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Influence of Flower Head Aqueous Extract of *Sphaeranthus indicus* Linn. on Wound Healing in Albino Rats

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Abstract: The basic objective of the present work was to assess the wound healing activity of *Sphaeranthus indicus* flower head by providing better tissue formation and protection against microbial invasion. Various ointments of aqueous extracts in various proportions were prepared and subjected for assessment of wound healing activity in albino rats. Based on the comparison of wound healing activity of various formulations, the formulation comprising of 6% (w/w) aqueous extract of flower head of *Sphaeranthus indicus* found to be superior to that of control and standard formulation. In addition to greater hydroxyproline content found in healed wounds, the formulation also showed formation of epidermis, keratinization, connective tissue, vascular tissue and collagen at greater level as compared to control and standard formulation.

Key word: *Sphaeranthus indicus* · Hydroxyproline · Ointment · Tensile strength · Epithelization · Aqueous extract · Neomycin

INTRODUCTION

Sphaeranthus indicus Linn belongs to family Asteraceae. The plant is commonly known as Gorakhmundi in Hindi. It is an annual spreading herb, which grows approximately 15-30 cm in height. The plant is distributed through out the plains and wet lands in India, Sri Lanka and Australia. In folk medicine, the plant is reportedly used in treating epileptic convulsions, mental illnesses and hemicranias [1]. The external application of a paste of this herb is beneficial in treating pruritus and edema, arthritis, filariasis, gout and cervical adenopathy [2]. Essential oil, obtained by steam distillation of the whole herb, contains ocimene, α-terpinene, methyl-chavicol, α-citral, geraniol, α-ionone, ß-ionone, d-cadinene, p-methoxycinnamaldehyde [3] and an alkaloid sphaeranthine [4].

Normal wound healing response begins the moment the tissue is injured. The healing cascade begins immediately following injury when the platelets come into contact with exposed collagen. As platelet aggregation proceeds, clotting factors are released resulting in the deposition of a fibrin clot at the site of injury. The fibrin clot serves as a provisional matrix and sets the stage for the subsequent events of healing. The inflammatory cells also arrive along with the platelets at the site of injury and they provide key signals are known as cytokines or growth factors [5]. The fibroblast is the connective tissue cell responsible for collagen deposition that is needed to repair the tissue injury. Collagen is the most abundant protein in the animal kingdom, accounting for 30% of the total protein in the human body [6]. In normal tissues collagen provides strength, integrity and structure. When tissues are disrupted following injury, collagen is needed repair the defect and restores anatomic structure and function. Hence we set out to investigate wound contraction, tensile strength measurement and determination of hydroxyproline content and histopathological studies in rats. There was no previous report on wound healing activities of *Sphaeranthus indicus* in literature to the best of our knowledge and in this paper, we report for the first time, the efficacy of *Sphaeranthus indicus* flower head aqueous extract in the treatment and management of wounds.

MATERIALS AND METHODS

Collection and Identification: *Sphaeranthus indicus* flower head were collected from K.C. Jain traders, Lalitpur and identified from at the Department of Ethnobiology, Jiwaji University, Gwalior.

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Preparation of Extract: The flower head was shade dried, powdered mechanically and sieved by using a mesh (size no. 10/44). It was extracted with distill water in a soxhlet extractor. The concentrated material was reduced to a thick mass at room temperature and water was removed by placing it in a desiccators. The weight of the dried mass was recorded and used for experimental studies [7].

Preparation of Ointments: The general method of preparation of various ointments of aqueous extract was as follows: Dried extract was taken in glass mortar and triturated first. Then small parts of PEG-400 were added with triturating to dissolve or to suspend the drugs. Portions of PEG-6000 (melted at 70°C) were added to above dispersion with triturating to form a homogenous mass of desired consistency [8].

Evaluation of Ointments for Physicochemical Parameters

pH: The pH of all the ointments was determined using digital pH meter. 0.5 g of the weighed formulation was dispersed in 50 ml of distilled water and the pH was noted [9].

Homogeneity: All the developed ointments were tested for homogeneity by visual inspection. They were tested for their appearance with no lumps [10].

Skin Irritation Test: The test for irritation was performed on human volunteers. For each cream, five volunteers were selected and 1 g of weighed formulation was applied on an area of 2 sq. inch to the back of the hand and covered with cotton. The volunteers were asked to report after 24 hours to observe for any reaction or irritation [10].

Evaluation of Wound Healing Activity of Various Prepared Formulation

Experimental Animals: Male Albino rats of wistar strain (150-250 g) were housed under standard conditions of temperature, 12 hour light / dark and fed with standard pellet diet and water *ad libitum*. Animals were acclimatized to laboratory conditions at least 24 hours before conducting the experiments (CPCSEA Registration No.-915/ac/05/CPCSEA).

Wound Models

Excision Wound Model

Wound Contraction Studies: A circular piece (300 mm^2 in area) of full thickness skin was excised from the dorsal interscapular region [11]. Wound contractions were monitored by measuring wound area, on alternate days till the wound were completely healed. To have uniform parameters for comparison of the effects of different drugs was calculated by Litchfield and Wileoxon method [12]. The time taken for epithelialization was measured in days required for full epithelialization was indicated by fall of scale leaving no raw wound behind. The progressive changes in wound area are monitored planimetrically by tracing the wound margin on graph. To determine the changes in healing of wound measurement of wound area on graph paper is expressed as unit (mm^2) [13].

Resutured Incisional Wound Model: Incision wound were inflicted by the method of Ehrlich and Hunt [14]. Groups of animals containing six in each group are anaesthetized and two paravertebral long incisions of 2.5 cm length are made through the skin and cutaneous muscles at a distance of about 1.5 cm from midline on each side of the depilated back of rat. After mopping the wound dry, intermittent sutures were applied by surgical nylon thread and curved needle No.11, 0.5 cm apart. On the 8^{th} day sutures were removed and on 10^{th} day, the tensile strength was measured by the method of Lee [15].

Tensile Strength Measurement: Tensile strength (the force required to open the healing skin) was used to measure the extent of healing. The model used for this purpose consists of wooden board with a pulley that was fixed in one side of edge of board. Two Allis forceps, one is fixed to the opposite side of pulley edge and another is tied with and hanged with rope that is attached to the pan through pulley on which the weights are placed. The weights are increased slowly till it breaks the healed wound. One day before performing this experiment the sutures are removed from the stitched wound of rats after recovery [16].

Determination of Hydroxyproline Content in Granular Tissue by Colorimetry: Hydroxyproline is an amino acid present in the collagen fibers of granulation tissue. Its estimation helps clinically to understand progress rate at which the healing process is going on in the connective tissue of the wound. The hydroxyproline contents of the granulation tissue were calculated from standard curve [17].

Histopathological Studies: On the 10^{th} post wounding day, small pieces of skin were excised from the rats under light ether anesthesia in such a way that each piece represented the skin surrounding the incision originally made. The sections of the skin were stained with eosin

and hemotoxylin and were examined microscopically for keratinization, epithelization, fibrosis, collagenation and neovascularizaion [18].

Treatments: First group (Group I) was topically treated with Neomycin ointment (F_1), Second group (Group II) remained untreated that acted as diabetic control (F_2); third group was treated with 2% aqueous extract (F_3), fourth group was treated with 4% aqueous extract (F_4) and fifth group was treated with 6% aqueous extract (F_5).

Statistical Analysis: The data was statistically analyzed by one-way ANOVA followed by Dennett multiple comparison test with equal sample size. The difference was considered significant when $p<0.001$. All the values were expressed as mean ± standard deviation (S.D.).

RESULTS

There is a report that *Sphaeranthus indicus* flower head extracts possesses excellent wound healing property. The wound healing property of *Sphaeranthus indicus* extracts are presumably because of its constituents promote cell division and therefore facilitates the healing of wound.

Selection of topical base was important to prepare topical formulations with optimum flow, spreadability and release properties. All the developed ointments were stored in tightly closed containers and evaluated for physical characteristics such as pH, Homogeneity, Spreadability and skin irritation test (Table 1).

Table 2 records the reduction of wound area of different groups over the period of 16 days. It was observed that fastest healing of wound took place in the group of animals treated with F_5 formulation i.e. wound were cured within 10 days. Treatment with the standard formulation (SF) was also found satisfactory but the rate of healing was comparatively slower than the formulation of herbal extracts.

The tensile strength of the healed skin treated with different formulation for 10 days. From the results, it is observed that the wounds treated with the test formulation show increase in tensile strength compared to untreated control group and standard (Table 3). During the healing of wound, collagen is synthesized which is one of the constituents of growing cell. Constituents of hydroxyproline are a measure of concentration of collagen. Higher concentration of hydroxyproline indicates faster rate of wound healing. Table 4 records the concentration of hydroxyproline in the tissue of animals,

Table 1: Evaluation data of developed ointment

Sr. No.	Group models and formulations	pH	Homogeneity	Skin irritation
1.	Group-I (F_1)	6.28	Good	x
2.	Group-II (F_2)	6.86	Good	x
3.	Group-IX (F_3)	6.58	Good	x
4.	Group-X (F_4)	6.89	Good	x
5.	Group-XI (F_5)	6.60	Good	x

x = Indicates ointment does not produce any irritation.

Table 2: Records the wound area (mm^2) of different groups over a period of 16 days

Post Wounding Days	Group I (F_1)	Group II (F_2)	Group III (F_3)	Group IV (F_4)	Group V (F_5)
0 Day	304.42± 2.6(O)	300.4 ± 4.6(0)	284.4 ± 5.1(0)	315.1 ± 6.3(0)	226.8 ± 3.2(0)
2 Day	290.3 ± 1.9(4.6)	285.5 ± 3.6(4.9)	160.6± 4.2*(43.5)	162.4± 2.8*(48.4)	111.8±1.8*(50.7)
4 Day	266.2± 3.0(12.5)	255.6± 3.2(14.9)	144.9± 1.4* (49.03)	64.8± 1.3*(79.4)	57.2±1.06*(74.8)
6 Day	176.1± 3.7*(42.1)	222.2± 2.8(26.03)	63.9± 0.5*(77.5)	24.4± 0.6*(92.25)	16.5±0.2*(92.71)
8 Day	93.7± 1.7*(69.2)	177.2± 3.2(41.02)	28.8± 0.4*(89.8)	12.2± 0.5*(96.1)	3.4±0.2*(98.5)
10 Day	37.9± 0.8*(87.52)	13.4± 1.5(55.2)	18.0± 0.4*(93.7)	5.7± 0.2*(98.2)	0.2±0.12*(99.9)
12 Day	18.08± 0.46*(93.8)	97.1± 0.7(67.7)	10.08± 0.26*(96.4)	0.25± 0.08*(99.9)	0.0±0.0*(100.0)
14 Day	0.0± 0.0*(100.0)	61.2± 0.8(79.6)	4.3± 0.2*(98.5)	0.0± 0.0*(100.0)	-
16 Day	-	41.9± 0.8(86.05)	1.95± 0.05*(99.3)	-	-

Values are Mean ± S.D. of six animals in each group. * $p<0.001$ as compared to control. The values shown in ( ) are the % reduction of wound area.

Table: 3 Indicate Tensile strength value in healed tissue

S. No.	Group Models	Tensile strength of skin (g)
1.	Group -I (F_1)	418.4 ± 6.4*
2.	Group -II (F_2)	266.6 ± 2.6
3.	Group -III (F_3)	357.68 ± 10.8*
4.	Group -IV (F_4)	437.56 ± 7.9*
5.	Group-V (F_5)	494.65 ± 16.5*

Values are Mean ± S.D. of six animals in each group. *$p<0.001$ as compared to control.

Table 4: Indicate Hydroxyproline value in healed tissue

S. No.	Group Models	Hydroxyproline (µg/g)
1.	Group -I (F_1)	604.8 ± 4.6*
2.	Group -II (F_2)	124.6 ± 2.8
3.	Group -III (F_3)	665.8 ± 6.6*
4.	Group -IV (F_4)	794.13 ± 3.4*
5.	Group-V (F_5)	886.45 ± 4.5*

Values are Mean ± S.D. of six animals in each group. *p<0.001 as compared to control

Table 5: Histopathological evaluation of healed wounds at end of 10 days

Sr. No.	Parameters	Group I(F_1)	Group II(F_2)	Group III(F_3)	Group IV(F_4)	Group V(F_5)
1.	Keratinization	4.56 ± 1.2	4.14 ± 0.6	4.43 ±0.6	4.55 ± 0.55	4.73 ± 1.5
2.	Epithelization	4.14 ± 0.6	1.52 ± 1.6	4.45 ± 1.1	4.66 ± 0.25	4.86 ± 1.8
3.	Fibrosis	4.24 ± 0.6	2.42 ± 1.2	4.28 ± 0.9	4.46 ± 0.80	4.70 ± 0.9
4.	Collagenation	4.84 ± 0.6	2.80 ± 0.5	4.22 ± 0.5	4.38 ± 0.30	4.48 ± 0.4
5.	Neovascularizaion	4.42 ± 1.14	0.88 ± 0.6	3.03 ± 0.2	3.41 ± 0.9	3.65 ± 0.7

Values are mean ± SD from 6 readings each. A value 5 refers to maximum similarity and 0 refers for least similarity of wound from the normal tissue. All values are significant at P<0.001.

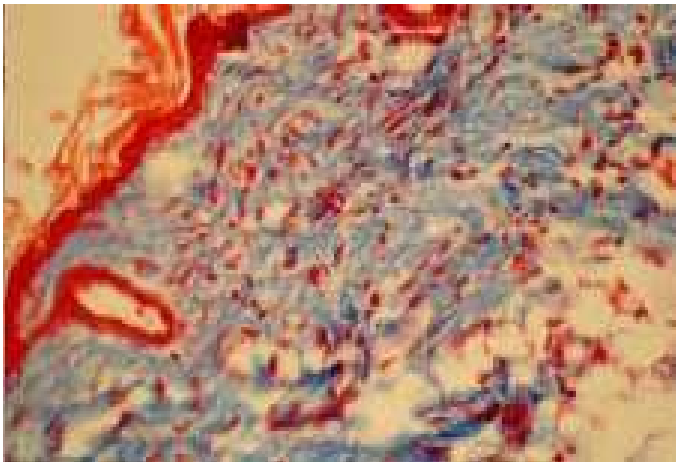

Fig. 1: Photomicrograph of the tissue removed on 10th day from animals, which were treated with standard formulation (F_1). In this section proliferation of epithelial tissue covering the wound area was seen. In the dermis area multiplication fibrous connective tissue was also seen, but union was not perfect, section revealed complete healing; however keratinization was poor in dermal layer.

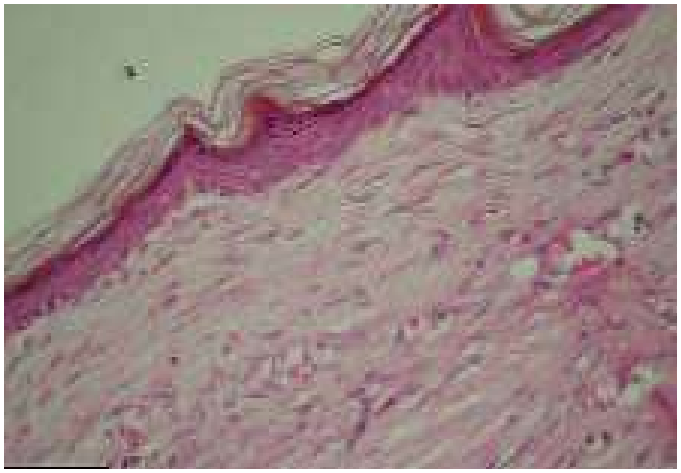

Fig. 2: Photomicrograph of the section of tissue removed on 10th day from animals, which received no treatment (F_2). In this section proliferation of epithelial tissue covering the wound area was seen. In the dermis area multiplication fibrous connective tissue was also seen, but union was not perfect, dermis was without keratin layer.

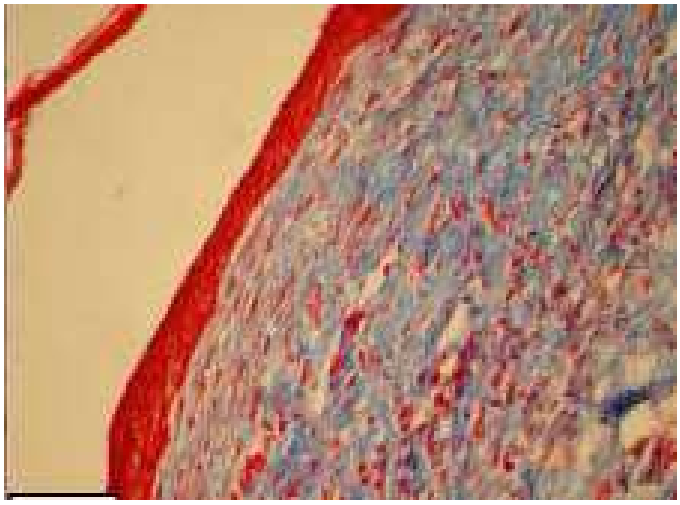

Fig. 3: Photomicrograph of tissues removed on 10th day from animals cured with 6% aqueous extract (F_5). The epidermal tissue of normal thickness and well-formed keratin was noticed. Scattered inflammatory cells were also present. Normal vascular tissue was observed. In the dermis area multiplication fibrous connective tissue was also seen.

which were treated with different formulation up to 10 days. Highest concentration of hydroxyproline (886.4 μg / g) was observed in the group of animals treated with F_5. Table 5 records the histopathological parameters evaluation and Figure 1-3 showed better keratinization, epithelization, collagenation and fibrosis of the test formulations of aqueous extract of *Sphaeranthus indicus* as compared with control and standard formulation. However neovascularizaion was not very prominent when compared with untreated control and standard.

DISCUSSION

Proper and timely wound healing is a vexing problem faced by all clinicians. In majority of patients normal healing established tissue integrity quickly and effectively. However at times this healing is delayed and the ability to accelerate the wound healing becomes a highly desirable objective [19]. Wound healing involves a highly dynamic integrated series of cellular, physiological and biochemical processes, which occur in living organism. Repair through regeneration is very common in unicellular and the lower metazoan animal groups while it is highly restricted in the higher animals [20]. In excision wound study the test formulation of *Sphaeranthus indicus* showed better and fast healing compared to untreated control group. The *Sphaeranthus indicus* treated group showed much greater contraction of wounds than those treated with neomycin 0.3% w/w as the reference standard. In incision wound study, increase in tensile strength is indicative of improved collagenation, which significantly contributes to better and effective healing with *Sphaeranthus indicus* formulations. The histopathological observation revealed better keratinization in *Sphaeranthus indicus* extract formulation. Epithelization improved with test formulation application that may be due to proliferation of epithelial tissue over wound area.

It may be concluded that *Sphaeranthus indicus* extract formulation promotes keratinization, epithelization and fibrosis comparable with neomycin treatment. Interestingly the visual examination of wounds inflected during “wound healing ability” experiments revealed that the wounds treated with *Sphaeranthus indicus* aqueous extracts were relatively clean and free from any inflammatory reaction like swelling and redness. This offers a very interesting dimension to treatment of wounds by *Sphaeranthus indicus* extracts.

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