

EVALUATION OF WOUND HEALING ACTIVITY OF LEAF EXTRACT OF *ALSTONIA SCHOLARIS* LINN. IN RATS

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ABSTRACT

Alstonia scholaris Linn. (Apocynaceae) is a medium sized tree known for its pharmacological activities like antimicrobial, antimalarial, anticholeric properties etc. The present study was aimed to assess the wound healing potential of crude methanol extract of *A. scholaris* (leaf) using three types of wound models in rats as incision wound, excision wound and dead space wound. The results were obtained with respect to the parameters such as wound contraction, epithelialization time, tensile strength, hydroxyproline content and granuloma weight. Enhanced wound contraction and decreased epithelialization time were observed in extract-treated animals in excision wound model. The tensile strength of the incision wound was significantly increased in comparison to control group. The granulation tissue weight and hydroxyproline content in the dead space wounds were also increased significantly in treated animals compared with control.

Keywords: Wound, Rats, Herbal medicine, Plant extract

INTRODUCTION

Wounds arise from injury by various agents. Healing of wounds is an important part of the reparative process. Like the alchemist's dream of turning base metal into gold, efforts aimed at achieving a perfect wound healing has pushed many researchers into trying various therapeutic options which were thought to aid or accelerate the wound healing process. Wound care can be traced back to early civilizations, and many of these treatments were based on the use of herbal remedies. Approximately one-third of all traditional medicines in use are for the treatment of wounds and skin disorders, compared to only 1-3% of modern drugs [1]. Abundant scientific literature is available about the involvement of medicinal plants in affecting various phases of the wound healing process, such as coagulation, inflammation, fibroplasia, collagenation, epithelialization and wound contraction are [2-5]. However, many traditional remedies are based on systematic observations and methodologies and have been time-tested but for many of them, scientific evidence is lacking. The cheaper and more effective the agent, the better for the patient.

Alstonia scholaris, popularly known as the "Saptaparni" or 'Devil's tree' is widely distributed in dried forests of India, Western Himalayas, Western Ghats and in the Southern region. It is a well known remedy for the treatment of various types of disorders in the ayurvedic, homoeopathic and folklore system of medicine in India [6]. The aim of the present investigation was to evaluate the wound healing potential of leaf extract of *A. scholaris* using different experimental models.

MATERIALS AND METHODS

Plant material and preparation of the extract

The fresh young leaves of *A. scholaris* were collected from local areas in Davanagere district, Karnataka during early summer. The plant material was shade dried and then powdered using a mechanical grinder. 100 grams of pulverized plant part was extracted successively in 500 ml of petroleum ether, ethyl acetate and methanol (LR grade, Merck, India) using Soxhlet apparatus. At the end of extraction, extracts were filtered under vacuum through a Whatman No. 1 filter paper and the process repeated until all soluble compounds had been extracted. The filtrate obtained was concentrated *in vacuo* using a Rotavapor (Buchi Flawil, Switzerland). The extracts were stored at 4 °C in air tight bottle until further use.

Animals

Healthy inbred Albino rats of Wistar strain, weighing about 150-200 g of either sex were obtained from Venkateshwara enterprises, Bangalore. All animals were housed, fed and treated in accordance with the in-house guidelines for animal protection. Animals were kept for 2 weeks to be acclimatized prior to the investigation. During this time they were given standard pellet diet and water *ad libitum*. Also, they were periodically weighed before and after experiments. Animals were closely observed for any infection; those which showed signs of infection were separated and excluded from the study. The rats were anaesthetized prior to infliction of the experimental wounds. Acute toxicity study was performed by stair case method [7]. 200 mg/kg body weight was taken as the therapeutic dose of the leaf methanol extract of *A. scholaris*. The study was performed with due permission from Institutional Animal Ethics committee (SETCP/IAEC/07/462).

Wound healing activity

The animals were kept under starvation for 12 hrs prior to wounding. Wounds were made on the animals under light ether anaesthesia. Twenty rats in all were used in the study.

They were divided into three groups containing six animals each as follows:

Group 1: The control group. The animals in this group had their wounds treated with normal saline.

Group 2: Animals in this group had their wounds treated with 100 mg/kg b.w. crude methanolic extracts of *A. scholaris*.

Group 3: Animals treated with 200 mg/kg b.w. crude methanolic extracts of *A. scholaris*.

Control group of animals were given 1ml of normal saline; test group animals received the suspension of methanolic extract by gavage from the day of wounding. The wound healing study was undertaken in excision wound, incision wound and dead space wound models.

Excision wound model

Under light ether anaesthesia each animal was secured to operation table in its natural position. An impression was made on the depilated dorsal thoracic central region of the rats, 5.0 cm away from the ears by using a round seal of 2.5 cm diameter. The extract was given everyday up to 16th day [8].

Incision wound model

Each animal was secured to operation table in its natural position under light ether anesthesia. Two Para-vertebral straight incisions of 6.0 cm each were made on the depilated back of the animals by cutting through the entire skin with the help of a sterilized scalpel. After complete haemostasis, the wounds were closed (sutured) using 2-zero silk threads as interrupted sutures about 1.0 cm apart with the help of a straight round bodied needle. The sutures were removed on 8th post wounding day [9].

Dead space wound model (Granuloma studies)

Under light ether anesthesia, dead space wounds were created by subcutaneous implantation of sterilized cylindrical grass piths (2.5cm X 0.3cm), one on either side of the dorsal paravertebral surface of rat [10]. The granulation tissues formed on the grass piths were excised on 10th post wounding day and the breaking strength was measured. Simultaneously, granulation tissue so harvested was subjected to hydroxyproline estimation [11].

Wound healing evaluation parameters

Wound contraction and epithelialization time

An excision wound margin was traced after wound creation by using transparent paper and area measured by graph paper. Wound contraction was measured in each 4 days interval, until complete wound healing and expressed in percentage of healed wound area. The percentage of wound closure was calculated. The period of epithelialization was calculated as the number of days required for falling of the dead tissue without any residual raw wound.

Collagen content from regenerated tissues of excision wound

The regenerated tissue collected from the excision wounds were cut into two pieces. They were washed with 0.5 M sodium acetate and then suspended in ten parts (w/v) of 0.5M acetic acid and stirred intermittently for 48 hrs. The solution was centrifuged at 5600 rpm for 2 hrs intermittently in the micro centrifuge, and then sodium chloride (5% w/v) solution was added to precipitate the collagen. The collagen so precipitated was filtered using a preweighed Whatman filter paper No.1. The weight of the collagen precipitate obtained was calculated by taking difference between the initial and final weight of the filter paper. The same procedure was followed for the animals of the control and both the test groups.

Measurement of tensile strength

Tensile strength is the resistance to breaking under tension. It indicates how much the repaired tissue resists to breaking under

tension and may indicate in part the quality of repaired tissue. Sutures were removed on the day 8 after wound creation and the tensile strength was measured. The skin breaking strength of the 10-day-old wound was measured by continuous constant water technique of [12]. The skin breaking strength is expressed as the minimum weight (in grams) of water necessary to bring about the gapping of the wound.

Hydroxyproline estimation

Tissues were dried in a hot air oven at 60-70 °C to constant weight and were hydrolyzed in 6 N HCl at 130 °C for 4 hrs in sealed tubes. The hydrolysate was neutralized to pH 7.0 and was subjected to Chloramine-T oxidation for 20 min. The reaction was terminated by addition of 0.4 M perchloric acid and color was developed with the help of Ehrlich reagent at 60 °C [13] and measured at 557 nm using a spectrophotometer.

Granuloma weight

The granulomas were excised from the surrounding tissue on 10th post wounding day and were dried at 60 °C to obtain constant dry weight [14].

Histological Study

Granulation tissues obtained on day 10 from the test and control group animals were sectioned for histological study and stained for collagen with Van Gieson's stain.

Statistical analysis

Results, expressed as mean \pm SEM were analyzed statistically using the student's t-test to identify the differences between the treated and control. The data were considered at $p < 0.01$.

RESULTS AND DISCUSSION

Wound contraction and epithelialization time

Significant wound healing activity was observed in both the group of animals treated with different doses of methanol extract. The percentage of closure of wound was significant in the animals treated with 200 mg/kg b.w. of methanol extract (90.18 \pm 1.01) on day 16th and (98.75 \pm 0.15) on day 20th, respectively. While in control animals it was (87.51 \pm 0.54) and (93.15 \pm 0.33), respectively. Also, the group treated with 100 mg/kg b.w. of extract showed significant value (97.35 \pm 0.30) on 20th day in comparison with control group. It was found that the mean time taken for complete epithelialization of the excision wound in 200 mg/kg dose of methanol extract treated group was less than the animals treated with 100 mg/kg of extract and the data are shown in Table 1.

Table 1: The effect of leaf methanol extract of *A. scholaris* on excision wound model

Treatment	Percentage of closure of excision wound area					Epithelialization in days
	Day 4	Day 8	Day 12	Day 16	Day 20	
Control (200 mg/kg b.w.)	20.12 \pm 0.65	61.31 \pm 0.53	79.45 \pm 0.66	87.51 \pm 0.54	93.15 \pm 0.33	24.63 \pm 0.65
MethanolExtract(200 mg/kg b.w.)	31.75 \pm 1.24**	67.14 \pm 0.69**	85.20 \pm 0.56**	90.18 \pm 1.01**	98.75 \pm 0.15**	20.67 \pm 0.76
	27.80 \pm 1.8*	65.10 \pm 0.22	81.02 \pm 0.26**	90.48 \pm 0.36	97.35 \pm 0.30**	22.01 \pm 0.21

Values are mean \pm S.E.; $n = 6$ in each group. * $P < 0.01$ is compared to control.

Collagen content from regenerated tissues of excision wound (mg/kg)

The collagen content was estimated from regenerated tissue for control as well as treated groups. There was a significant increase in collagen content on 4th, 8th, 12th, 16th and 20th day in 200 mg/kg extract treated group compared to the 100 mg/kg extract treated and control group. The increase in collagen content in 100 mg/kg

extract treated group was also significant except for the 20th day compared to the control group.

Statistical analysis of the results by ANOVA followed by student's t test showed that there was a significant difference between all the groups ($p < 0.001$) and the ethanol extract was found to be highly effective than aqueous extract (Table 2).

Table 2: The effect of leaf methanol extract of *A.Scholaris* on collagen content from regenerated tissues of excision wound (mg/kg)

Treatment	Day 4	Day 8	Day 12	Day 16	Day 20
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Control	10.15±0.82	17.79±0.97	23.63±1.32	32.23±0.87	40.97±0.70
Ethanol extract	22.52±1.68**	31.90±0.96**	39.76±0.66**	45.59±0.94**	51.58±0.64**
Aqueous extract	15.93±0.65*	22.46±0.8141*	30.91±0.6652*	33.68±0.3569*	42.71±0.7554

Values are mean±S.E.; n = 6 in each group. *P < 0.01 is compared to control.

Table 3: Wound healing effect of *A. scholaris* leaf extract on incision wound model

Treatment	Tensile strength (g)
Control	272.67±1.76
Ethanol extract	421±2.08**
Aqueous extract	326.33±2.03**

Values are mean±S.E.; n = 6 in each group. *P < 0.01 is compared to control.

Measurement of tensile strength

In incision wound model, significant increase in the tensile strength was observed in animals treated with 200 mg/kg of methanol extract followed by 100 mg/kg of extract treated group, indicating the effect of *A. scholaris* leaf extract in maturation of collagen fibers

(Table 3). The values were highly significant when compared to control group (p < 0.0001).

Hydroxyproline estimation and Granuloma weight

Treated group showed significant increased hydroxyproline level when compared to control group (P < 0.01) in Table 4. Granuloma weight of treated animal groups was found to be increased when compared with control group.

Histological Study

Histological sections of granulation tissue from extract treated rats showed increased and well-organized bands of collagen, more fibroblasts and few inflammatory cells (Fig. 2 & 3). Granulation tissue sections obtained from control rats revealed more inflammatory cells and less collagen fibers and fibroblasts (Fig. 1).

Table 4: The effect of *A. scholaris* leaf extract on dead space wound model

Treatment	Hydroxyproline content (µg)	Tensile strength (g)	Granuloma dry weight (mg/100g)
Control	6.2±0.1033	287.4±4.23	35.17±2.973
Ethanol extract	8.917±0.1352**	378.4±19.12*	47.48±2.082*
Aqueous extract	6.917±0.2330*	312.6±5.372*	45.28±1.896*

Values are mean±S.E.; n = 6 in each group. *P < 0.01 is compared to control.

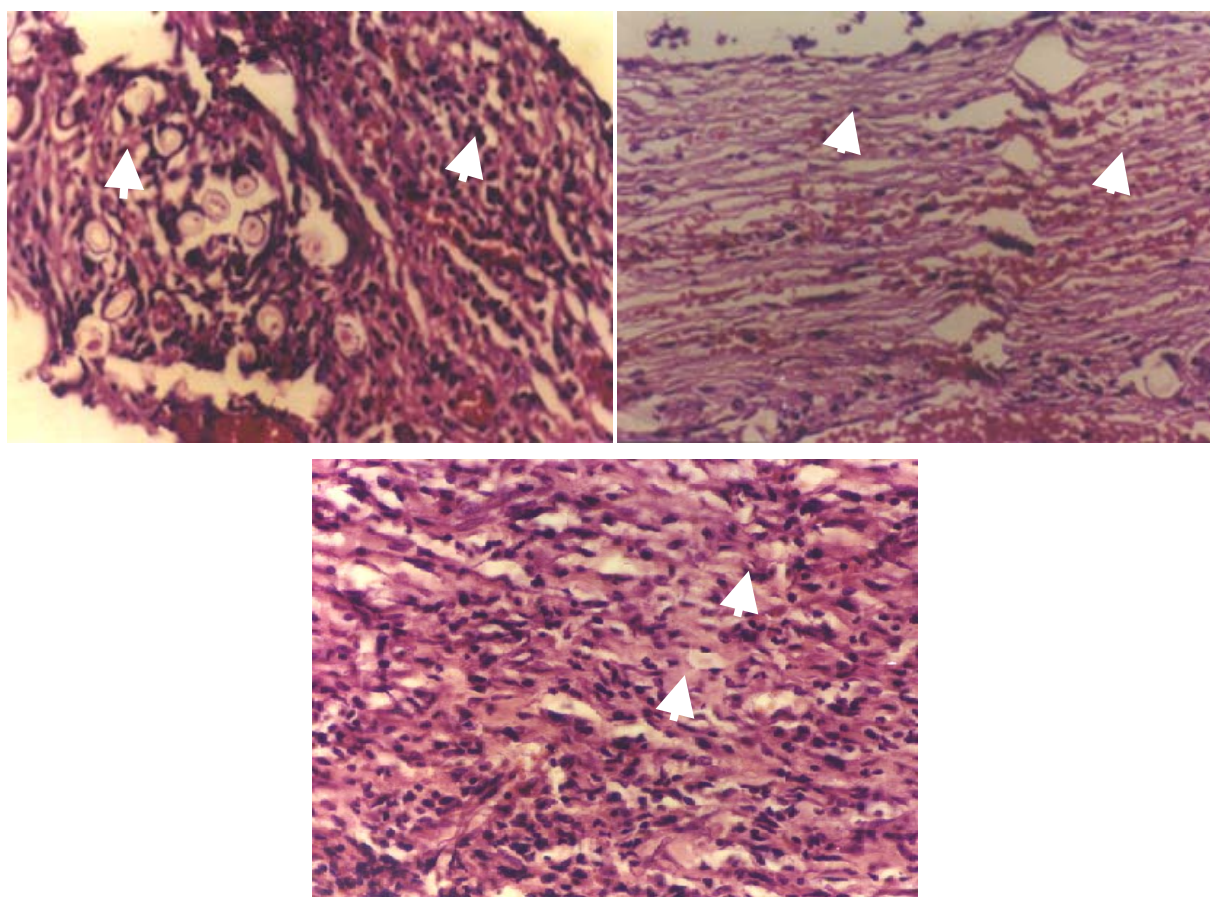


Fig. 1: a) Histology of the granulation tissue obtained from the control rats received plain water (Van Gieson stain) b) Histology of the granulation tissue obtained from the test group rats treated with 200 mg/kg b.w. methanol extract (Van Gieson stain) c) Histology of the granulation tissue obtained from the test group rats treated with 100 mg/kg b.w. methanol extract (Van Gieson stain)

Wound represents a major health problem both in terms of morbidity and mortality. Wound healing is a fundamental response to tissue injury that results in restoration of tissue integrity. It mainly depends on the repairing ability of the tissue, type and extent of damage and general state of the health of the tissue [15]. A therapeutic agent selected for the treatment of wounds should ideally improve one or more phases of healing without producing deleterious side effects [16].

In excision wound model significant wound healing activity was observed in the animals treated with different doses of methanol extract of *A. scholaris*. Significant decrease in the period of epithelialization and increase in wound contraction rate were observed in these groups of animals. In both extract treated animals, epithelialization was completed on 20th and 21st post wounding day respectively. While in control animals, the rate of wound contraction was slow and the complete epithelialization of the excision wound was extended up to 24th post wound day.

The tensile strength depicts the strength of a healing wound and is measured experimentally by the amount of force required to disrupt it. The tensile strength increases rapidly as collagen deposition increases and cross linkages are formed between the collagen fibers, than in the beginning where a wound will be having little breaking strength because the clot will alone will be holding the edges together. In the present investigation, significant increase in the tensile strength was observed in the animals treated with the plant extracts on the 10th post wounding day. Similar observations have been reported by Shirwaikar et al. (2003) [14] and [17].

Granulation tissue formed in the final part of the proliferative phase is primarily composed of fibroblasts, collagen, edema and new small blood vessels. The increase in dry granulation tissue in the treated groups is an indication of higher protein content. The constituents present in the plant extracts may be responsible for promoting the collagen formation at the proliferative stage of wound healing. The methanol extract of *A. scholaris* demonstrated a significant increase in the hydroxyproline content in the granulation tissue indicating increased collagen turnover. Collagen, which strengthens and supports the extra cellular tissue is composed of amino acid, hydroxyproline, which has been used as a biochemical marker for tissue collagen [18].

Wound healing is a complex and dynamic process of restoring the tissue integrity in damaged tissue as closely as possible to its normal state. It has four phases; inflammation, wound contraction, epithelialization and granulation tissue formation. Inflammation starts immediately after the disruption of tissue integrity. The platelets adhere to the clotting factors and form haemostatic plug to stop bleeding from the vessels. The prostaglandins (PGE1 and PGE2) are released in the inflammation area and seem to be the final mediators of acute inflammation and may play a haemostatic role for white cells and fibroblasts. The active motile white cells migrate into the wound and start engulfing cellular debris, at the initial stages wound contraction begin slowly but became rapid after 3 or 4 days. The myofibroblasts present in the margin of the wound appear to constitute the machinery for the wound contraction. These are responsible for overlaying debris. The epithelialization of the wound mainly occurs by proliferation and migration of the marginal basal cells lying close to the wound margin. The hematoma within the wound may be replaced by granulation tissue, which consists of new capillaries and fibroblasts. The fibroblasts are responsible for production of the mucopolysaccharide ground substance. The lymphatics develop new nerve fibers and there is also formation of scar tissue in which collagen turn over increases. The increase in the formation of collagen fibers in the present study could be attributed to the effect of the methanol extract of *A. scholaris*. At the later stage the tensile strength of the wound increases correspondingly to the increase of collagen content.

CONCLUSION

The results of the present study do indicate the healing effect of crude methanol extract of powdered leaves of *A. scholaris*. The potency of the plant in healing the wounds may be attributed to the phytoconstituents like flavonoids, tannins, saponins, glycosides, steroids, and triterpenoids present in it, which may be either due to their individual or additive effect, hastening the process of wound healing. The present investigation offers scientific evidence to the folkloric accounts of the use of leaf extract of *A. scholaris* in treating cuts and wounds. However, it needs further evaluation in clinical settings before consideration for the treatment of wounds. Also, studies with purified constituents are needed to understand the complete mechanism of wound healing activity of *A. scholaris*.

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