Int. J. Chem. Sci.: 7(3), 2009, 1857-1866

EVALUATION OF WOUND HEALING ACTIVITY OF *NARAVELIA ZEYLANICA* LEAVES

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ABSTRACT

Present study is about the wound healing activity of ethanol and aqueous extracts of N. *zeylanica* 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 used with 5% of aqueous and ethanol extracts. For oral administration, 1% gum tragacanth suspension with 250 mg/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 N. *zeylanica* possesses better wound healing property compared to the aqueous extract.

Key words : Dexamethasone, Wound contraction, Wound breaking strength, Period of epithelialization, Wound models.

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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].

N. *zeylanica* (Ranunculaceae) is a climbing shrub with tuberous roots; wiry stem, strong tendrils. The plant is richly available all around south India[6]. In Ayurveda, the plant has been extensively used by native peoples as an astringent, bitter, antipruritic and anti-inflammatory. It is also useful in pitta, helminthiasis, dermatopathy, leprosy, rheumatalgia, odontalgia, cephalalgia, colic inflammation and wound healing and ulcer protection. The root and stem have a strong penetrating smell and is used to relieve malarial fever and headache. Root and stem paste is applied externally for psoriasis, itches and skin allergies. N. *zeylanica* is used as a source of drug for intestinal worms, skin disease and toothache, particularly in Kerala. The traditional medicine practitioners in Karnataka are using the leaf and stem juices for treating psoriasis and dermatitis[7].

A review of the literature mentioned the wound healing property of this plant. 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 N. *zeylanica* on various animal wound models in Wistar rats.

EXPERIMENTAL

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

Preparation of extracts

The leaves of N. *zeylanica* were collected from Udupi, Karnataka, during October. It was authenticated by Dr. Gopalakrishna Bhat, Department of Botany, Poorna Prajna College, Udupi, Karnataka, India. A voucher specimen (H. S. 198) was deposited in the herbarium of our institute.

Extraction

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. The yield was about 13% w/w.

For aqueous extract, 250 g of powdered leaves was macerated with 1000 mL of distilled water for three days with intermittent stirring, filtered and concentrated (yield 11.8% w/w). The dried extract was stored at 4ºC until used. Both the extracts were subjected to preliminary phytochemical tests.

Drug formulations

For topical separate ointment formulation 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, 250 mg/mL of aqueous and ethanolic of leaf extracts.

Animal care and handling

This was done as per the guidelines set by the Indian National Science Academy New Delhi, India. Twelve- week-old healthy Wistar rats (150–200 g) of either sex procured from the Indian Institute of Sciences, Bangalore, Karnataka were selected for the study. They were housed under controlled conditions of temperature of 23 ± 2°C, humidity of 50 ± 5% and 10–14 h of light and dark cycles, respectively. The animals were housed individually in polypropylene cages containing sterile paddy husk (procured locally) as bedding throughout the experiment and had free access to sterile food (animal chow) (M/s Hindustan Lever Ltd.) and water *ad libitum.* The study was undertaken after obtaining the approval of Institutional Animal Ethical Committee. Acute toxicity study was conducted for both the extracts by the stair-case method[8].

Study design

Acute toxicity studies: Healthy Wistar rats of either sex were chosen and were divided into four groups (n = 6). They were administered single dose of ethanolic extract of N. *Zeylanica* orally with increasing doses of 100, 300, 1000 and 2500 mg/Kg body weight, respectively. The dose up to 2.5 g/Kg was well tolerated without producing any signs of toxicity and mortality. 10% of the maximum tolerated dose i. e. 250 mg/Kg was selected for the study.

Excision wound

The rats were inflicted with excision wounds as described by Morton and Malone[9] 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 1st were left untreated and considered as the control. Animals of group II and III were treated with 50 mg of ointment prepared from aqueous and ethanolic leaf extract of N. *zeylanica*. The ointment was topically applied once a day, 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 and 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. And also the granulation tissue formed on day 4, 8 and 12 was used to estimate some biochemical parameters like protein, DNA, collagen and lipid peroxides.

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 as described by Ehrlich and Hunt[10]. 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. The ointment was topically applied once in a day. The sutures were removed on the 8th post wound day. The skin breaking strength of the wounds was measured on the 10^{th} day as described in the method of Lee[11].

Dead space wound

The animals were divided into three groups of 6 rats in each group. Group I served as the control, which received 1 mL of 1% gum tragacanth/Kg body weight, post orally. The animals of group II and III received oral suspensions of aqueous and ethanol leaf extracts, respectively (250 mg/Kg, body weight, post orally). 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[12]. The granulation tissues formed on the grass piths were excised on the 10^{th} post wounding day and the breaking strength was measured. Simultaneously, granulation tissue so harvested was subjected to hydroxyproline estimation following the method of Woessner[13].

RESULTS AND DISCUSSION

Significant promotion of wound-healing activity was observed in both; aqueous and ethanolic leaf extracts in all the three wound models excision, incision and dead space wound. The preliminary phytochemical analysis of aqueous leaf extract revealed the presence of flavonoids, saponins, tannins and glycosides whereas ethanolic extract showed positive test to flavonoids, saponins, tannins, glycosides, sesquiterpenes and triterpenoids. The LD_{50} of aqueous and ethanol leaf extracts were found to be 2500 mg/Kg, body weight. One tenth of the dose was selected[8] for the evaluation of wound-healing activity i. e., 250 mg/Kg, body weight. 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 ethanolic leaf extract treated animals showed faster epithelialisation of wound (17.86 ± 0.19) than the animals treated with aqueous leaf extract (19.03 ± 0.59).

Table 1. Effect of topical application of aqueous and ethanol leaf extracts of N. zeylanica 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)	463.69 ± 1.49* (8.48)	343.40 ± 0.54* (32.22)	271.26 ± 0.54* (46.46)	148.46 ± 0.57* (70.69)	66.22 ± 0.60* (86.93)	7.50 ± 0.43* (98.51)	19.03 ± 0.59*
Ethanol extract	508.81 ± 1.51* (0.00)	443.20 ± 1.18* (12.89)	331.64 ± 0.58* (34.82)	268.25 ± 0.55* (47.27)	141.40 ± 0.43* (72.28)	18.50 ± 0.43* (96.36)	0* (100.00)	17.86 ± 0.19*
One-way F	12.87	25.19	15.54	12.70	10.52	12.31	15.43	11.59
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

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. There was a significant reduction in lipid peroxides on the day 4, 8 and 12 of healing in treated rats in comparison to control.

In incision wound model, ethanol and aqueous leaf extracts treated animals showed increase in breaking strength (494.41 ± 4.30) and (463.74 ± 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 (184.46 ± 0.49) and breaking strength (387.72 ± 3.25) 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 (2250.00 ± 0.57) followed by the aqueous leaf extract treated group (1979.33 ± 0.80). However, the control group showed less hydroxyproline content (1398.66 ± 1.02).

Table 2 : Effect of aqueous and ethanol leaf extracts of N. *zeylanica* 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	5.06 ± 0.11*	4.92 ± 0.09*
	8th day	7.07 ± 0.08	8.63 ± 0.10*	8.45 ± 0.08*
	12th day	6.02 ± 0.09	7.70 ± 0.09$	7.59 ± 0.07$
DNA (mg/100mg wet weight)	4th day	1.55 ± 0.07	1.89 ± 0.07	1.91 ± 0.08
	8th day	5.39 ± 0.10	5.98 ± 0.08	5.75 ± 0.16
	12th day	4.26 ± 0.13	4.93 ± 0.12	4.66 ± 0.12
Collagen (mg/100mg wet weight)	4th day	2.34 ± 0.11	3.95 ± 0.12*	3.78 ± 0.14*
	8th day	4.82 ± 0.16	7.04 ± 0.27$	6.56 ± 0.10$

Biochemical parameters	Post wounding day	Group I	Group II	Group III
Lipid peroxides (n mole MDA/100 mg wet weight)	12th day	3.78 ± 0.16	5.35 ± 0.27*	5.25 ± 0.17*
	4th day	1357 ± 30	573 ± 13#	648 ± 15#
	8th day	978 ± 15	374 ± 16#	413 ± 10#
	12th day	692 ± 24	201 ± 07#	302 ± 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 N. *zeylanica* 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	146.34 ± 0.61	347.12 ± 3.53	1979.33 ± 0.80
Ethanol extract	184.46 ± 0.49	387.72 ± 3.25	2250.0 ± 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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Accepted : 22.05.2009