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The wound healing effects of a new polyherbal formulation

G. K. Dash*[1] and P. N. Murthy[2]

[1]*Institute of Pharmacy and Technology, Salipur, Cuttack district, Odisha-754202, India*
[2] *Royal College of Pharmacy and Health Sciences, Berhampur, Odisha-760002, India*

ABSTRACT

Six polyherbal ointments containing methanol leaf extracts of Ageratum conyzoides Linn. (Asteraceae), Argemone mexicana Linn. (Papaveraceae), Heliotropium indicum Linn. (Boraginaceae) and bark extract of Alstonia scholaris (L.) R. Brown. (Apocynaceae) were formulated and tested for wound healing activity in rats using excision and incision wound models and skin irritation study. The effects of test samples on the rate of wound healing were assessed by the rate of wound closure, period of epithelialisation, wound breaking strength and determination of hydroxyproline. Nitrofurazone (0.2% w/w) in respective ointment bases was used as reference standard for the activity comparison. All the groups of animals treated with various formulations exhibited significant ($P<0.01$) increase in the percentage of wound contraction as compared to the respective control group of animals on different days of the study commencing from Day 8. The hydroxyproline content in the treatment groups were found to be significantly higher compared to the respective control group of animals. The skin breaking strength in the animals of all formulation treatment groups was significantly greater than that of the animals of the corresponding control group, thus showing enhanced collagen synthesis. The tested ointments did not show any severe type of irritation and there was no evidence of any noticeable inflammation on the skin. Our study indicates that a combination of 2.5% w/w each of the methanol extracts of A. conyzoides, A. mexicana, A. scholaris and H. indicum can be useful in wound contraction, improvement of tensile strength and augmentation in hydroxyproline content or collagen content. These properties together make this combination a potential candidate for wound healing activities. The present work justifies the use of these plant materials for wound healing as claimed in the folklore literature.

Keywords: *Ageratum conyzoides* Linn., *Argemone mexicana* Linn., *Heliotropium indicum* Linn., *Alstonia scholaris*, polyherbal formulation, Wound healing.

INTRODUCTION

Wound healing is the process of repair that follows injury to the skin and other soft tissues. The capacity of a wound to heal depends in part on its depth, as well as on the overall health and

nutritional status of the individual. Wound healing involves a complex series of interactions between different cell types, cytokine mediators, and the extracellular matrix. The phases of normal wound healing include hemostasis, inflammation, proliferation, and remodeling. Each phase of wound healing is distinct, although the wound healing process is continuous, with each phase overlapping the next. Because successful wound healing requires adequate blood and nutrients to be supplied to the site of damage, the overall health and nutritional status of the patient influences the outcome of the damaged tissue.

Several medicinal plants have been used since time immemorial for treatment of cuts, wounds and burns and showed promising effects. Some very common plants like *Aloe vera*, *Azadirachta indica*, *Carica papaya*, *Celosia argentea*, *Centella asiatica*, *Cinnamomum zeylanicum*, *Curcuma longa*, *Nelumbo nucifera*, *Ocimum sanctum*, *Phyllanthus emblica*, *Plumbago zeylanica*, *Pterocarpus santalinus*, *Terminalia arjuna* and *Terminalia chebula* have been extensively reported in Ayurveda, Siddha and Unani systems of medicines for their wound healing potential [1].

Ethnobotanical information on plants used in India for treatment of cuts, wounds and burns is widely scattered with reports in leading journals devoted to ethnobotany and traditional medicine. A few such lesser-known plants indigenous to India but extensively used by various tribes of Odisha state since time immemorial in their traditional medicine include *Ageratum conyzoides* Linn. (Asteraceae), *Argemone mexicana* Linn. (Papaveraceae), *Heliotropium indicum* Linn. (Boraginaceae) and *Alstonia scholaris* (L.) R. Brown. (Apocynaceae) respectively. The leaf juice of *A. conyzoides*, *A. mexicana*, *H. indicum* and bark paste of *A. scholaris* have been widely used by different tribes of Cuttack district of Odisha in treating fresh cuts and wounds and claim for their promising activity. These important plant materials are reported to have significant anti-bacterial, anti-inflammatory activities which are complimentary to wound healing process. The combination is used in order to enhance the wound healing activity. This has inspired this present study where we have investigated a formulation of the methanol extracts of the selected plant materials in a simple ointment base for wound healing activity.

MATERIALS AND METHODS

The fresh plant materials of *Ageratum conyzoides* Linn. (Leaves), *Heliotropium indicum* Linn. (Leaves), *Argemone mexicana* Linn. (Leaves) and *Alstonia scholaris* (L.) R. Brown (Barks) were collected from young matured plants and authenticated by the taxonomists of the Botanical Survey of India, Shibpur, Howrah.

Monographic analysis of selected plant materials

The monographic analysis of the plant materials were performed according to the procedure laid out in Indian Pharmacopeia [2] and presented in Table 1. The parameters studied included determination of Foreign organic matter, Extractive values, Ash values and Loss on drying respectively.

Table 1: Monographic analysis of selected plant materials

Parameters	Obtained value			
	A. conyzoides	*A. mexicana*	*A. scholaris*	*H. indicum*
Foreign organic matter (% w/w)	0.6	0.8	1.1 (Not more than 2)	0.6
Ethanol soluble extractive (% w/w)	5.2	4.2	5.6 (Not less than 4)	3.8
Water soluble extractive (% w/w)	13.6	10.3	14.8 (Not less than 12)	11.2
Total ash (% w/w)	10.8	8.13	5.9 (Not more than 11)	8.7
Acid insoluble ash (% w/w)	3.2	2.65	1.38 (Not more than 3)	2.4
Water soluble ash (% w/w)	6.12	4.8	3.23	4.6
Sulphated ash (% w/w)	11.6	10.9	6.65	9.4
Loss on Drying (% w/w)	7.3	7.5	6.2	8.6

Values in parentheses indicate pharmacopoeial standards (Ayurvedic Pharmacopeia of India)

Preparation of extracts

The dried powdered plants materials (500 g each) were separately defatted with petroleum ether followed by extraction with methanol using a Soxhlet extractor for 48 h. The methanol extracts of all selected plant materials were separately filtered and concentrated in rotary evaporator and finally air-dried. The extractive values were determined with respect to the dried plant material. Preliminary phytochemical studies were carried out to find out the nature of phytoconstituents present in different methanol extracts.

Preparation of polyherbal formulations

At least six polyherbal ointments (F-I to F-VI) were prepared by taking all the plant materials under study in different proportions. Three types of ointment bases viz. Simple ointment I.P. [3], Emulsifying ointment I. P. [3] and Macrogol ointment B. P. [4] were selected. The plant extracts were incorporated into the bases.

Preparation of formulation bases

The ointment bases (100 g each) were separately prepared as per the procedure laid down in individual monographs with their compositions as is given in Table 2. The ingredients were melted together and stirred until cold to get uniform consistency.

Table 2: Preparation of formulation bases

Ingredients	Quantity (g)
Simple ointment I. P.	
Wool fat	5
Hard paraffin	5
Cetosteryl alcohol	5
White soft paraffin	85
Emulsifying Ointment I.P.	
Emulsifying wax	30
White soft paraffin	50
Liquid paraffin	20
Macrogol ointment B. P.	
Macrogol 4000	35
Macrogol 300	65

Method of preparation of Medicated ointments

The trituration method was employed in the preparation of the medicated ointments. The required quantity of the ointment bases were weighed separately and melted at a temperature of about 70° C in a hot water bath. The designated quantity of each extract was added to the melted base at 40°C, mixed and triturated gently and continuously until a homogenous dispersion is obtained. The compositions of different formulations are presented in Table 3. The prepared formulations were designated as F-I, F-II, F-III, F-IV, F-V and F-VI respectively.

Table 3: Preparation of Medicated ointments

Ingredients	F-I (% w/w)	F-II (% w/w)	F-III (% w/w)	F-IV (% w/w)	F-V (% w/w)	F-VI (% w/w)
Methanol extract of *A. conyzoides*	1.25	2.5	1.25	2.5	1.25	2.5
Methanol extract of *A. mexicana*	1.25	2.5	1.25	2.5	1.25	2.5
Methanol extract of *A. scholaris*	1.25	2.5	1.25	2.5	1.25	2.5
Methanol extract of *H. indicum*	1.25	2.5	1.25	2.5	1.25	2.5
Simple ointment I. P.	95	90	-	-	-	-
Emulsifying Ointment I.P.	-	-	95	90	-	-
Macrogol ointment B. P.	-	-	-	-	95	90

Wound Healing activity

The prepared formulations were separately evaluated for their wound healing activity in rats using excision and incision wound models. The effects of test samples on the rate of wound healing were assessed by the rate of wound closure, period of epithelialisation, wound breaking strength and determination of hydroxyproline. Nitrofurazone (0.2% w/w) in respective ointment bases was used as reference standard for the activity comparison.

Healthy Wistar albino rats of either sex and of approximately the same age, weighing between 150–250 g were used for the study. They were individually housed, maintained in clean polypropylene cages and fed with commercially pellet diet (M/s Hindustan Lever Ltd., Mumbai) and water *ad libitum*. The experimental protocols were subjected to scrutiny of Institutional Animal Ethics Committee for experimental clearance (No. 1025/C/07/CPCSEA). One day before the study, animals were removed from their cages and were put in polypropylene cages for randomization and were divided into groups and numbered. After allocation of the numbers to animals, the cages were given respective identification numbers and six animals were housed per cage.

Excision model

The selected albino rats were randomly divided in to eight groups of six animals in each for each study. Animals were anesthetized prior to and during creation of the wounds, with 1 ml of intravenous ketamine hydrochloride (10 mg/kg). The rats were inflicted with excision wounds as described by Morton and Malone [5] and suggested by Kamath *et al.* [6]. An impression was made on the dorsal thoracic region 1 cm away from vertebral column and 5 cm away from ear on the anaesthetized rat. The dorsal fur of the animals was shaved with an electric clipper and the anticipated area of the wound to be created was outlined on the back of the animals with methylene blue using a circular stainless steel stencil. A full thickness of the excision wound of circular area of 500 mm^2 and 2 mm depth was created along the markings using toothed forceps, scalpel and pointed scissors. Haemostasis was achieved by blotting the wound with cotton swab

soaked in normal saline. The entire wound was left open [7]. All surgical procedures were performed under aseptic conditions. The wounds on each animal of various groups were treated topically with the vehicles (bases), nitrofurazone (0.2% w/w) in respective bases and the prepared formulations as applicable in a similar manner.

The wound closure rate was assessed by tracing the wound on days 4, 8, 12 and 16 post wounding days using transparent paper and a permanent marker. The wound areas recorded were measured using graph paper [8]. The percentage of wound healing was calculated of original wound size for each animal of group on predetermined days for final analysis of results. The number of days for complete epithelialization was noted. The acid hydrolysis of the dry granulation tissue formed on the wound was studied for the concentration of hydroxyproline [9]. The results of the study are presented in Table 4.

Table 4: Effect of various formulations on percentage (%) wound closure (Excision Wound Model)

Treatment	Conc. (w/w)	Percentage (%) wound closure				Period of epithelization (no. of days)	Concentration of hydroxyproline (mg/100 g dry tissue)
		4th day	8th day	12th day	16th day		
Control (SO)	-	27.58±2.21	43.47±3.31	60.61±4.11	79.74±2.48	24.66±1.17	2978.63±412.55
Control (EO)	-	27.37 ±5.18	42.38±3.11	59.55±2.88	77.77±3.02	22.66±0.55	2853.16±382.27
Control (MO)	-	27.28±4.83	45.61±4.01	57.93±3.11	77.19±5.03	23.83±1.92	2958.16±362.27
Nitrofurazone in SO	0.2%	39.61±1.22*	77.90±2.62**	89.41±3.11**	100±00**	13.66±1.05**	6819.91±387.16**
Nitrofurazone in EO	0.2%	36.30±4.82*	75.80±3.08**	87.91±1.03**	100±00**	14.5±0.42**	6560.77±365.08**
Nitrofurazone in MO	0.2%	36.90±1.84*	76.56±2.11**	88.35±3.06**	100±00**	13.83±1.16**	6452.22±365.08**
GKD I	5%	31.82±2.66	73.42±3.11**	93.53±2.78**	97.48±0.11**	17.5±1.17**	5432.03±214.83**
	10%	38.34±3.11*	75.52±3.44**	94.46±2.33**	100±00**	14.5±1.25**	6845.71±417.66**
GKD II	5%	30.65±3.69	71.57±4.22**	92.03±1.69**	97.02±0.61**	17.16±0.7**	4167.98±523.45*
	10%	33.71±2.98	74.46±3.65**	93.91±1.13**	100±00**	15.16±0.94	6008.06±601.17**
GKD III	5%	34.90±4.02	72.82±3.91**	93.01±1.22**	97.87±0.11**	18.5±0.76**	4082.98±523.45*
	10%	37.47±2.11*	75.07±1.22**	93.73±0.88**	100±00**	15.5±1.17**	6143.77±514.43**

SO-Simple ointment I.P., EO- Emulsifying ointment I. P., MO- Macrogol ointment B. P.
Values are expressed as mean ± S.E. (n = 6). All co lumns are significant using ANOVA.
** P<0.05, ** P<0.01 when compared to control; Dunnet's t-test.*

Incision wound model

The selected rats were anaesthetized prior to and during creation of the wounds, with 1 ml of intravenous ketamine hydrochloride (10 mg/kg). The dorsal fur of the animals was shaved with an electric clipper. A longitudinal paravertebral incision of 6 cm long was made through the skin and cutaneous tissue on the back as described by Ehrlich and Hunt [10]. After the incision, the parted skin was sutured 1 cm apart using a surgical thread and curved needle. The wounds were left undressed [11]. Extracts were topically applied to the wound once a day. The sutures were removed on 8th post wound day and continued the application of the extract. The wound breaking strength was measured by the method of Reddy *et al.*, 2002 [12] on the 10th day evening after the last application. The results are depicted in Table 5.

Skin irritation study

This study was carried out as suggested by *Gfeller et al.*, 1985 [13] on selected rats. An area measuring about 500 mm^2 on the dorsal fur of the animals was shaved. The prepared ointments were applied separately to different group of animals. After 4 h, the skins were observed for signs of inflammation.

Table 5: Effect of various formulations on wound breaking strength (Incision Wound Model)

Treatment	Concentration	Breaking strength (g)
Control (SO)	-	312.11±8.11
Control (EO)	-	317.09 ±17.03
Control (MO)	-	318.71±14.23
Nitrofurazone in SO	0.2% w/w	498.21±16.26**
Nitrofurazone in EO	0.2% w/w	455.15 ±8.33**
Nitrofurazone in MO	0.2% w/w	468.31±22.13**
GKD I	5% w/w	388.18±10.22**
	10% w/w	453.2±14.9**
GKD II	5% w/w	373.96±12.6*
	10% w/w	442.71±8.31**
GKD III	5% w/w	381.18±9.66**
	10% w/w	448.14±11.8**

SO-Simple ointment I.P., EO- Emulsifying ointment I. P., MO- Macrogol ointment B. P. Values are expressed as mean ± S.E. (n = 6). All co lumns are significant using ANOVA.
** P<0.05, ** P<0.01 when compared to control; Dunnet's t-test.*

RESULTS

The monographic analysis of the plant materials was performed according to the Indian Pharmacopeia and the values obtained were presented in Table 1. It was observed that the plant *A. scholaris* is official in Ayurvedic Pharmacopeia of India [14], where the monographic standards for the barks have been listed. Our plant material also confirmed to all the parameters listed in the Pharmacopoeia and it was found that all the values were with in the pharmacopeial limits. However, for other plant materials, obtained experimental values are listed, since there are no pharmacopoeial standards available for them. The analysis reports will help the future investigators for proper identification of the plants. The findings will also enable pharmacognostical standardization of the plant materials. Preliminary phytochemical studies of the methanol extracts of the selected plant materials revealed presence of alkaloids, flavonoids, saponins, tannins and terpenoids.

Wound contraction ability of the prepared polyherbal formulations in excision model was measured at different time intervals till complete wound healing took place. Table 4 depicts the effect of topical application of prepared ointment variants on percentage wound contraction in excision wound model. All the groups of animals treated with various formulations exhibited significant ($P<0.01$) increase in the percentage of wound contraction as compared to the respective control group of animals on different days of the study commencing from Day 8. The hydroxyproline content which is indicative of the collagen turnover was determined in the treatment groups and control group and the values were found to be significantly higher compared to the respective control group of animals.

The results of the measurement of skin breaking strength on 10^{th} post wounding day in incision wound healing model are depicted in Table 5. The skin breaking strength in the animals of all formulation treatment groups was significantly greater than that of the animals of the corresponding control group, thus showing enhanced collagen synthesis. The breaking strength

ultimately depicts the tensile strength, thus showing a significant increase in the tensile strength of the skin tissues in animals of the formulation treated groups.

In the skin irritation study, The tested ointments did not show any severe type of irritation and there was no evidence of any noticeable inflammation on the skin.

DISCUSSION

Conclusively, the various ointments prepared with the selected plant materials exhibited a good wound healing effect comparable to that of nitrofurazone irrespective of the type of the base used.

For effective evaluation of the formulated ointment variants (F-I to F-VI), we used excision and incision wound healing models and studied suitable and relevant parameters such as wound contraction, hydroxyproline content (collagen content) and skin breaking strength which generally indicate the rate of tissue cell regeneration, amount of collagen or rate of collagenation and tensile strength of the skin. The normal response of an organism to injury or wound is either regeneration (the complete restoration of the damaged part) or repair (the reconstruction of the injured region). The scar tissue is appropriately covered, by an epithelium at the site of injury. When skin is injured or wounded the dermis responds primarily to repair while the epidermis responds to regeneration; the collective response of the skin to injury is termed as wound healing. We have ensured authenticity and chemical consistency of all the materials used. We hypothesized that such a combination would have a synergistic activity and put all the four test materials through appropriate experimental tests. The base formulation did not show statistically significant activity in any of the test models. Amongst other treatment groups, groups of animals, treated with 10% combination of total extracts, showed significantly greater wound contraction, higher collagen content and increased skin breaking strength as compared to 5% combination of total extracts formulations and the respective control groups.

Thus, our study indicates that a combination of 2.5% w/w each of the methanol extracts of *A. conyzoides*, *A. mexicana*, *A. scholaris* and *H. indicum* can be useful in wound contraction, improvement of tensile strength and augmentation in hydroxyproline content or collagen content. These properties together make this combination a potential candidate for wound healing activities.

Several phytoconstituents like alkaloids, saponins are known to promote wound healing process due to their anti-oxidant and anti-microbial properties [15]. The study reveals that the wound healing activity of polyherbal formulation may be due to the combined action of phytoconstituents like alkaloids, flavonoids, saponins, tannins and terpenoids present in the methanol extracts of the selected plants.

CONCLUSION

The present work justifies the use of these plant materials for wound healing as claimed in the folklore literature.

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