EVALUATION OF WOUND HEALING ACTIVITY OF PHALLUSIA ARABICA

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ABSTRACT

Wound healing is a complex process and has been the subject of intense research for a long time. The ultimate goal for wound healing is a speedy recovery with minimal scarring and maximal function. The aim of this research is to investigate the wound healing potential of ethanolic extract of simple ascidian Phallusia arabica by excision, incision and dead space wound models. Significant increase in skin, granuloma breaking strength, wound contraction and hydroxyl proline content was observed. Epithelialization period was also found to be highly significant when compared with that of the standard, in both the models.

KEYWORDS: wound healing, Phallusia arabica, ethanolic extract, hydroxyl proline.

INTRODUCTION

Wound may be defined as disruption of anatomic/functional continuity of living tissue; the causative factors for wound may be physical, chemical, thermal and electrical.[1] Research on wound healing agents is one of the developing areas in modern biomedical sciences. Many traditional practitioners across the world particularly in countries like India and China with age old traditional practices have valuable information of many lesser known hither to unknown wild plants used by the traditional healers for treating wounds and burns. Several drugs of plant, mineral, marine and animal origin are described in the traditional texts of Indian systems of medicine like Ayurveda for their healing properties under the term
‘Vranaropaka’. Besides the classical systems of Indian Medicine, the folk and the tribal medicine also employ a number of plants and animal products for treatment of cuts, wounds and burns.

The ascidians, commonly called sea squirts (Subphylum: Urochordata, Class: Asciidiacea) are dominant organisms in many marine communities, having a wide geographic distribution. In India, work on marine ascidians are restricted to their biology and classification. So far, about 372 species of ascidians have been reported from the Gulf of Mannar. Phallusia arabica is an important simple ascidian which contains a wealth of interesting pharmacological substances. Up to date, no pharmacological study has been reported for its wound healing activity. The present study was therefore, undertaken to evaluate the effect of ethanolic extract of Phallusia arabica on wound healing activity for the synthesis of new generation drugs.

MATERIALS AND METHODS

Collection and identification

Phallusia arabica (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. A voucher specimen (AS 2276) was deposited in the Museum of the Department of Zoology, A.P.C. Mahalaxmi College for Women, Tuticorin 628002, Tamilnadu, India.

Cleaning and extraction

Phallusia arabica was washed several times with sterile sea water, dried under shade and powdered. 100 g powder was exhaustively extracted with ethanol in a Soxhlet apparatus, concentrated in a rotary vacuum evaporator when 15 g of a brown sticky mass was obtained.
Three formulations of the extract ointment 10, 20 and 40% (w/w) were prepared by incorporating 10 mg, 20 mg and 40 mg of extract in 100 mg of simple ointment base BP respectively for topical application.

**Experimental animal**

Adult male wistar albino rats weighing about 180-200 g were obtained from Central Animal House, Annamalai University, Chidambaram, Tamil Nadu, India. They were housed in standard environmental conditions of temperature at 24±1°C under a 12 h dark-light cycle, and allowed free access to drinking water and standard pellet diet. Rats were deprived of food except water 16-18 hour prior to the experiments. The rules and regulations of the Animal Ethical Committee, Government of India were followed.

**Acute oral toxicity studies**

Acute oral toxicity study was performed according to the Organization of Economic Co-operation and Development (OECD) guideline.[13] Six rats were administrated with a single oral dose of 2,000 mg/kg body weight ethanolic extract of *Phallusia arabica* while the control group received saline. They were placed under continuous observation for gross behavioral changes like irritability, tremor, labored breathing, staggering, convulsion and death for the first 2 h and then frequently during the next 24 h after which the number of dead rats if any were recorded.

**Subchronic oral toxicity**

A total of thirty six mature albino rats were used in this study. These were divided into six groups of six each. Group I received normal saline and group II, III, IV, V and VI were treated with 200, 400, 800, 1600 and 2000 mg/kg body weight of the extract respectively for fourteen days. Food and water intake were monitored daily. They were observed for toxic signs and symptoms like morbidity and mortality at an interval of 2, 4, 8 12, 16, 24 h and thereafter twice daily till the end of the experiment. 24 h after the last dose and 18 h of fasting, blood samples were collected through cardiac puncture, under chloroform anaesthesia into heparinised tube for haematological studies and non heparinised tube for liver and kidney function test. Total count, RBC, platelets were performed using Neubauer haemocytometer and estimation of haemoglobin by Sahli’s haemoglobinometer.
Wound healing activity
Excision, incision and dead space wound models were used to determine the wound healing activity.

Excision wound model
Excision wound was inflicted on the rats according to methods described by Morton and Malone, under light ether anesthesia. The dorsal fur of the animals were shaved with an electric clipper. Full skin thickness was excised from the marked area to get a wound measuring about 500 mm² by using toothed forceps and pointed scissors. The animals were then divided into five groups (n = 6/group); Rats in: group 1 (ointment control) received a topical application of the simple ointment BP; group II was treated with standard drug 1% framycetin sulphate cream; group III, IV and V were treated with 10, 20 and 40% of ethanolic extract ointment respectively. All the formulations were applied once a day, starting from the day of wounding till complete epithelialization.

Determination of wound contraction
Wound contraction was noted by following the progressive changes in wound area planimetrically, excluding the day of the wounding. Wound contraction which contributes for wound closure or reduction in the wound area was expressed as percentage reduction of the original wound area (500 mm²). The percentage wound contraction was determined using the following formula

Percentage Wound Contraction = Healed area
                                 Total wound area

(Healed area = Original wound area – recent 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.

Incision wound model
Five groups of animals containing six in each group were taken. The animals were anaesthetized under light ether anaesthesia. One full thickness paravertebral incision of 6 cm length was made including the cutaneous muscles of the depilated back of each rat. Full septic measures were not taken and no local or systematic antimicrobials were used through out the experiment. After the incision was made, the parted skin was kept together and
stitched with sutures, 1 cm apart. 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 Simple ointment BP; group II was treated with standard drug 1% framycetin sulphate cream; group III, IV and V were treated with 10, 20 and 40% ethanolic extract of Phallusia arabica respectively.

**Dead space wound model**

The dead space wounds were produced 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. Animals were then divided into five groups (n = 6). Drugs were administered for 10 days. Rats in: group 1 (ointment control) received a topical application of 50 mg of the simple ointment BP; group II was treated with standard drug 1% framycetin sulphate cream; group III, IV and V were treated with 10, 20 and 40% ethanolic extract of Phallusia arabica respectively. The tensile strength of the wound was measured as described by Lee. Weight of granulation tissue and hydroxyl proline content were recorded.

**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 in paraffin at 40\(^0\)-60\(^0\) C. Microtome sections of 10 \(\mu\) thickness were taken. The processed sections were stained with hematoxylin and eosin and observed under the light microscope.

**Statistical analysis**

Results obtained from all the wound models have been expressed as mean ± standard error. P values were calculated by Student’s t-test by comparing treated groups with the control.

**RESULTS AND DISCUSSION**

Determination of toxicity is usually an initial screening step in the assessment and evaluation of the toxic characteristics of all compounds. Acute and sub chronic oral toxicity of the ethanolic extract of Phallusia arabica showed that there is no obvious toxicity. No mortality was observed even at a dose of 2000 mg/kg. Severe gross behavioral changes like irritability, tremor, laboured breathing, staggering and convulsion were noted at a dose of 1600 mg/kg body weight and above only. Hence sub-lethal doses of 100, 200 and 400 mg/kg bw doses of the extract were selected for the following experiments.
Wound contracture is a process that occurs throughout the healing process, beginning from the fibroblastic stage when the wound area undergoes shrinkage. In the final stage of wound healing, the wound undergoes contraction resulting in a smaller amount of apparent scar tissue.

The ethanolic extract ointment of *Phallusia arabica* showed a significant increase in wound closure in excision wound model (Table 1).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Epithelialization Period (days)</th>
<th>Percentage of Wound contraction in post wounding days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Group I (Control)</td>
<td>22.76±1.23</td>
<td>23.68±0.87</td>
</tr>
<tr>
<td>Group II Standard 1%</td>
<td>16.67±0.75*</td>
<td>27.18±0.56*</td>
</tr>
<tr>
<td>Group III 10%</td>
<td>20.48±0.16</td>
<td>19.46±1.09</td>
</tr>
<tr>
<td>Group IV 20%</td>
<td>18.88±0.21*</td>
<td>31.60±0.67**</td>
</tr>
<tr>
<td>Group V 40%</td>
<td>14.57±0.28**</td>
<td>34.97±0.63**</td>
</tr>
</tbody>
</table>

Data represented as mean ±SEM, (n=6). Significance between control and extract treated groups. **P <0.05; **p <0.01; ***p <0.001.

The effect of ethanolic extract of *Phallusia arabica* 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 (40% w/w). On day 16, group V (40% w/w) showed 99.13%, wound contraction which is higher than that of the standard (97.17%). Epithelialization period was also found to be highly significant in group V (10.31±0.13) when compared to that of the standard (12.91±0.24).
### Table - 2: Effect of ethanolic extract of *Phallusia arabica* on incision wound

<table>
<thead>
<tr>
<th>Groups</th>
<th>0 day</th>
<th>4th day</th>
<th>8th day</th>
<th>12th day</th>
<th>16th day</th>
<th>Epithelization period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wound area (mm²)</td>
<td>Percentage of wound contraction</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
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</tr>
<tr>
<td>Group I (Control)</td>
<td>420.16±3.46</td>
<td>392.54±3.16 (6.27)</td>
<td>379.54±4.16 (9.66)</td>
<td>361.16±5.11 (14.04)</td>
<td>354.25±3.84 (15.68)</td>
<td>28.43±1.93</td>
</tr>
<tr>
<td>Group II Standard 1%</td>
<td>315.60±5.63</td>
<td>172.16±1.81* (45.45)</td>
<td>112.65±1.29* (64.30)</td>
<td>50.25±0.73** (84.05)</td>
<td>8.91±0.18*** (97.17)</td>
<td>12.91±0.24***</td>
</tr>
<tr>
<td>Group III 10%</td>
<td>384.15±4.22</td>
<td>306.16±2.40 (20.30)</td>
<td>269.11±1.93 (29.94)</td>
<td>131.65±3.84* (65.72)</td>
<td>21.40±1.03*** (94.42)</td>
<td>20.13±0.91*</td>
</tr>
<tr>
<td>Group IV 20%</td>
<td>340.25±3.15</td>
<td>210.16±1.84* (35.29)</td>
<td>160.15±1.84* (52.93)</td>
<td>78.15±1.81** (77.03)</td>
<td>14.16±0.31*** (95.83)</td>
<td>13.16±0.81**</td>
</tr>
<tr>
<td>Group V 40%</td>
<td>354.16±4.13</td>
<td>163.15±1.91* (53.93)</td>
<td>106.11±0.93** (70.03)</td>
<td>42.16±0.68** (88.09)</td>
<td>3.06±0.13*** (99.13)</td>
<td>10.31±0.13***</td>
</tr>
</tbody>
</table>

Data represented as mean ±SEM, (n=6). Significance between control and extract treated groups. *P <0.05; **p <0.01; ***p <0.001. Values in parenthesis represent wound closure (%) calculated relative to the wound diameter on day 0.

In dead space model, significant increase in the wet, dry weight, tensile strength and hydroxyproline content of the granulation tissue were observed in the animals treated with the ethanolic extract of *Phallusia arabica* (Table 3). There is a significant increase with the granuloma wet weight when we move from lower to higher dose. Group V shows the highest activity. Hydroxyproline content was found to be maximum in group V (113.81 ± 2.80) compared to that of standard (93.65 ± 2.85).

### Table - 3: Effect of ethanolic extract of *Phallusia arabica* on dead space wound

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I Control</th>
<th>Group II Standard 1%</th>
<th>Group III 10%</th>
<th>Group IV 20%</th>
<th>Group V 40%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tensile strength (g)</td>
<td>313.28 ±2.91</td>
<td>355.5 ±5.18*</td>
<td>460.1 ± 6.29***</td>
<td>496.5 ± 6.51**</td>
<td>542.6 ± 5.90***</td>
</tr>
<tr>
<td>Wet weight of granulation tissue (mg)</td>
<td>93.65 ±3.14</td>
<td>163.2 ± 4.84*</td>
<td>149.3 ± 4.52***</td>
<td>184.5 ±3.80**</td>
<td>199.3 ± 6.80***</td>
</tr>
<tr>
<td>Dry weight of granulation tissue (mg)</td>
<td>21.85 ± 3.84</td>
<td>28.16 ±1.05*</td>
<td>18.45 ± 0.18*</td>
<td>22.50 ± 0.65ns</td>
<td>25.15 ±0.93ns</td>
</tr>
<tr>
<td>Hydroxyproline (mg/kg)</td>
<td>42.16 ± 1.84</td>
<td>93.65 ± 2.85**</td>
<td>76.50 ± 2.15**</td>
<td>92.56 ± 2.83**</td>
<td>113.81 ± 2.80**</td>
</tr>
</tbody>
</table>

Data represented as mean ±SEM, (N=6). Significance between control and extract treated groups. *P <0.05; **p <0.01; ***p <0.001.
Fig. 2. Photomicrograph showing histopathological changes in the granulation tissues

Histological profiles of the granulation tissues of the control and the ethanolic extract treated animals are presented in Fig. 2. Fig. 2a shows control showing less collagenation, more macrophages and lymphocytes; 2b indicates the standard, Framycetin sulphate (1%) treated
granuloma showing more amount of collagen formation, tissue infiltration with macrophages and lymphocytes; 2c represents the 10% extract treated granuloma tissue showing less collagen fibers and infiltration of tissue: 2d contains the 20% extract treated granulation tissue containing moderate collagen, fibroblasts, blood capillaries; 2e shows the 40% extract treated granuloma showing more collagen and fibroblasts with absence of inflammatory cells. Increased collagen formation was observed in the ethanolic extract treated groups compared to that of control. The ethanolic extract of *Phallusia arabica* at the dose of 40% was more effective in promoting collagen formation.

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.[17] Deposition of newly synthesized collagen at the wound site increases the collagen concentration per unit area and hence, the tissue tensile strength.[18]

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.[19] 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 ethanolic extract of *Phallusia arabica* showed profound wound healing activity against various experimental wound models, affecting all the phases—wound contraction, proliferative and remodeling phases of wound healing.

Preliminary chemical analysis revealed the presence of flavonoids, terpenoids and steroids. The flavonoids are reported to have therapeutic uses due to their anti-inflammatory, antifungal, anti-oxidant and wound healing properties.[20,21] 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.[22,23] The ethanolic extract of *Phallusia arabica* is reported to contain antioxidant compound, n-Hexadecanoic acid.[6] Similar observations have been reported from the methanolic extract of *Phallusia nigra*.[24] Hence, the wound healing activity of *Phallusia arabica* could be attributed to the presence of flavonoids and antioxidant compounds.

**CONCLUSION**

The present study indicated the wound healing nature of ethanolic extract of *Phallusia arabica* on experimental rats. The topical application of *Phallusia arabica* gel significantly accelerated the wound contraction and marked wound closure. It may be concluded that *Phallusia arabica* is very effective on open wounds and a promising drug. Attempts will be made in future to isolate and identify the chemical constituents of the ethanolic extract responsible for the wound healing activity.

**ACKNOWLEDGEMENTS**

The authors thank the University Grants Commission, Hyderabad for Financial assistance (No: F. MRP-4218/12) (MRP/UGC-SERO) and to Dr. S. Sampath Raj, Samsun Clinical Research laboratory, Thirupur for providing assistance to carry out the pharmacological studies.

**REFERENCES**


