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Research Article

WOUND HEALING ACTIVITY OF POLY HERBAL FORMULATION IN ALBINO RATS USING EXCISION WOUND MODEL, INCISION WOUND MODEL, DEAD SPACE WOUND MODEL AND BURN WOUND MODEL

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ABSTRACT

The present study was conducted to investigate the wound healing activity of the selected Indian medicinal plants. The present study was designed to evaluate wound healing activity of poly herbal formulation (Curcuma longa, Eclipta alba, and Tridax procumbens) in Wistar albino rats using excision wound model, incision wound model, dead space wound model and burn wound model at two different dose levels of 250 and 500 mg/kg. The suitable gel formulation has been selected by cellophane membrane study to apply topically. The following parameters were checked in the present study to assess the effects of poly herbal formulation extract on wound healing. Collagenation phase of healing (for this incision and dead space wound model)Wound contraction (excision wound model and burn wound model) Epithelization phases of healing (excision wound model and burn wound model). Wound healing was monitored on Days 3, 6, 9 and 14 and histological evaluation was carried out on the samples. In the excision wound and burn wound model, rat treated with poly herbal formulation leaf extract showed significant difference in period of epithelisation,wound contraction 50%. In incision wound model, poly herbal formulation extract treated rat showed significant increase in breaking strength. In dead space wound model, poly herbal formulation shows significant increase in breaking strength, dry weight and hydroxyproline content of the granulation tissue. It can be concluded that poly herbal formulation topically (5% and 10% gel formulation) and orally (250 mg and 500 mg/Kg body weight) have wound healing activity, which may be due to its epithelization, thereby justifying the traditional claim.

Keywords: poly herbal formulation, wound healing, Excision wound, Hydroxyproline, Incision wound.

INTRODUCTION

Wounds are inescapable events of life, which arise due to physical or chemical injury or microbial infections. The healing of wounds often deviates from a normal course and under-healing, over-healing or failure of wounds to heal is common. Research on drugs that increase wound healing is a developing area in modern biomedical sciences. Several drugs obtained from plant sources are known to increase the healing of different types of wounds [1]. Herbal medicine has become an integral part of standard healthcare, based on a combination of time honoured traditional usage and ongoing scientific research. Medicinal plants are coming into prominence because of the over-use of conventional medicines such as antibiotics which has resulted in the development of resistance in many infectious organisms. Thus, herbal preparations can be more effective than conventional medicines and their non- toxic nature means that they can be administered over long periods [2].

A greater deal of research has been carried out to evaluated scientific basis for the claimed wound healing activity of herbal agents as single agent or in formulation. The plant herbal formulation under study contain plant ingredients like ethanolic extract of Curcuma longa, Eclipta alba, and Tridax procumbens.The form of extract whether

hydroalchol or ethanol and contact in dose are based on the traditional knowledge and reports present on these plants. Formulations are developed based on Ayurvedic principles where plants are included for antioxidant activity, wound healing activity, bio availability enhancement and specific activity in modulation of different liver disease conditions as many of these herbal ingredients are known to have liver modifying activity[3].We have undertaken this study to evaluate the efficacy of these formulations in experimental animals in which wound healing activity was induced by Excision wound model.

MATERIALS AND METHODS:

Plant Material: All parts of Curcuma longa, Eclipta alba, and Tridax procumbens were collected from in and around Moinabad, telangana, India. The dried plant materials were extracted in its entire form by Soxhalation, Maceration & Percolation.

Phytochemical Screening: The poly herbal formulation (500g) was subjected to successive extraction with different solvents like ethanol. The dry extracts were subjected to various chemical tests in order to detect the presence of different phytoconstituents. Qualitative tests for the presence of plant secondary metabolites such as carbohydrates, alkaloids, tannins, flavonoids, saponins and glycosides were carried out on the bark powdered using standard procedures [4].

Animals: Wistar albino rats of either sex, weighing about 150–250 each, were used for the study. They were fed with standard food and wate adlibitum. They were housed in polypropylene cages maintained under standard conditions (12 hour light - dark cycle; 25 ± 3°C; 35–60% humidity) [5,6]. The experimental protocol was subjected to the scrutiny of the Institutional Animal Ethics Committee, and was cleared by same before beginning the experiment.

Acute Toxicity Studies: Healthy adult albino rats of either sex, fasted overnight, were divided into 6 groups (n = 6 per cage) and were fed with increasing doses (1, 2, 4, and 5 g/kg body wt.) of the ethanol extract. The total ethanol extract, administered orally in doses of up to 5000mg/kg body wt., did not produce any evident sign of toxicity or mortality in rats up to 14 days after administration.

Wound Healing Models: Wound healing activity was studied using three models viz. i) excision wound model ii) incision wound model iii) dead space wound model.

i) Excision Wound Model: Wister male albino rat weighing between 250-275 gm body weigh were divided into four groups, each group consisting of 6 rats and each animal kept separately under laboratory condition. They had free access to commercial pallet diet and ad libitum.

Group I (control group): animal of this group received 7.5% HPMC gel (2% tween 80).

Group II (standard group): animal of this group received Aloe vera (90%) gel formulation.

Group III (test group): animal of this group received poly herbal formulation (5%) in HPMC (7.5%) gel formulation.

Group IV (test group): animal of this group received poly herbal formulation (10%) in HPMC (7.5%) gel formulation.

The animals were anesthetized by using 30mg/kg rbw, i.p. 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 particular skin area was shaved one day prior to the experiment. The skin of impressed area was excised to the full thickness to obtain a wound area of about 500 mm^2. Haemostasis was achieved by blotting the wound with cotton swab soaked in normal saline. Contractions, which contribute for wound closure, was studied by tracing the raw wound. Wound area was measured by tracing the wound on a millimeter scale graph paper. The percentage of wound healing was calculated of original wound size (500mm2) for each animal of group and mean on predetermined days i.e, 2, 4, 8, 10, 12, 14, 16, 18, 22, 22 for final analysis of results. Falling of scar leaving no raw wound behind was taken as end point of complete epethilization and the days required for this was taken as period of epithelization.

ii) Incision Wound Model: Wister male albino rat weighing between 250-275 gm body weigh were divided into four groups same as followed in excision wound model. Para vertebral straight incision of 6 cm length each were made through the entire thickness of the skin, on either side of the vertebral column with the help of a sharp scalpel. After complete haemostasis the wound were closed by means of interrupted sutures placed at equidistance points about 1 cm

apart. Animals were treated daily with drugs, from 0 day to 9th post-wounding day the wound breaking strength were estimated on 10th day of wounding by continuous, constant water flow technique. Allis forceps were firmly applied on either side of incision wound 3 mm away from wound margin on adjacent normal skin [7]. The forceps on one side was hooked to a fixed metal rod while the other forcep was attached to a thread suspended by weights running over a pulley. As soon as gapping of the wound occurred, addition of weights was stopped and simultaneously the weights were lifted so as to avoid opening of the entire wound. The weights required to produce gapping were noted.

iii) Burn Wound Model: Wister male albino rat weighing between 250-275 gm were divided into four groups same as mentioned earlier in excision wound. Partial thickness burn wounds were inflicted, on overnight-starved animals under pentobarbitone (30 mg/Kg, i.p.) anesthesia, by pouring hot molten wax at 800 into a cylinder of 300 mm^2 circular openings placed on the shaven back of the animal until the wax get solidified. Which took about 10-12 minutes then the cylinder with wax adhered to the skin was removed which left demarked partial thickness circular burn wound of 300mm^2. Immediately after the injury and on subsequent days the drugs or vehicle was applied topically. Apart from the drug under investigation no local/systemic chemotherapeutic cover was provided to the animals.

iv) Dead Space Wound Model: Wister male albino rat weighing between 250-275 gm body weigh were divided into three group, each group consisting of 6 rats and each animal kept separately under laboratory condition. They had free access to commercial pallet diet and ad libitum.

Group I (control group): animal of this group received (2% tween 80 solution).

Group II (test group): animal; of this group received poly herbal formulation (250mg/kg rbw, p.o.).

Group III (test group): animal; of this group received poly herbal formulation (500 mg/kg rbw, p.o.).

This type of wound was created by implanting subcutaneously a 2.5x0.5 cm polypropylene tube in the lumber region of dorsal side. Animals received drug from 0 day to 9th post woundering day, on the 10th post wounding day, granulation tissue harvested on the implanted tube, was carefully dissected out along with the tube. The tubular granulation was cut along its length to obtain a sheet of granulation tissue. The breaking strength was measured as described under incision wound model. The pieces of granulation tissue were collected, dried at 60^0C for 24 hours, to get a constant weight and weighed[10].After noting down the dry weight of granulation tissues, they were used for the determination of hydroxyproline content.

Determination of Hydroxyproline: The granulation mass, which had dried at 600^0C for about 24 hours was weight and placed in a selected tube containing 10 ml of 6 N HCl. Heating the sealed tubes at 110^0C for 24 hours hydrolyzed the tissues. The hydrolysate was cooled and excess of acid was neutralized with 10N NaOH using methyl red as indicator. The volume of neutral hydrolysate was made up to 20ml with distilled water. From this 0.1 ml was used to estimate hydroxyproline.

Hydroxyproline estimation: 0.1 ml of hydrolysate sample was pipetted out into clean test tubes, volume made upto 0.5 ml with distilled water. From the stock solution of standard hydroxyproline 1.6 ml was taken and diluted up to 100 ml. from this 0.5 (8 µg) was pipetted out into a clean test tube. To this 1 ml each of 2.5 N NaOH, 0.01 M $CuSO_4$ and 6% H_2O_2 were added. Immediately, the tubes were placed in a water bath at 800^0C for 16 minutes and then cooled for 5 minutes [9].To this 2 ml of freshly prepared 5% solution of para-dimethylamino-benzaldehyde in n-propanol, and 4 ml of 3N H_2SO_4 were added. Test tubes were once again placed in a hot water bath at 800 C for 15 minutes and then cooled for 5 minutes. The optical density (O.D.) of the pink colour of these test samples were compared to that of standard hydroxyproline of known concentration samples at 540 nm using Backman Du-64 spectrophotometer for the estimation of hydroxyproline.

v) Skin Irritation Study: Ratings corresponding to the following definitions were derived from data obtained from the test methods as described in 16 CFR 1500.41 and/or NAS Publication 1138, and categories of toxicity as described in 16 CFR 1500.3.The rabbit was shaved the skin in three different position of dorsal side, each about 500 mm^2. The rabbit was kept in rabbit holder and the 1st area was kept as control, to which vehicle was applied. 2nd area was applied with poly herbal formulation (5%) and the 3rd area treated with poly herbal formulation (10%). After 4

hour the skins was observed and compare to control the score was given.

Score:

(1)Practically Non-Irritating: The undiluted product causes no noticeable irritation, or causes slight inflammation (edema and erythema skin reaction values of 0 or 1) of intact or abraded skin of rabbits during the study period. Primary Irritation Index of 0- 1.9.

(2) Moderately Irritating: The undiluted product causes well-defined inflammation (edema and erythema skin reaction values of 2) during the study period. Primary Irritation Index of 2-4.9.

(3)Primary Skin Irritant: The undiluted product causes moderate to severe inflammation (edema and erythema skin reaction values of 3 or 4) of the intact or abraded skin of rabbits during the study period. Primary Irritation Index of 5 or more.

(4)Corrosive: The undiluted product causes visible destruction or irreversible alterations of the tissue structure at the site of contact on intact or abraded skin of rabbits during the study period.

Histopathological study: Samples of healing tissue were taken on Days 3, 6, 9 and 14 from all four groups of animals and were processed for histological study. The samples were fixed in formalin and installed on slides, stained with Hematoxylin and Eosin and then analysed under a light microscope. The recorded parameters were scars, inflammatory cells, angiogenesis, congestion, fibroblast collagen density, fibrin and fibroblastic aggregation.

Statistical Analysis: Results are expressed as mean±S.E.M. The differences between experimental groups were compared by one-way Analysis of Variance (ANOVA) (control vs. treatment) followed by Bonferroni's test and were considered statistically significant when $P<0.05$.

RESULTS:

Acute Oral Toxicity Study: Poly herbal formulation upto a dose of 2000 mg/kg body weight did not showed any mortality. Hence 1/4th and 1/8th of this dose i.e. 500 mg/kg and 250- mg/kg body weight of Echinacea Angustifolia were used for wound healing activity. The results are tabulated in Table: 1

Selection of Gel Base: The result showed that both the rate and extent of tannin delivered from the vehicle under sink condition was maximum for the gel prepared with HPMC. There was no apparent retarding effect of the gel on the active moieties; hence HPMC was used for wound healing activity.

Wound healing models:

Excisoin Wound Model:

A significant decrease in period of epithelization was observed after poly herbal formulation (5%) and poly herbal formulation (10%) and application.

Treatment with Aloe vera (std.) also significantly reduced period of epethelization as compared with control group. At the same time poly herbal formulation (5%) and poly herbal formulation (10%) and Aloe vera also decreased the wound contraction (50%) as compared with control. Comparative analysis revealed that poly herbal formulation (5%), poly herbal formulation (10%) and Aloe vera had almost equal wound healing activity.

Incision Wound Model:

The breaking strength of 10 day wound was significantly increased in all treatment groups when compared to control. (Table: 3)

Burn wound model:

Both poly herbal formulation (5%) and poly herbal formulation (10%) gel applied topically shorten the period of epithelization significantly when compared with control. Aloe vera gel applied topically shortens the period of epithelization and all the three also decreased the wound contraction (50%) significantly as compared with control (Table: 4).

Dead Space Wound Model: The breaking strength of 10 days old granulation tissue was significantly promoted by poly herbal formulation (250mg/kg) and poly herbal formulation (500mg/kg).The dry tissue weight also significantly increased in poly herbal formulation (250mg/kg) and poly herbal formulation (500mg/kg) when compared with control group. The hydroxyproline content was significantly more in poly herbal formulation. High dose treated animal compared to poly herbal formulation low dose treated animal. From the above findings it is clear that poly herbal formulation promotes the breaking strength by promoting the wound collagen content. (Table: 5)

Skin Irritation Study: Low dose does not showed any severe type of irritation, there was no evidence of showing any

Table 1: Acute oral toxicity studies

Sl. No.	Treatment	Dose mg/kg body weight				Inference
		175	550	2000	5000	
1	Control (Tween 80, 2%)	0	0	0	0	Stop Dosing
2	poly herbal formulation	0	0	0	x	Stop Dosing

0 = Survived X = Died

Table 2: Effect of Poly herbal formulation on period of epithelization and wound contraction in excision wound model.

Parameter studied	Epithelization period (days)	Wound contraction Wc-50% (days)
Control (7.5% Hpmc Gel)	21.00 + 0.3651	8.608 + O.457
Aloe Vera Gel (90%)	15.833 + 0.542***	6.660+ 0.375**
poly herbal formulation (5%) In 7.5%	15.00 + 0.577***	5.72 + 0.264***
Hpmc Gel	16.00 + 0.516***	6.07 + 0.141***

All values are mean + SEM, n=6, ** p<0.01, *** p<0.001 vs. control

Table 3: Effect of poly herbal formulation on breaking strength in incision wound model

Parameter studied	Breaking strength
Control (7.5% HPMC Gel)	283.33 + 8.333
Aloe vera gel (90%)	391.66 + 16.667***
poly herbal formulation.(5%)	400 + 10.035***
poly herbal formulation.(10%)	370.83 + 6.455***

All Values Are Mean + SEM, N=6 , *** P<0.001 Vs. Control

Table 4: Effect of Poly herbal formulation on period of epithelization and wound contraction in burn wound model

Parameter Studied	Epithelization Period (Days)	Wound Contraction Wc-50% (Days)
Control (7.5% HPMC Gel)	20.50 + 0.500	7.87 + O.320
Aloe Vera Gel (90%)	16.16 + 0.401***	5.37 + 0.275***
poly herbal formulation (5%) in 7.5% HPMC GEL	14.33 + 0.614***	4.31 + 0.225***
poly herbal formulation (10%) in 7.5% HPMC Gel	16.50 + 0.670***	5.22 + 0.274***

All values are mean + SEM, n=6 , *** p<0.001 vs. control

Table 5: Effect of Poly herbal formulation on breaking strength, dry tissue weight and hydroxyproline content in dead space wound model.

Parameter Studied	Breaking strength	Dry tissue weight	Concentration of Hydroxyproline
Control	337.5 + 16.968	90.566 + 2.596	2933.33 + 133.33
poly herbal formulation 250mg/kg Rbw	597.5 + 18.062***	189.166+ 6.020***	6066.66 + 190.90***
poly herbal formulation 500 mg/kg rbw	636.66 +14.240***	185.583 + 5.746***	7600.00 +273.25***+++

All values are mean + SEM, n=6, *** p<0.001 vs. control +++ p<0.001 vs. low dose

Table 6: Results of skin irritation study

Group	Sign	Score
Contol	–	0
poly herbal formulation (5%)	Not noticeable inflammation but slight redness	0.5
poly herbal formulation (10%)	Well defined redness and moderate type of inflammation	3.5

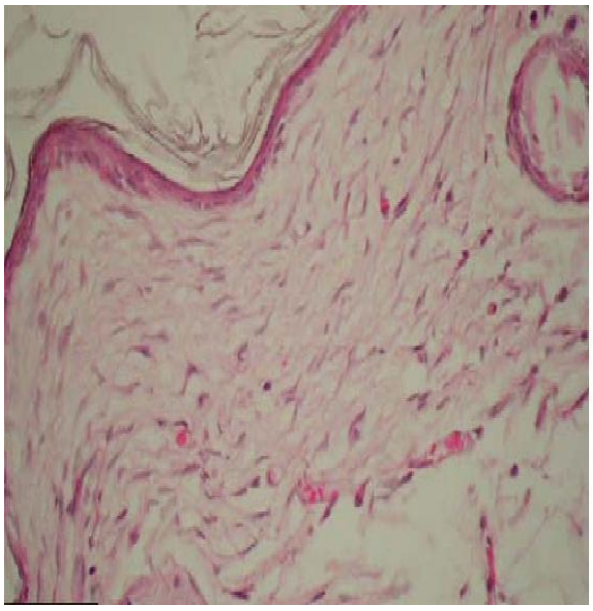

Fig. 1

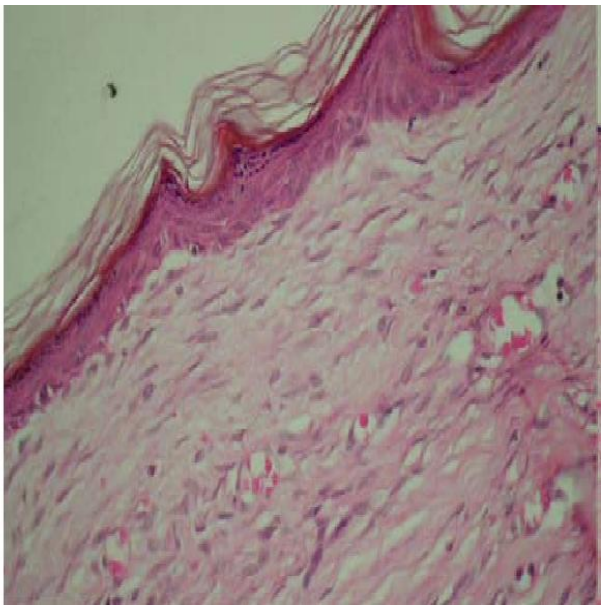

Fig. 2

Fig. 1: Control (H&E 400×) showing well formed but thick granular cell layer, theunderlying dermis contains deposited collagen fibers formation in the dermis and no inflammatory cells.

Fig. 2: Animals treated with *Kigelia pinnata* (H&E 400×) showing thin well-formed epidermis with hair follicle formation in the dermis and no inflammatory cells in a well organized dermis.

noticeable inflammation but slight redness was observed in case of poly herbal formulation low dose gel formulation. On the other hand high dose of poly herbal formulation showed well-defined redness and moderate type of inflammation.

Histopathological studies:

A section of the granuloma tissue was subjected to histopathological examination to determine the pattern of lay-down for collagen using two special stains i.e. Van Gieson and Masson Trichrome[14].

DISCUSSION:

Wound healing process consists of different phases such as granulation, collagenation, collagen maturation and scar maturation which are concurrent but independent to each other. Hence in the present study two different wound models were used. In the incision wound model, a significant increase was observed in the skin tensile strength of the ethanol extract treated group, at both dose levels (Table 3). The drug animals at both dose levels of the dead-space wound model showed a significant increase in dry granuloma weight, granuloma breaking strength and the level of hydroxyproline content (Table 5). In the excision wound model, animals treated with the ethanol extract of poly herbal formulation showed a significant decrease in the epithelization period, as evidenced by the shorter period for the fall of eschar compared to control. The drug extract also facilitated the rate of wound contraction significantly at both dose levels (Table 2). The histopathological study revealed increased collagen deposition in the drug, treated group (Figs. 1, 2), as compared to control.

Phytochemical work reveals that ethanol extract of leaf of Echinacea Angustifolia contains high amount of free ferulic acid and 6 methoxymelein naphthoquinone lapachol, stigmasterol, β sitosterol ,implied that β sitosterol is one of the active compounds which may be responsible for the epithelization activity. It can be concluded that poly herbal formulation topically (5% and 10% gel formulation) and orally (250 mg and 500 mg/Kg body weight) have wound healing activity, which may be due to its epithelization, thereby justifying the traditional claim.

CONCLUSION:

The hydro-alcoholic extract of poly herbal formulation exhibited significant wound healing activity in excision, incision, burn and dead space wound model, which is comparable to the marketed Aloe vera gel formulation. This finding provides an insight into the usage of the poly herbal formulation in traditional treatment of wounds or burns associated with bacterial infections.

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