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Wound Healing Activity of Ethanolic Extract Of *Hemidesmus Indicus* (Linn) R.Br Leaves In Rats

M Mohammed Moideen*, Ruby Varghese, E Krishna Kumar, CK Dhanapal

Azath street, Ulundurpet, Villupuram district-606107, Tamil Nadu, India

ABSTRACT

The study was carried out to evaluate the wound healing activity of ethanolic extract of *Hemidesmus indicus* leaves in rats. Twenty four healthy animals of Wister strain were assigned to four groups, containing six animals each. Group 1 (positive control) was provided with nitrofurantoin ointment, group 2 (solvent control) with 70% ethanol, group 3 (test dose I) with 5% w/w *Hemidesmus indicus* ointment and group 4 (test dose II) with 10% w/w *Hemidesmus indicus* ointment. The total exposure of the study was 16 days. The groups were compared for the percentage of wound healing. It was observed that the group treated with nitrofurantoin ointment showed an increase in the rate and percentage of wound contraction and period of epithelization compared to the ethanol treated group. The alcoholic extract of *Hemidesmus indicus* (5% and 10% ointment) increased rate of wound contraction and period of epithelization than solvent and control groups. Comparison between test dose I and test dose II showed that the percentage and rate of wound healing was increased at test dose I than test dose II. The study evaluated the wound healing activity of the alcoholic extract of *Hemidesmus indicus* and identified it to possess significant wound healing activity.

Keywords: Hemidesmus indicus, wound healing, epithelization, ethanolic extract, positive control, solvent control.

Corresponding author

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INTRODUCTION

The history of wound care spans from prehistory to modern medicine. As wounds naturally heal by themselves, regardless of whether recovery from the scar or recovery from lost body tissue was a possibility, hunter-gatherers would have noticed several factors and certain herbal remedies which would speed up or assist the process, especially if it was grievous. In ancient history, this was followed by the realization of the necessity of hygiene and the halting of bleeding, where wound dressing techniques and surgery developed.

Tracing its origins to ancient Egypt and Greece, the treatment of acute and chronic wounds is an ancient area of specialization in medical practice, with a long and eventful clinical history. The Ebers papyrus, circa 1500 BC details the use of lint, animal grease and honey as topical treatments for wounds. As noted in the Berlin papyrus, the Egyptians believed that closing a wound preserved the soul and prevented the exposure of the spirit to infernal beings. The Greeks, who had a similar perspective on the importance of wound closure were the first to differentiate between acute and chronic wounds, calling them 'fresh' and 'non-healing', respectively [1]. Galen of Pergamum, a Greek surgeon who served Roman gladiators, circa 120-201 AD, made many contributions to the field of wound care [2]. There were limited advances that continued throughout Middle Ages and the Renaissance but the most profound advances, both technological and clinical, came with the development of microbiology and cellular pathology in the 19th century.

Hemidesmus indicus Linn. (Family: Apocynaceae) [3], commonly referred to as Indian sarsaparilla, Anantamool or Nannari is a commonly available perennial climbing plant, used as the main ingredient in the preparation of the cool and refreshing drink Nannari sherbat. It is a native of India and also found in south tropical Asian countries such as Pakistan and Sri Lanka [4].

The plant is well known for its antioxidant and anti-inflammatory activity [5]. Other uses of the plant are against syphilis, leucorrhoea, bronchitis, chronic rheumatism, urinary diseases, leprosy, leucoderma and as purgative, diaphoretic, diuretic, antipyretic and antidiarrheal [6,7]. It has also been used in combination with other drugs for snake bite [8]. The plant is used in traditional medicine for biliousness, respiratory disorders, eye diseases, epileptic fits in children, kidney and urinary disorders, loss of appetite and burning sensation [9, 10]. The major chemical constituents are coumarin, hemidesmine, hemidine, hemidesine and rutin [11]. In the present study, leaves of *Hemidesmus indicus* were selected to assess the wound healing activity.

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MATERIALS AND METHODS

PLANT MATERIAL

Plant collection and authentication

The leaves of *Hemidesmus indicus Linn.* (4 kg) were collected from Cuddalore district of Tamil Nadu in the months of April – May. The plant was identified and authenticated by Prof. P. Jayaraman, Ph.D., Director, Plant Anatomy Research Centre (PARC), Tambaram, Chennai and a voucher specimen (PARC/2009/318) was deposited at the Pharmacognosy institute.

Extraction of plant material

The coarse dried powder of leaves of *Hemidesmus indicus* was used for the extraction procedure. The coarse powder of shade dried leaves of *Hemidesmus indicus* (500g) was extracted with 2000 ml of ethanol by cold maceration method in a narrow mouthed bottle with occasional shaking for four days. It was filtered and solvent was removed by distillation under reduced pressure. The residue was then weighed and yield was recorded. The yield of ethanolic extract of *Hemidesmus indicus* seeds was found to be 18.7%.

Preliminary phytochemical screening was conducted which included test for phenol, flavanoids, flavones, alkaloids, lignins, glycosides, tannins, steroids and saponins.

Formulation of Drug

Hard paraffin (5g) and cetosteryl alcohol (5g) were melted on a water bath. To this wool fat (5g) and white soft paraffin (85g) were incorporated and stirred until all the ingredients were melted. The contents were examined for any foreign particles. It was then decanted and stirred until the mixture was thoroughly cold. Two types of drug formulations were prepared from the isolated compound. For topical administration, 5% w/w and 10% w/w were prepared with the use of simple ointment base by spatulation method.

Experimental Animals

Adult rats of both strains (Albino and Wister) of either sex weighing 150-250 gms were obtained from the animal house. Clearance to carry out the work was obtained from the Institutional Animal Ethical Committee bearing no. 991/C/06/CPCSEA. The animals were placed in a controlled room, in which temperatures were maintained at 25±3ºC and 35-60% humidity. Normal rat feed and water was provided at regular intervals. All the animals were housed in polypropylene cages having a measurement of 43×27×15 cm. The animals were acclimatized to laboratory conditions before experimental procedures were started.

Acute oral toxicity studies were conducted using the acute toxic class method. The procedure was followed by using OECD 423 guidelines. The method used defined doses (5, 50, 300, 2000 mg/kg body weight) and the results allowed the substances to be ranked and classified according to the Globally Harmonized System (GHS) for the classification of chemical which causes acute toxicity. From the LD_{50} determination, $1/10^{th}$ of the dose was focused as the medial for pharmacological screening.

Administration of dose

The test substance was administered in a single dose by gavages using a stomach tube or a suitable intubation canula. In the unusual circumstance that a single dose was not possible, the dose was given in smaller fractions over a period not exceeding 24 hours. Animals were fasted prior to dosing but water was provided ad libitum. Following the period of fasting, the animals were weighed and the test substance administered. The dose level to be used as the starting dose was selected from one of four fixed levels 5, 50, 300 and 2000 mg/kg body weight.

Experimental design

Table 1. Treatment schedule

Group	Category	Drug administered
1	Positive control	Nitrofurantoin ointment
2	Solvent control	70% v/v ethanol
3	Test (Dose 1)	5% w/w *Hemidesmus indicus* ointment
4	Test (Dose 2)	10% w/w *Hemidesmus indicus* ointment

Twenty four healthy animals of Wister strain were assigned to four groups. Six animals were randomly selected to one solvent control group that are treated with 70% ethanol. Eighteen animals were randomly selected to three experimental groups of six animals. Since 2000 mg/kg of ethanolic extract of *Hemidesmus indicus* did not produce any toxicity, $1/10^{th}$ of the dose was fixed. The extract was assigned in two dose concentration levels (5% and 10% ointment) as shown in Table 1. Total exposure of the study was 16 days. All of the drugs and extracts were applied topically in an ointment base. The ointment base selected was simple ointment.

Method of creation of wound

Animals were thoroughly washed and dried and the hair was removed by using an electric shaver. Then they were transferred to the aseptic area to create the wound. Excision wounds were made as described by Morton and Malone by excising the full thickness circular skin (approx. 500 mm^2) from the nape of the neck under ether anesthesia. Wound closure rate and epithelization time were assessed by tracing the wound on polythene sheet from wounding day, followed on 2, 4, 8, 12, 14^{th} days and subsequently on alternate days till complete epithelization. Similarly, scars were traced on complete epithelization to assess wound contraction by noting scar size and shape.

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Evaluation of haematological parameters such as RBC, WBC and haemoglobin content was performed. The percentage of wound healing was calculated by using the formula

Percentage of wound contraction = healed area/total area × 100
(Healed area = original wound area – present wound area)

The present wound area and total wound area were measured from the tracings that had been done. The percentage of wound contraction helped to evaluate the rate of wound contraction and the period of epithelization. Histopathological examination was conducted wherein the skin of the rats were excised and stored in a small container containing 10% formalin solution and sent to the pathologist. The above mentioned parameters were analyzed using one-way ANOVA followed by Dunnette's multiple comparison test.

RESULTS

Extraction of the plant material *Hemidesmus indicus* leaves using ethanol by cold maceration method gave a yield value of 18.7%.

Table 2. Qualitative analysis of ethanolic extract of *Hemidesmus indicus*

S.No.	Phytochemical screening	Ethanolic extract of *Hemidesmus indicus*
1	Phenol	+
2	Flavanoids	+++
3	Alkaloids	+
4	Coumarin	—
5	Glycosides	+
6	Tannins	+
7	Fixed oil &fats	—
8	Lignins	+
9	Steroids	+
10	Proteins & Free amino acids	—
11	Gums&Mucilage	—
12	Saponins	+

+ indicates presence of constituent, - indicates absence

Phytochemical screening of the ethanolic extract of *Hemidesmus indicus* revealed that phenols, flavanoids, alkaloids, glycosides, tannins, lignins, steroids and saponins were present in the extract whereas constituents like coumarin, fixed oils and fats, proteins and free amino acids, gums and mucilage were found to be absent (Table 2).

Acute toxicity studies were performed with three animals and the observations seen after the studies were:

a) Maximum of 2000 mg/kg body weight of dose was given.
b) At the maximum dose level no mortality rate was estimated.

ISSN: 0975-8585

c) Depending on the LD_{50} determination, $1/10^{th}$ of the dose was fixed as the medial dose for pharmacological screening on animals.

Table 3: Functional observatory parameters

Parameter	Animal no. 1	Animal no. 2	Animal no. 3
Eye ball movement	+	+	+
Salivation	+	_	_
Muscle strength	+	+	+
Diarrhoea	_	_	_
Frequent urination	+	+	+
Fear	_	_	_
Body temperature	Optimum	Optimum	Optimum
Respiration	Normal	Normal	Normal
Vagueness	_	+	_
Hyperactivity	+	+	_
Insomnia	_	_	+
Nasal discharge	+	_	_

(+) indicates presence, (-) indicates absence

Some of the functional observatory parameters that were observed in the dose level of 2000 mg/kg body weight are indicated in Table 3. In all the three animals, body temperature and respiration was found to be optimum and normal, respectively.
The percentage of wound contraction was calculated by using the formula

Percentage of wound contraction = healed area/total area × 100
(Healed area = original wound area – present wound area)

Table 4: Percentage of wound contraction

Group	Day 0 (%)	Day 2 (%)	Day 4 (%)	Day 6 (%)	Day 8 (%)	Day 10 (%)	Day 12 (%)	Day 14 (%)	Day 16 (%)
Positive group	0	15.867 ± 2.009	40.267 ± 3.517	56.362 ± 2.574	71.283 ± 1.528	80.568 ± 1.093	85.667 ± 1.014	92.182 ± 0.625	95.152 ± 0.253
Solvent group	0	32.533 ± 1.314	38.900 ± 1.135	43.438 ± 0.848	57.883 ± 4.811	80.215 ± 1.839	85.750 ± 1.896	92.233 ± 0.526	95.361 ± 0.162
Test dose I	0	25.317 ± 0.581	40.333 ± 1.976	54.417 ± 1.468	81.860 ± 0.850	85.150 ± 1.005	89.983 ± 1.060	95.117 ± 0.946	98.812 ± 0.583
Test dose II	0	14.683 ± 1.360	30.433 ± 3.380	43.712 ± 1.517	60.400 ± 2.707	68.550 ± 2.497	77.500 ± 2.278	87.305 ± 1.220	93.834 ± 0.785

Values are given as mean ± SEM of six rats.
Statistical analysis was done by one way analysis of variance (ANOVA).

Test dose I was found to be having the most wound healing potential compared to the other groups (Table 4).

Haematological parameters such as RBC, WBC and haemoglobin count were estimated by standard laboratory analytical procedures (Table 5). Statistical analysis was done by one way analysis of variance (ANOVA).

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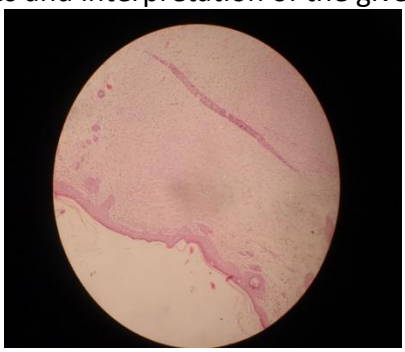
Table 5: Haematological observation

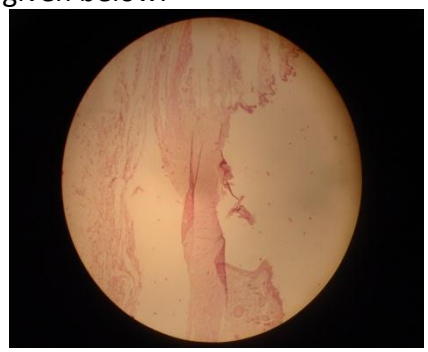
Group	RBC (million/cumm)	WBC (million/cumm)	Haemoglobin (million/cumm)
Positive control	4.333 ± 0.061	7668.33 ± 35.158	12.533 ± 0.080
Solvent control	4.800 ± 0.003	7638.33 ± 20.883	14.517 ± 0.040
Test dose I	4.300 ± 0.002	8333.33 ± 40.139	10.333 ± 0.033
Test dose II	3.433 ± 0.003	8510.00 ± 27.689	10.517 ± 0.0091

Values are given as mean ± SEM of six rats.
Statistical analysis was done by one way analysis of variance (ANOVA).

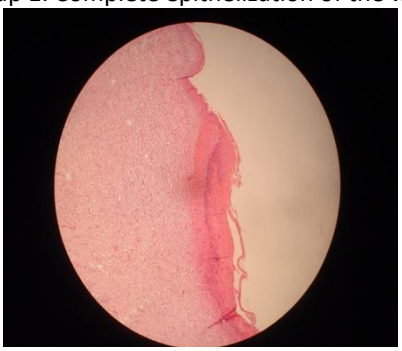
The histopathological examination of the skin was done by using standard laboratory staining techniques and interpretation of the given samples is given below:



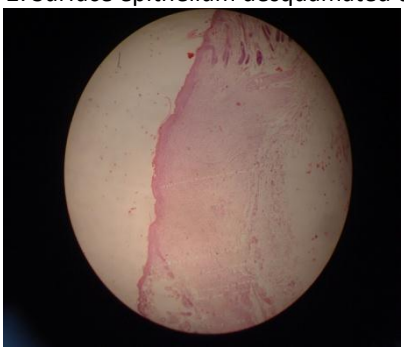
Group 1: Complete epithelization of the wound



Group 2: Surface epithelium desquamated area



Group 3: Wound surface area curved with necrotic tissue. Dermoepidermal junction infected with neutrophil and oedema fluid.



Group 4: Complete epithelization of wound area. Dermoepidermal junction revealed moderate infiltration of lymphocytes.

a) Positive control (Nitrofurantoin ointment): At the wound area the surface of the skin was slightly depressed and the epidermis was thick. Dermatoepidermal junction revealed diffuse scattered infiltration of eosinophils and a few lymphocytes. Dermis revealed moderate fibrovascular tissue. Moderate lymphatic infiltration with a few scattered macrophages was also seen in the dermis. Sub-cutis was hyperemic congested with mild diffuse infiltration and few scattered eosinophils and macrophages.

b) Solvent control (70% ethanol): At the wound area, the surface of the skin was severely depressed and the epidermis was desquamated. Dermoepidermal junction was moderately infiltrated with lymphocytes with a few scattered eosinophils and macrophages. Dermis

revealed high amount of fibrovascular tissue with more amount of collagen. Moderate lymphatic infiltration with a few scattered eosinophils and macrophages were also evident in the dermis. Sub-cutis was congested with a few scattered infiltration of lymphocytes, eosinophils and macrophages.

c) Test dose I (5% Nannari ointment): At the normal wound area, the surface of the skin was moderately depressed and epithelization was just indicated. The middle of the wound was curved with necrotic tissue. Dermoepidermal junction revealed diffuse severe infiltration with necrotic and oedema fluid. Dermis revealed severe fibrovascular proliferation. Diffuse severe infiltration with lymphocytes and mild infiltration with macrophages were seen and collagen deposition was found to be moderate.

d) Test dose II (10% Nannari ointment): The wound area was completely curved with thickened epidermis. Dermoepidermal junction revealed diffuse moderate infiltration of lymphocytes with a few scattered macrophages. Dermis revealed moderate fibrovascular proliferation with high amount of collagen deposition and moderate infiltration with lymphocytes. A few scattered eosinophils and macrophages were also evident (Figure 1).

DISCUSSION

The present study was undertaken to establish the wound healing effect of *Hemidesmus indicus*. It was observed that the group treated with nitrofurantoin ointment showed an increase in the rate and percentage of wound contraction and in the period of epithelization when compared to the ethanol treated group. It was also observed that the total WBC count was increased in both the above mentioned groups whereas RBC and haemoglobin count was decreased in both.

The alcoholic extract of *Hemidesmus indicus* which was formulated as 5% and 10% ointment was found to increase the rate of wound contraction and period of epithelization than the control and solvent groups. The RBC and haemoglobin count was found to be decreased in the extract treated groups. Increase of total WBC count in animals treated with ointment prepared from the alcoholic extract was observed.

The comparison between test dose I and test dose II showed that the percentage and rate of wound contraction was increased at the first dose level (5%). Period of epithelization also showed that the effect in healing of wounds by 5% ointment was more than 10% ointment extract.

The histopathological examination revealed that the positive group (nitrofurantoin treated animals) and the test dose II group (10% ointment of alcoholic extract treated animals) showed complete epithelization of wound area. Test dose I group (5% ointment of alcoholic

extract treated animals) showed formation of granulation tissue in the dermis and curved wound surface area with necrotic tissue.

CONCLUSION

The study concluded that the leaves of *Hemidesmus indicus* possess marked wound healing activity and could play a promising role in the treatment of wounds especially chronic wounds and in diabetic and cancer patients. Test dose I was found to have moderate action whereas test dose II showed a highly effective activity. However, further studies are required to identify and isolate the active principle which was responsible for this effect.

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