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Research Article

EVALUATION OF WOUND HEALING ACTIVITY OF *HELIOTROPIUM INDICUM* LEAVES

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ABSTRACT
Present study is about the wound healing activity of ethanol and aqueous extracts of H.indicum leaves in wistar rats. Three wound models viz incision, excision and dead space wound were used in this study. The biophysical parameters studied were breaking strength in case of incision wounds and granulation tissue dry weight, breaking strength and hydroxyproline content in dead space wound model. In excision wound model, rate of contraction and number of days for epithelialization and also the granulation tissue formed on day 4, 8 and 12 were used to estimate some biochemical parameters like protein, DNA, collagen and lipid peroxides. For tropical application, 2% w/w sodium alginate ointment was prepared with 5% of aqueous and ethanol extracts of leaves. For oral administration 1% gum tragacanth suspension with 500mg/ml of extract was used. In excision and incision wound models, the control groups of animals were left untreated and in dead space wound model the animals were treated with 1 ml of 1% gum tragacanth per Kg, body weight orally.
Aqueous and ethanol leaf extracts induced significant wound-healing activity against all the wound models studied. High rate of wound contraction, decrease in the period for epithelialisation, high skin breaking strength and granulation strength, increase in dry granulation tissue weight were observed in treated animals when compared to the control group of animals. There was significant increase in hydroxyproline, protein, collagen contents and decrease in lipid peroxide level in treated animals. Results of the study confirmed the prominent wound healing activity of the test extracts. Ethanol extract of H.indicum possesses better wound healing property compared to the aqueous extract.

KEY WORDS: Heliotropium indicum, hydroxyproline, wound contraction, collagen, wound models.

INTRODUCTION

Wound may be defined as a loss or breaking of cellular and anatomic or functional continuity of living tissue[1]. Wound healing is a process by which a damaged tissue is restored as closely as possible to its normal state and wound contraction is the process of shrinkage of area of the wound[2]. Three different phases constitute the physiologic process of wound-healing; (i) substrate phase, (ii) proliferative phase and (iii) remodeling phase. All these steps are orchestrated in controlled manner by a variety of cytokines including growth factors. Some of these growth factors like platelet derived growth factor (PDGF), transforming growth factor B (TFG-B), fibroblast growth factor (FGF) and epidermal growth factor (EGF) etc. have been identified in self healing wounds. In chronic wounds the application of some growth promoting agents or some compounds which can enhance the in situ generation of these growth factors is required to augment the healing process[3]. Today wound healing abnormalities are among the greatest causes of disability and deformity. "I dressed the wound, God healed it" wound healing involves multiple

complicated events[4]. The understanding of the mechanism of wound healing has increased dramatically during last few years.

Herbs have been used as a source of drugs to combat diseases since time immemorial. The effectiveness, easy availability, low cost and non-toxic nature of herbal remedies are main reasons for its popularization. Ayurveda describes several drugs of plant, mineral, and animal origin for their wound healing properties under the term Vranaropaka. Most of these drugs are derived from plant origin[5].

Heliotropium indicum (Boraginaceae) - commonly called as Indian Turnsole, is a herb with slightly woody at base. It is distributed in the tropical and temperate regions of the world and found throughout India[6].

The whole plant is claimed to possess medicinal properties. In ayurveda the juice of leaves applied on boils, pimples, ulcers, sores and wounds to cure. In Belize, the plant used for diarrhea, malaise or vomiting in infants. The leaves are used for the treatment of ophthalmic disorders, erysipelas, pharyngodynia, anti-tumor and anti-inflammatory. The roots are used as astringent, expectorant and febrifuge. The extract of leaves was proved to be active against Schwart's leukemia and anti-inflammatory activities[7].

However there is no scientific data available to authenticate the folklore claim. Hence the present study was undertaken to evaluate the wound healing property of ethanol and aqueous extract of H.indicum on various animal wound models in Wistar rats.

MATERIALS AND METHODS

Dexamethasone, Pentobarbitone, Hydroxyproline, chloramines T, thiobarbituric acid, 1,1,3,3, tetra methoxy propane, bovine serum albumin and calf thymus DNA were obtained from Sigma Aldrich Chemical Company, St Louis, USA. All other chemicals used were of analytical grade.

Plant Material

The leaves of H.indicum were collected from Udupi, Karnataka, during October. It was authenticated by Department of Botany, Poorna Prajna College, Udupi, Karnataka, India. A voucher specimen (H.I.119) was deposited in the herbarium of our Institute.

Preparation of Extract

Leaves were shade dried and powdered mechanically. The powder was loaded into soxhlet extractor in 8 batches of 250 g each and was subjected to extraction for about 30–40 h with 95% ethanol. After extraction the solvent was distilled off and the extract was concentrated under reduced pressure using a rotary flash evaporator (Buchi, Flawil, Switzerland) to a syrupy consistency. Then it was dried in the dessicator.

For aqueous extract, 250g of powdered leaves was macerated with 1000 ml of distilled water for three days with intermittent stirring, filtered and concentration. The dried extract was stored at 4°C until used. Both the extracts were subjected to preliminary phytochemical tests.

Drug Formulations

For topical application ointment of the extract was prepared using 2% sodium alginate as aqueous base containing 5% w/w of drug extracts. Oral suspension was prepared using 1% gum tragacanth containing, 500 mg/ml of aqueous and ethanolic leaf extracts.

Animals

Twelve week-old healthy Wistar rats (150–200 g) of either sex procured from Indian Institute of Sciences Bangalore were used for this study. They are maintained under standard conditions (temperature 22 ± 2^{o}C, relative humidity 60±5% and 12 h light/dark cycle).The animals were housed in sanitized polypropylene cages containing sterile paddy husk as bedding. They had free access to standard pellet diet and water ad libitum. Experiments were conducted between 9:00 to14:00 h. Each rat was used only once. The Institutional Animal Ethics Committee approved the experimental protocol. All the animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the "National Academy of Sciences" and published by the "National Institute of Health".

Excision Wound

The rats were inflicted with excision wounds under light ether anesthesia. A circular wound of about 500 sq. mm was made on depilated ethanol sterilized dorsal thoracic region of the rats. The animals were divided into three groups of six each. The animals of group I was left untreated and considered as the control. Animals of group II and III were treated with 50 mg of ointment prepared from ethanolic and aqueous leaf extract respectively. The ointment was topically applied once daily starting from the day of the operation till complete epithelialisation. The parameters studied were wound closure and epithelialisation time. The wound were traced on mm^2 graph paper on days 3, 6, 9, 12, 15, 18 and thereafter on alternate days until healing was complete. The percentage of wound closure was calculated. The period of epithelialisation was calculated as the number of days required for falling of the dead tissue remnants of the wound without any residual raw wound. Also the granulation tissue formed on day 4, 8 and 12 was harvested to estimate some biochemical parameters like protein, DNA, collagen and lipid peroxides[8].

Incision Wound

In incision wound model, 6 cm long paravertebral incisions were made through the full thickness of the skin on either side of the vertebral column of the rat. The wounds were closed with interrupted sutures of 1 cm apart. The animals were divided into three groups of six animals each. Group I animals were control and left untreated. Animals in groups II and III were treated with 50 mg of ointment prepared from aqueous and ethanolic leaf extract respectively. The ointment was topically applied once in a day. The sutures were removed on the 8^{th} post wound day. The skin breaking strength of the wounds was measured on the 10^{th} day[9].

Dead Space Wound

The animals were divided into three groups of 6 rats in each group. Group I served as the control, which received 1ml of 1% gum tragacanth/Kg body weight orally. The animals of group II and III received oral suspensions of ethanol and aqueous leaf extracts respectively (500 mg/Kg, body weight). Under light ether anesthesia, dead space wounds were created by subcutaneous implantation of sterilized cylindrical grass piths (2.5 cm x 0.3 cm), one on either side of the dorsal paravertebral surface of the rat[10]. The granulation tissues formed on the grass piths were excised on the 10^{th} post wounding day and the breaking strength was measured. Simultaneously, harvested granulation tissue was subjected to hydroxyproline estimation[11].

RESULTS

Significant promotion of wound-healing activity was observed in both aqueous and ethanol leaf extracts in all the three wound models namely excision, incision and dead space wound. In excision wound model, the mean percentage closure of wound area was calculated on the 3, 6, 9, 12, 15 and 18 post wounding days. The ethanol leaf extract treated animals showed faster epithelialisation of wound than the animals treated with aqueous leaf extract as shown in **(Table-1).**

There was a significant increase in protein and collagen contents on the day 4 and 8 of healing in aqueous and ethanolic extract treated animals in comparison to control and a decrease by day 12. Increase in total protein contents correlated well with increased collagen content in the granulation tissue in the treated groups. There was also an increase in DNA content on the day 4 and 8 of healing in treated rats and a decrease on day 12 but effect is not significant. The reduction in lipid peroxides was very significant on all the days in treated animals when compared to control group as shown in **(Table-2).**

In incision wound model, ethanol and aqueous leaf extract treated animals showed increase in breaking strength (496.41±4.30) and (465.74±3.63) respectively when compared to the control (240.46±3.28).

In dead space wound model, ethanol leaf extract treated animals showed significant increase in dry weight of granulation tissue and breaking strength followed by aqueous leaf extract treated group of animals. Estimation of hydroxyproline content in the granulation tissue revealed that the animal groups treated with ethanol leaf extract had high hydroxyproline content followed by the aqueous leaf extract treated group as shown in **(Table-3).**

DISCUSSION

Granulation, collagen maturation and scar formation are some of the many phases of wound healing which run concurrently but independent of each other. The use of single model is inadequate and no reference standard exists that can collectively represent the various phases of wound healing. Hence three different models have been chosen in our study to assess the effect of H.indicum on wound healing.

Wound healing is a fundamental response to tissue injury that results is restoration of tissue integrity, which is due to the synthesis of connective tissue matrix. Collagen is a major protein of the extracellular matrix and is the component that ultimately contributes to wound strength. Breakdown of collagen liberates free hydroxyproline and its peptides. Measurement of hydroxyproline could be used as an index for collagen turnover. The data depicted in table-3 reveal that the hydroxyproline content in granulation tissue of the treated animals was significantly increased when compared to control group indicating increased collagen turnover. Increase in breaking strength of granulation tissue of treated animals indicated the enhanced collagen maturation by increased cross linking. In addition, increase in dry granulation tissue weight also indicated the presence of higher protein content[11].

In the present investigation, preliminary phytochemical analysis of leaf extract revealed the presence of alkaloids, saponins and tannins. Any drug that inhibits lipid peroxidation is believed to increase the viability of collagen fibrils by increasing the strength of collagen fibres, increasing the circulation, preventing the cell damage and by promoting the DNA synthesis[12]. Tannins also known to promote the wound-healing process mainly due to their astringent and antimicrobial property, which seems to be responsible for wound contraction and increased rate of epithelialisation[13]. Thus, wound-healing potency of H.indicum may be attributed to the phytoconstituents present in it, which may be either due to their individual or additive effect that fastens the process of wound healing. Between the two extracts studied, the ethanol leaf extract was found to possess better wound-healing property. However which components of the extract are responsible for this effect was not investigated. Further phytochemical studies are in progress where the ethanol extract will be subjected to further fractionation and purification to identify and to isolate the active compound(s) responsible for these pharmacological activities. The present findings provide scientific evidence to some of the ethno medicinal properties of H. indicum.

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Table 1: Effect of topical application of Aqueous and ethanol leaf extracts of *H. indicum* on healing of excision wound model

Group	Post wounding days							Period of epitheliasation
	0 day	3rd day	6th day	9th day	12th day	15th day	18th day	
Control	511.91± 0.46 (0.00)	484.53± 1.49 (5.34)	404.21± 1.14 (21.03)	357.62± 0.58 (30.14)	277.85± 0.72 (45.72)	191.16± 0.54 (62.65)	88.32± 0.50 (82.74)	24.29±0.23
Aqueous extract	506.68 ±2.12* (0.00)	462.69 ± 1.49* (8.48)	342.40 ± 0.54* (32.22)	280.26 ± 0.54* (46.46)	147.46 ± 0.57* (70.69)	65.22 ± 0.60* (86.93)	7.40 ± 0.43* (98.51)	19.13±0.59*
Ethanol extract	508.81± 1.51* (0.00)	444.20± 1.18* (12.89)	332.64± 0.58* (34.82)	269.25± 0.55* (47.27)	142.40± 0.43* (72.28)	18.60± 0.43* (96.36)	0* (100)	17.76±0.19*
One-way ANOVA F	12.87	25.19	15.54	12.70	10.52	12.31	15.43	11.59
One-way ANOVA P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Values are expressed as mean ±SEM; df=3, 20; n=6 animals in each group; Number in parenthesis indicate percentage of wound contraction; *P< 0.001 when compared to control

Table 2: Effect of aqueous and ethanol leaf extracts of *H. indicum* on biochemical parameters in excision wound model

Biochemical parameters	Post wounding Day	Group I	Group II	Group III
protein (mg/100mg wet weight)	4th day	3.57±0.12	5.05 ± 0.11*	4.93±0.09*
	8th day	7.07± 0.08	8.62 ± 0.10*	8.46 ± 0.08*
	12th day	6.02 ± 0.09	7.69 ±0.09$	7.60 ±0.07$
DNA (mg/100mg wet weight)	4th day	1.55 ± 0.07	1.98 ± 0.07	1.92 ± 0.08
	8th day	5.39 ± 0.10	5.97 ± 0.08	5.76 ± 0.16
	12th day	4.26 ± 0.13	4.90 ±0.12	4.67 ± 0.12
Collagen (mg/100mg wet weight)	4th day	2.34 ± 0.11	3.91 ± 0.12*	3.79 ±0.14*
	8th day	4.82 ± 0.16	7.03 ± 0.27$	6.57 ± 0.10$
	12th day	3.78 ± 0.16	5.34 ± 0.27*	5.26 ± 0.17*
Lipid peroxides (n mole MDA/100 mg wet weight)	4th day	1357 ± 30	570 ± 13#	649 ± 15#
	8th day	978 ± 15	372 ± 16#	414 ± 10#
	12th day	692 ± 24	200 ± 07#	303 ± 10#

Values are expressed as mean ±SD; n=6 animals in each group;
Analysis done with One-Way ANOVA and Newman-Keuls Multiple Comparison Test
Values are significant at *P<0.05, $P<0.01, #P<0.001 when compared to control

Table 3: Effect of aqueous and ethanol leaf extracts of *H. indicum* on healing of dead space wound model

Group	Granulation tissue dry weight (mg/100g)	Breaking strength (g)	Hydroxyproline (mg/100g)
Control	87.94 ±0.61	230.46 ± 2.57	1398.66 ± 1.02
Aqueous extract	145.34 ± 0.61	346.12 ± 3.53	1970.33 ± 0.80
Ethanol extract	185.46 ± 0.49	388.72 ± 3.25	2252.0 ± 0.57
One-way F	11.02	10.89	15.03
ANOVA P	<0.001	<0.001	<0.001

Values are expressed as mean ±SEM; df=2, 15; n=6 animals in each group; *P<0.001 when compared to control

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