.66	95.27±0.32
Group IV (10% w/w)	14.93±0.26*	27.27±0.13*a	51.27±0.33*	65.14±0.43*	88.27±0.91*	90.67±0.31*	93.21±0.46*	95.55±0.43
Group V (15% w/w)	12.31±0.14*	29.16±0.31*a	57.23±0.68*	70.25±0.69*a	90.33±0.34*a	92.56±0.37*	95.57±0.43*	97.25±0.34*

* P < 0.05 compared to control; a P < 0.05 compared to standard. Values are mean ± SEM (n =6)

Table - 2: Effect of methanolic extract of *Phallusia nigra* on incision wound

	Wound area (mm^2) and percentage of wound contraction				
Groups	4th day	8th day	12th day	16th day	Epithelization period
Group I (Control)	386.33±2.34 (22.73)	354.78±1.65 (29.04)	204.23±2.34 (59.15)	131.34±1.98 (73.73)	24.34±2.11
Group II Standard (2%) (FSC)	204.34±1.98* (59.13)	141.33±2.11** (71.73)	51.67±0.89** (89.66)	09.45±1.33*** (98.11)	12.34±1.06*
Group III (5% w/w)	296.45±1.67 (40.71)	234.67±1.78* (53.06)	148.33±1.05* (70.33)	36.23±1.45** (92.75)	18.34±0.98*
Group IV (10% w/w)	234.56±2.11 (53.08)	173.54±1.45* (65.29)	73.34±1.23** (85.33)	20.34±1.06*** (95.93)	15.56±0.56*
Group V (15% w/w)	302.12±1.34 (39.57)	172.89±2.02* (65.42)	32.33±0.89*** (93.53)	1.02.±0.01*** (99.98)	11.34±0.23**

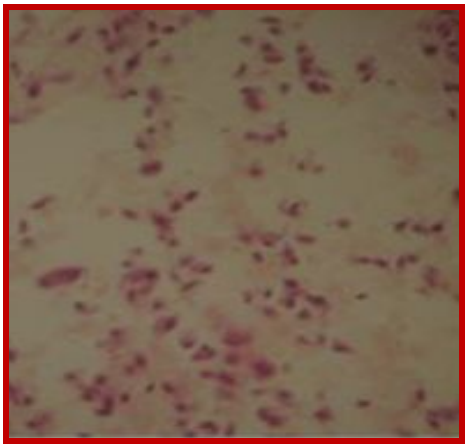
n = 6; *: p<0.05;** P<0.01;*** P <0.001: Compared to control. Values of wound diameter shown are Mean ± SEM; Values in parenthesis represent wound closure (%) calculated relative to the wound diameter on day 0.

Table - 3: Effect of methanolic extract of *Phallusia nigra* on dead space wound

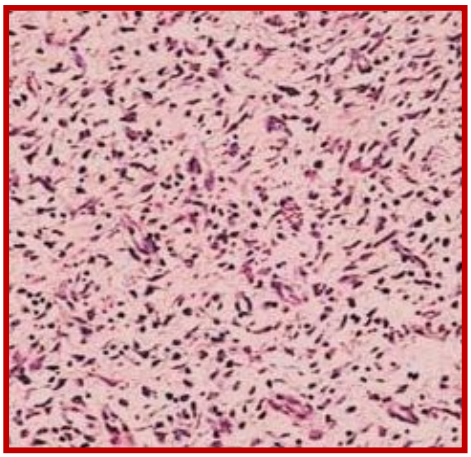
Parameter	Control	Standard 2% w/w	5% w/w	10% w/w	15% w/w
Tensile strength (g)	320.13 ±3.23	370.5 ± 4.16	440.0 ± 4.53*	490.0 ± 3.40 *	520.0 ± 4.10**
Wet weight of granulation tissue (mg)	87.1 ± 5.20	128.2 ± 4.20	135.7 ± 4.10*	177.3 ± 2.90 **	190.0 ± 3.40**
Dry weight of granulation tissue (mg)	13.0 ± 2.40	19.0 ± 0.68	20.0 ± 0.30 *	21.0 ± 0.48*	23.5 ± 1.30*
Hydroxyproline (mg/kg)	33.6 ± 2.90	90.1 ± 3.32	81.3 ± 3.42*	83.6 ± 3.10*	93.6 ± 3.40**

Values are expressed as mean ± SE, n = 6 animals in each group, *P<0.05; ** P < .001 when compared to control

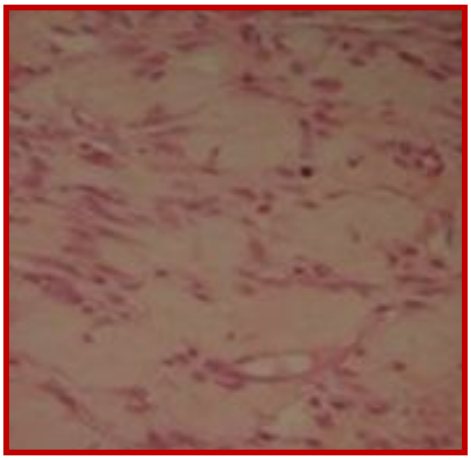
The granulation tissue of the wound is primarily composed of edema, fibroblast, collagen and new blood vessels. The mesenchymal cells of the wound area adjust themselves into fibroblast then begin migrating into the wound gap together with the fibrin strands. The collagen is the main constituent of extra cellular tissue, which is responsible for support and strength. Free hydroxyproline and its peptides are released with collapse of collagen. Thus, measurement of the



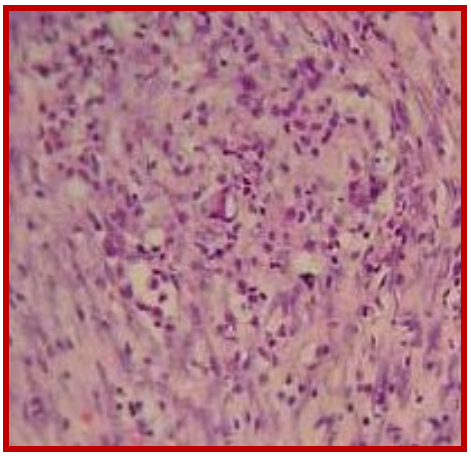
(a) Control (Normal saline treated)



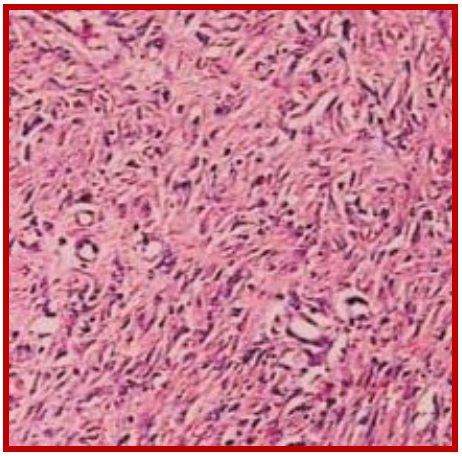
(b) FSC Oinment (2% w/w treated)



(c) *Phallusia nigra* extract (5% w/w)



(d) *Phallusia nigra* extract (10% w/w)



(e) *Phallusia nigra* extract (15% w/w)

Fig. 2. Photomicrograph showing histopathological changes in the granulation tissues from control, standard and methanolic extract (*Phallusia nigra***)** treated animals

hydroxyproline could be used as an indicator for collagen turnover. Furthermore, increase in dry tissue also indicates the presence of elevated protein content.

In the inflammatory phase, bacteria and debris are phagocytosed and removed and factors are released that cause the migration and division of cells involved in the proliferative phase. The proliferative phase is characterized by angiogenesis, collagen deposition, granulation tissue formation, epithelialization and wound contraction. In epithelialization, epithelial cells crawl across the wound bed to cover it [11]. The wound is closed by a combination of all these and by the process of wound contraction. During wound contraction, the wound is made smaller by the action of myofibroblasts which establish a grip on the wound edges and contract themselves using a mechanism similar to that in smooth muscle cells. In the maturation and remodeling phase, collagen is remodeled and realigned along tension lines and cells that are no longer needed are removed by apoptosis. Increased wound contraction in extracts treated rats might be due to an enhanced activity of fibroblasts in regenerated wound tissues. This is also supported by the increase in the tensile strength.

In dead space wound model, increase in tensile strength of treated wounds may be due to an increase in collagen formation per unit area and stabilization of the fibers [12]. Deposition of newly synthesized collagen at the wound site increases the collagen concentration per unit area and hence, the tissue tensile strength [13].

In dead space wound model, granulation tissue formation is indicative of proliferative and remodeling phase of wound healing process. The granulation tissue of the wound is primarily composed of edema, fibroblasts, collagen and new blood vessels. The mesenchymal cells of the wound area adjust themselves into fibroblasts then begin migrating into the wound gap together with the fibrin strands [14]. Test groups significantly increased the granulation weight, indicating that there might be increased protein synthesis and improvement of both proliferative and remodeling phases of the wound healing.

The methanolic extract of *Phallusia nigra* showed profound wound healing activity against various experimental wound models, affecting all the phases-wound contraction, proliferative and remodeling phases of wound healing. The flavonoids are reported to have therapeutic uses due to their anti-inflammatory, anti-fungal, anti-oxidant and wound healing properties [15,16]. Flavonoids are also known to endorse the wound healing process primarily due to their anti-microbial and astringent properties which appears to be responsible for wound contraction and elevated rate of epithelialization [17,18]. Preliminary phytochemical screening and HPTLC analysis of *Phallusia nigra* revealed the presence of flavonoids which have antimicrobial activity. Also methanolic extract of *Phallusia nigra* were reported to contain antioxidant compound, n-Hexadecanoic acid [19]. Hence, the wound healing activity of *Phallusia nigra* could be attributed to the presence of flavonoids and antioxidant compounds.

4. CONCLUSION

The present investigation reveals that the methanolic extract of *Phallusia nigra* is found to have better wound healing properties. Attempts will be made in future to isolate and identify the phytochemical constituents of the methanolic extract responsible for the wound healing activity.

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