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Evaluation of Flower Head Alcoholic Extract of *Sphaeranthus indicus* Linn. On Wound Healing in Diabetic Albino Rats

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ABSTRACT
The basic objective of the present work was to assess the wound healing activity of alcoholic extract of *Sphaeranthus indicus* flower head by providing better tissue formation and protection against microbial invasion. The flower heads of *Sphaeranthus indicus* were subjected for extraction with ethanol. Various ointments of extracts in various proportions were prepared and subjected for assessment of wound healing activity using four parameters i.e., Wound Contraction Studies (Excision Wound), Tensile Strength Measurement (Incision Wound), Hydroxyproline Content Determination (Incision Wound) and Histopathological Studies (Incision Wound).
Based on the comparison of wound healing activity of various formulations, the formulation comprising of 2 % (w/w) alcoholic extract found to be superior to that of control and standard formulation (0.3 % w/w Neomycin standard ointment). The present studies evidenced the significant wound healing activity of *Sphaeranthus indicus* by increasing cellular proliferation, formation of granulation tissue, synthesis of collagen and by increase in the rate of wound contraction.

Keywords: *Sphaeranthus indicus,* Wound healing, Extract, Ointment, Hydroxyproline content, Histopathological Studies, wound contraction, Tensile Strength.

INTRODUCTION

Despite the advances made in orthodox medicine, there has been a global resurgence of interest in traditional systems of medicine over past few years and used in medical practice since antiquity. [1] *Sphaeranthus Indicus* is an aromatic and medicinal herb, 30-60 cm tall, locally known as "Mundi". It is found abundantly in damp situations in the plains of India and is one of the ingredients in Unani medicines. As per Ayurveda, all parts of the plant are medicinally important. [2] It is used to treat vitiated conditions of hemicranias, jaundice, hepatopathy, diabetes, leprosy, fever, pectoralgia, cough, gastropathy, hernia, hemorrhoids, helminthiasis, dyspepsia and skin diseases. The external application of a paste of this herb is beneficial in treating pruritus and edema, arthritis, filariasis, gout and cervical adenopathy. It also treats piles and hepatitis. [3] The alcoholic extract of powdered flower head contains stigmasterol, ß-sitosterol, hentriacontane, sesquiterpene lactone, sesquiterpine glycoside, sphaeranthanolide, flavones and isoflavone glycosides. Recently, many medicinal properties have been attributed to the extracts, fractions and isolated constituents of *S. indicus* flowers, which include hypotensive, peripheral vasodilator and cathartic activity of alcoholic extract, and antimicrobial activity of alkaloidal and nonalkaloidal fractions of alcoholic extract. [4]

Wound may be defined as loss or breaking of cellular and anatomic or functional continuity of living tissues. Normal wound healing response begins the moment the tissue is injured. 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. [5] The alcoholic extract of the plant is traditionally important and have been used by traditional practioners in Bundelkhand region of India for its healing property. Keeping this in view it was worthwhile considered to investigate further for its traditionally claimed wound healing property.

MATERIAL AND METHOD

Collection & identification: *Sphaeranthus indicus* flower head were collected from K.C. Jain traders, Lalitpur and identified from Prof. A.K. Jain, Director, and Deptt. Of Ethnobiology, Jiwaji University, Gwalior.

Preparation of extract: The flower head were shade dried, powdered mechanically, and sieved by using a mesh (size no.

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10/44). It was extracted with ethanol (90 %) 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 desiccator. The weight of the dried mass was recorded and used for experimental studies. [6]

Preparation of ointments: The general method of preparation was as follows: Dried extract was taken in glass mortar and triturated first. Then small parts of PEG-400 were added with trituration to dissolve or to suspend the drugs. Portions of PEG-6000 (melted at 70˚C) were added to above dispersion with trituration to form a homogenous mass of desired consistency tested on alloxanised hyperglycaemic animals. [7]

Experimental animals: Male Albino rats of wistar strain (150-250 g) were housed under standard conditions of temperature, 12 h 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).

Alloxan induced Diabetes: Diabetes was induced by a single IP injection of 120mg/kg of alloxan monohydrate in a sterile saline. After 72 h of alloxan injection the diabetic rats (blood glucose level> 250mg/dl) were separated and used for study. [8]

Wound models:

Excision wound model: A circular piece (300 mm^2 in area) of full thickness skin was excised from the dorsal interscapular region. Wound contraction was monitored by measuring wound area, on alternate days till the wound were completely healed. 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). [9]

Wound contraction studies: The skin of the impressed area on the depilated back of each rat was excised to the full thickness under light ether anesthesia to obtain a circular wound area about 300 mm^2. Measuring wound area that was traced on transparent polythene paper monitored wound contraction. Later the wound area was assessed using a graph paper. Wound contraction was also expressed as the percentage decrease of original wound size (about 300mm^2) on every alternate day. [10]

Resutured incisional wound model: Incision wound was inflicted by the method of Ehrlich and Hunt. [11] Groups of animals containing six in each group were anaesthetized and two paravertebral long incisions of 2.5 cm length were 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 8th day sutures were removed and on 10th day, the tensile strength was measured by the method of Lee. [12] The average of six readings per animal of a group was taken as mean and SE was calculated.

Tensile strength measurement: Tensile strength of wound represents the promotion of wound healing. 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. [13]

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 neovascularization. [14]

Determination of Hydroxyproline content 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. [15-17]

Treatments: Rats were divided into five groups, of six rats each. First group (Group I) was topically treated with Neomycin ointment (F1), Second group (Group II) remained untreated that acted as control (F2); Third group was treated with 2 % alcoholic extract (F3), Fourth group was treated with 4 % alcoholic extract (F4) and Fifth group was treated with 6 % alcoholic extract (F5).

Statistical analysis: The data was statistically analyzed by one-way ANOVA followed by Dunnett 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* extracts possesses excellent wound healing property. The wound healing property of *sphaeranthus indicus* extracts are presumably because of its constituents, which promote cell division and therefore facilitate the healing of wound. Thus process of wound healing has two components one, formation of new tissue and two, protection from microbial invasion during the healing process.

Table 1: Records the wound area (mm^2) of different groups over a period of 16 days

Post Wounding Days	Group I	Group II	Group III	Group IV	Group V
0 Day	296.55± 1.8 (0)	288.8 ± 2.2 (0)	310.6 ± 9.2 (0)	267.5 ± 6.6 (0)	324.3 ± 0.8 (0)
2 Day	282.3 ± 2.5 (4.6)	265.3 ± 5.5 (8.1)	245.4 ± 3.7* (20.9)	211.2 ± 5.2* (21.1)	276.6 ± 6.2* (14.7)
4 Day	245.2± 1.4 (17.3)	221.4 ± 3.6 (23.4)	187.8 ± 6.1* (39.5)	191.7± 0.6* (28.4)	238.6 ± 4.6* (26.4)
6 Day	195.4± 5.6* (34.1)	199.6 ± 4.5 (30.9)	131.4 ± 5.4* (57.7)	149.2 ± 3.8* (44.2)	197.5 ± 2.6* (39.1)
8 Day	113.6± 3.9* (61.7)	156.5 ± 6.7 (45.8)	86.0 ± 6.2* (72.3)	101.6 ± 1.4* (62.1)	111.4 ± 1.9* (65.6)
10 Day	88.5± 1.0* (70.2)	98.7 ± 4.9 (65.8)	16.6 ± 5.8* (94.6)	73.6 ± 4.4* (72.5)	87.6 ± 4.6* (73.0)
12 Day	47.4± 3.2* (84.1)	77.8 ± 1.5 (70.6)	5.6 ± 1.2* (98.2)	26.5 ± 5.6* (90.1)	34.8 ± 4.3* (89.3)
14 Day	10.2± 2.5* (96.6)	59.4 ± 2.5 (79.4)	0.0± 0.0* (100)	7.7 ± 0.4* (97.1)	8.8 ± 3.3* (97.3)
16 Day	0.0± 0.0* (100)	52.4 ± 4.0 (81.9)	-	0.0± 0.0* (100)	1.2 ± 2.2* (99.6)

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 2: Tensile strength value of healed tissue.

S. No.	Group Models	Tensile strength of skin (μg)
1	Group -I (F_1)	376.5 ± 2.8*
2	Group -II (F_2)	294.6 ± 6.8
3	Group -III (F_3)	524.4 ± 1.4*
4	Group -IV (F_4)	410.6 ± 5.8*
5	Group -V (F_5)	385.2 ± 4.2*

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

Table 3: Hydroxyproline value of healed tissue

S. No.	Group Models	Hydroxyproline (μg/g)
1	Group -I (F_1)	515.6 ± 4.4*
2	Group -II (F_2)	178.4 ± 2.2
3	Group -III (F_3)	752.4 ± 0.8*
4	Group -IV (F_4)	698.2 ± 2.6*
5	Group -V (F_5)	551.1 ± 6.8*

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

Table 4: Histopathological evaluation of wounds at end of 10 days

Parameters	Group I	Group II	Group III	Group IV	Group V
Keratinization	4.21 ± 0.6	4.36 ± 0.9	4.68 ± 0.8	4.60 ± 1.4	4.41 ± 0.7
Epithelization	4.38 ± 0.3	1.78 ± 1.4	4.78 ± 0.45	4.71 ± 0.3	4.63 ± 0.4
Fibrosis	4.25 ± 0.8	2.50 ± 0.6	4.60 ± 0.5	4.50 ± 0.5	4.35 ± 1.9
Collagenation	4.55 ± 0.5	3.00 ± 0.7	3.46 ± 0.7	4.35 ± 0.2	4.28 ± 2.2
Neovascularization	4.30 ± 0.25	0.70 ± 0.8	3.53 ± 0.7	3.31 ± 0.6	3.06 ± 1.8

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.

Wound contraction indicates the rate of reduction of unhealed area during the course of treatment. Greater the reduction better is the efficacy of medication. Table 1 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 F3 formulation i.e. wound were cured within 12 days. Treatment with the standard formulation (F1) was also found satisfactory but the rate of healing was comparatively slower than the formulation of herbal extracts.

Table 2 comprises 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 thus promoting wound healing. 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 3 records the concentration of hydroxyproline in the tissue of animals, which were treated with different formulation up to 10 days. In the histopathological study, healed tissues were observed for the healing markers like neovascularization, keratinization, collagenation, epithelization and fibrosis. The test formulation showed better keratinization, epithelization, collagenation and fibrosis. However neovascularizaion was not very prominent when compared with untreated control. The results are shown in Table 4 and Figure 4.

DISCUSSION

Wounds may be defined as loss or breaking of cellular and

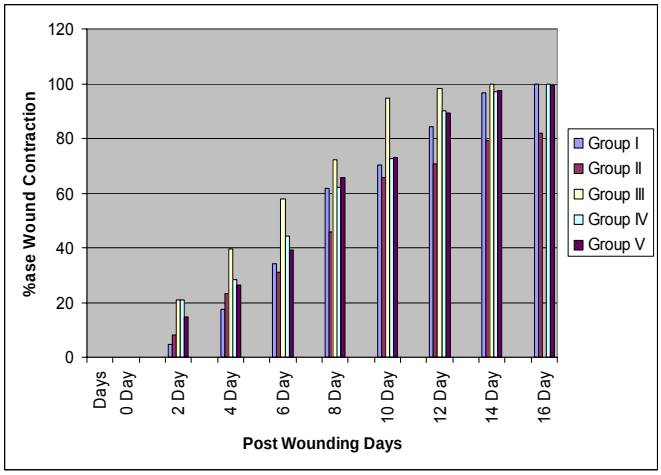

Fig. 1: %ase Wound contraction of excision wound of different groups

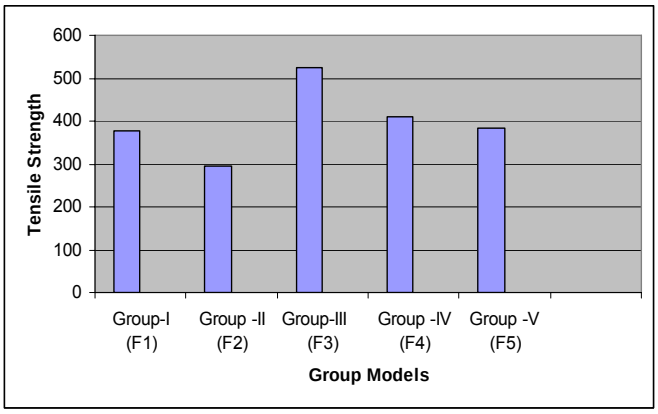

Fig. 2: Tensile strength of different groups

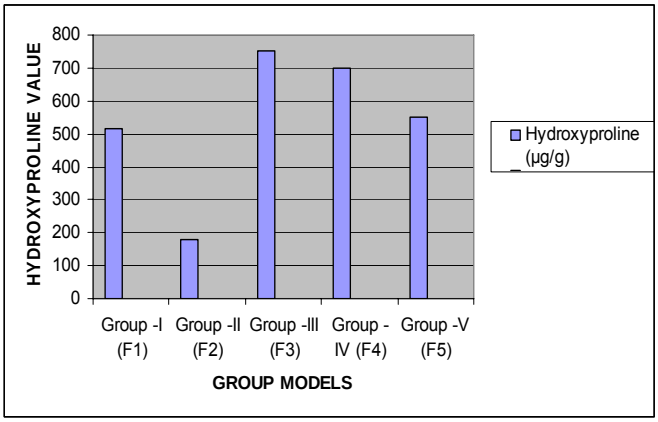

Fig. 3: Hydroxyproline values of different groups of healed tissues

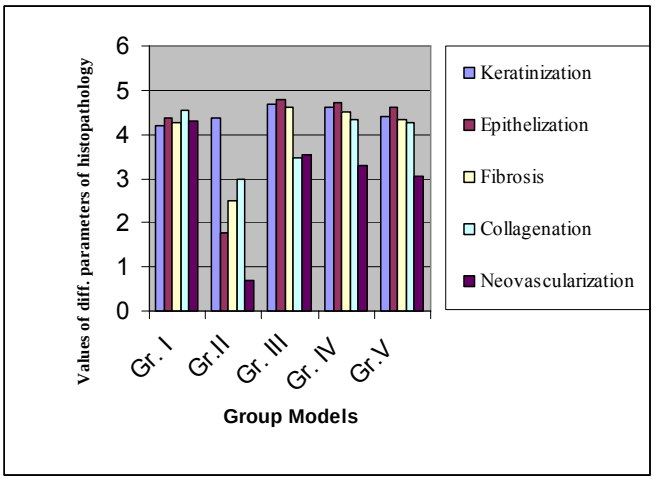

Fig. 4: Histopathological parameters values of different groups of healed tissues

anatomic or functional continuity of living tissues. [18] 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. [19] Wound healing involves different phases such as contraction, epithelization, granulation, collagenation. [20]

In excision wound study the test formulation of *sphaeranthus indicus* showed better and fast healing compared to untreated control group. The wound contraction ability of *sphaeranthus indicus* was so prominent initially but progressively the contraction ability of *sphaeranthus indicus* was slowed. The *sphaeranthus indicus* treated group showed much greater contraction of wounds than those treated with neomycin 0.3 % w/w as the reference standard.

Increase in tensile strength is indicative of improved collagenation, which significantly contributes to better and effective healing. Increase in hydroxyprolin content is indicative of improved collagen, which significantly contributes to better and effective healing.

The histopathological observation revealed better keratinization in *sphaeranthus indicus* extract formulation treated animals when compared with control group. Epithelization improved with test formulation application when compared with control group and Neomycin treatment that may be due to proliferation of epithelial tissue over wound area.

Interestingly the visual examination of wounds inflected during "wound healing ability" experiments revealed that the wounds treated with *sphaeranthus indicus* extracts were relatively clean and free from any inflammatory reaction like swelling and redness. Consequently it was observed that test formulation does exert remarkable anti-inflammatory action when applied to wounds. This offers a very interesting dimension to treatment of wounds by *sphaeranthus indicus* extracts.

In present investigation it was concluded that formulation F3, F4 and F5 are stable and promoted significant wound healing activity by increasing cellular proliferation, formation of granular tissue, synthesis of collagen and by increase in the rate of wound contraction as compared to control and standard formulations.

Further studies are needed to confirm whether the results are reproducible which may help to explain some of the ways in which *sphaeranthus indicus* promotes wound healing.

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