

Healing potential of *Anogeissus latifolia* for dermal wounds in rats

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Wound healing potential of ethanolic extract of *Anogeissus latifolia* bark (ALE) for treatment of dermal wounds in rats was studied on excision and incision wound models. HPTLC of the total extract was recorded for the purpose of standardization. Various parameters of incision wound, viz. epithelization period, scar area, tensile strength and hydroxyproline measurements along with wound contraction, were used to evaluate the effect of *A. latifolia* on wound healing. The results obtained indicate that *A. latifolia* accelerates the wound healing process by decreasing the surface area of the wound and increasing the tensile strength. Nitrofurazone ointment was used as a positive control. Complete epithelization was observed within 15 days with ALE. Measurements of the healed area and the hydroxyproline level were in agreement. Antibacterial activity of ALE was studied against Gram-positive (*Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*) compared to erythromycin and tetracycline. Moderate activity was observed against all organisms. The present study provides a scientific rationale for the traditional use of *Anogeissus latifolia* in the management of skin diseases such as sores, boils and itching.

Keywords: *Anogeissus latifolia* (Combretaceae), wound healing, antibacterial activity, HPTLC

Received June 22, 2004

Accepted November 24, 2004

Wounds are physical injuries that result in an opening or breaking of the skin. Proper healing of wounds is essential for the restoration of disrupted anatomical continuity and disturbed functional status of the skin. This is a product of the integrated response of several cell types to injury. Wound healing is a complex process that results in the contraction and closure of the wound and restoration of a functional barrier (1). Cutaneous wound repair is accompanied by an ordered and definable sequence of biological events starting with wound closure and progressing to the repair and remodeling of damaged tissue (2). Repair of injured tissues includes inflammation, proliferation, and

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migration of different cell types (3). Inflammation, which constitutes a part of the acute response, results in a coordinated influx of neutrophils at the wound site. In spite of tremendous advances in the pharmaceutical drug industry, the availability of drugs capable of stimulating the process of wound repair is still limited (4). Moreover, the management of chronic wounds is another major problem due to the high cost of therapy and the presence of unwanted side effects (5, 6).

There is general consent that reactive oxygen species (ROS) are deleterious to the wound healing process due to their harmful effects on cells and tissues. Topical applications of compounds with free radical scavenging properties in patients have been shown to improve significantly wound healing and to protect tissues from oxidative damage (7).

Anogeissus latifolia (Roxb. ex DC) Wall. ex Guill. & Perr. (*Combretaceae*), is a medium to large sized tree distributed throughout India in dry deciduous forests and in the sub-Himalayan region and hills of South India up to 1300 meters. It provides important timber, with the leaves and bark being used for tanning. The bark was first examined by Reddy *et al.* (8), who isolated (+)-leucocyanidin. Later, ellagic acids and two new glycosides of ellagic and flavellagic acids were reported (9). Ethnobotanically, the bark has been reported to be used in the treatment of various skin diseases such as sores, boils and itching (10), snake and scorpion bites, stomach diseases (11), colic (12), cough (13) and diarrhea (14) though to date no biological/pharmacological report on the plant or its extract has been published. We reported the antioxidant potential of the 50% aqueous alcoholic extract of *A. latifolia* (ALE) for the first time (15).

The search for »natural remedies« for healing has drawn attention to herbals. Proanthocyanidins or condensed tannins are a group of biologically active polyphenolic bioflavonoids that are synthesized by many plants. Proanthocyanidins and other tannins facilitate wound healing (16). Since the role of free radicals in the physiology of wound is clearly defined and the plant has been reported to contain anthocyanidins and tannoid principles with potent antioxidant activity, we have studied the wound healing potential of the *A. latifolia* extract. Also, since the plant has been used traditionally in the treatment of skin diseases, antibacterial activity of the extract has been also studied for scientific validation of ethnobotanical claims.

EXPERIMENTAL

Plant material and extraction

Anogeissus latifolia (*Combretaceae*) bark was collected from Chitrakoot (Madhya Pradesh, India) during October 2002. The plants were authenticated and a voucher specimen was lodged in the Pharmacognosy Division of the National Botanical Research Institute, Lucknow, India. The plant material was air dried at room temperature and powdered coarsely. The powder obtained was macerated with 50% aqueous ethanol for a period of 24 h and filtered. The extracts were pooled, concentrated at reduced temperature on a rotary evaporator (Büchi, USA) and then freeze-dried (Freezone[®] 4.5, Labconco, USA) under high vacuum (133×10^4 mBar) and at a temperature of -40 ± 2 °C to get 16.2 g (6.5%) of the dry extract (ALE). 300 mg of ALE was incorporated into a simple ointment

base to make up 1 g (17). ALE dissolved in 50% ethanol ($100 \mu\text{g mL}^{-1}$) was used for antibacterial screening.

High performance thin layer chromatography

The high performance thin layer chromatography (HPTLC) studies of ALE were carried out on a pre-coated silica gel plate (0.2 mm, Merck 60 F 254, Germany) as the stationary phase, and chloroform/methanol/formic acid (9:1:0.1) as the mobile phase. The extract was spotted as a band using a Camag Linomat IV applicator (CAMAG, Switzerland). The plates were observed in the visible region after derivatization using an anisaldehyde sulphuric acid reagent and were scanned on a CAMAG TLC scanner III using the Wincats software.

Animals

Male Sprague – Dawley rats (160–180 g) were purchased from the animal house of the Central Drug Research Institute, Lucknow. They were kept in the departmental animal house at $26 \pm 2^\circ\text{C}$ and relative humidity 44–55%, light and dark cycles of 10 and 14 h, respectively, for one week before the experiment. Animals were given the rodent diet (Amruth, India) and water *ad libitum*. All studies were conducted in accordance with the National Institute of Health »Guide for the Care and Use of Laboratory Animals« (18).

In the experiment, the rats were divided into three groups ($n = 6$). Group 1 was the control group which received the simple ointment base, group 2 was treated with the reference standard (0.2%, *m/m* nitrofurazone ointment), group 3 received ALE ointment (33.3%, *m/m* ALE) topically on wounds created on the dorsal back of rats daily until the wounds completely healed (19). Both ointments (100 mg) were spread over 500 mm^2 area.

Excision wound model

An impression was applied on the dorsal thoracic region 1 cm away from the vertebral column and 5 cm away from the ear using a round seal of 2.5 cm diameter on the anaesthetized rat. The skin of the impressed area was excised to its full thickness to obtain a wound area of about 500 mm^2 . Haemostasis was achieved by blotting the wound with a cotton swab soaked in normal saline. Contractions, which contribute to wound closure in the first 2 weeks, were studied by tracing the raw wound. The wound area was measured by retracing the wound on a millimeter scale graph paper. The degree of wound healing was calculated (20) and hydroxyproline was measured using the method of Neuman and Logan (21).

Incision wound model

Rats were anaesthetized and two paravertebral long incisions were made through the skin and cutaneous muscles at a distance of about 1.5 cm from the midline on each side of the depilated back of the rat. Full aseptic measures were not applied and no local or systemic antimicrobial was used throughout the experiment (4). All the groups were treated in the same manner as mentioned for the excision wound model. No ligature

was used for stitching. After the incision was made, the parted skin was kept together and stitched with black silk at 0.5 cm intervals. Surgical thread (No. 000) and a curved needle (No. 11) were used for stitching. Continuous threads on both wound edges were tightened for good closure of the wounds. The wound was left undressed, ALE ointment, along with the water-soluble base ointment (control) and nitrofurazone ointment, were applied topically twice a day for 9 days. When wounds were cured thoroughly, the sutures were removed on the ninth day and tensile strength was measured with a tensiometer.

Tensile strength

The tensile strength of a wound represents the degree of wound healing. Wound-healing agents usually provide a gain in tensile strength. The sutures were removed on the ninth day after wounding and the tensile strength was measured on the tenth day. The herbal ointment along with the standard and control were applied throughout the period, twice daily for 9 days. The mean tensile strength on the two paravertebral incisions on both sides of the animals were taken as the measures of the tensile strength of the wound for an individual animal. The tensile strength of ALE ointment treated wounds was compared with the control and nitrofurazone ointment as the standard. Tensile strength increment indicated better wound healing stimulated by the applied herbal formulation. Further epithelization period and scar area were measured daily for 25 days after tensile strength determination (20).

Antimicrobial activity

The antimicrobial activity of ALE was studied against pathogens, namely *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella* sp., *Pseudomonas aeruginosa* clinical isolates. All microorganisms were provided by IMTECH, Chandigarh (India). Cultures of these bacteria were grown in a nutrient broth at 37 °C and maintained on nutrient agar (Himedia, India) slants at 40 °C. The antibacterial property was studied by the disc diffusion method (1) using ALE (100 µg µL⁻¹). Control disks contained solvents only (50% aqueous ethanol). Erythromycin and tetracycline were used as standards. Minimum inhibitory concentration (MIC) was evaluated by the micro dilution method using 5 mL of liquid broth with different concentrations of ALE in ethanol.

Statistical analysis

Pharmacological data were subjected to statistical analysis using SPSS 11.0 for Windows. The values are represented as the mean ± SEM for six rats. Paired *t*-test was used for reporting the *p* value and significance with respect to the control group.

RESULTS AND DISCUSSION

The results showed that upon administration of ALE, there was a decrease in the epithelization period, along with a visibly decreased scar area (Table I). There was a sig-

nificant increase in the tensile strength and hydroxyproline content compared to the control group and comparable to the nitrofurazone group (Table I). The observations and results obtained in this study indicate that the alcoholic extract of *A. latifolia* significantly stimulated wound contraction (Table II).

ALE also exhibited a potential inhibitory effect on all the pathogens examined, in the following order: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *E. coli* MIC being 0.56, 0.78, 0.89, 1.33 mg mL⁻¹, respectively. Erythromycin and tetracycline were used as standards (Table III). The 50% aqueous ethanol (20 mL) served as a negative control and showed no inhibiting effect. Ellagic acid has been reported to possess potent antimicrobial activity (22). Since the extract of *A. latifolia* has been found

Table I. Effect of the *A. latifolia* extract ointment on incision wounds

Topical treatment	Epithelization period (day)	Tensile strength (g)	Scar area (mm ²)	Hydroxyproline (mg per 100 mg tissue)
Control	25.3 ± 1.6	291.5 ± 21.5	56.7 ± 4.4	7.05 ± 0.4
ALE (33.3%) ointment ^b	15.0 ± 0.97 ^d	416.6 ± 21.8 ^e	30.2 ± 3.2 ^e	9.30 ± 0.54 ^e
Nitrofurazone (0.2%) ointment ^c	14.5 ± 1.6 ^e	421.2 ± 21.7 ^e	31.9 ± 3.9 ^f	9.88 ± 0.47 ^e

^a Values are mean ± SEM for six rats; ^b 60 µg mm⁻²; ^c 0.4 µg mm⁻²
Statistically significant difference in comparison with the control group: ^d $p < 0.01$, ^e $p < 0.001$, ^f $p < 0.02$.

Table II. Effect of the *A. latifolia* extract ointment on excision wounds

Topical treatment	Percentage of close of excision wound area after days			
	4	7	15	21
Control	26.2 ± 2.44	46.9 ± 3.68	64.1 ± 3.98	73.3 ± 4.1
ALE (33.3%) ointment ^b	37.7 ± 2.97 ^d	64.7 ± 4.27 ^d	90.8 ± 5.20 ^e	96.2 ± 4.9 ^e
Nitrofurazone (0.2%) ointment ^c	38.1 ± 3.19 ^d	64.5 ± 4.18 ^d	91.7 ± 4.79 ^e	96.2 ± 5.0 ^e

^a Values are mean ± SEM for six rats; ^b 60 µg mm⁻²; ^c 0.4 µg mm⁻²
Statistically significant difference in comparison with the control group: ^d $p < 0.01$, ^e $p < 0.001$.

Table III. Antimicrobial activity of *A. latifolia* extract

Microorganism	Inhibition zone (mm)		
	ALE (100 µg mL ⁻¹) in 50% EtOH	Erythromycin (10 µg mL ⁻¹)	Tetracycline (10 µg mL ⁻¹)
<i>Escherichia coli</i>	16	20	19
<i>Pseudomonas aeruginosa</i>	15	26	21
<i>Klebsiella pneumoniae</i>	12	22	26
<i>Staphylococcus aureus</i>	10	18	15

to contain ellagic acid as one of the major constituent, the antibacterial activity of the extract may be due to the presence of the ellagic acid.

The significant antibacterial effect of ALE against all the four pathogens confirmed that the compounds present in the crude extract are responsible for the effective antimicrobial activity. Thin layer chromatography studies indicated the presence of more than ten different compounds (Fig. 1), further confirming the synergistic action.

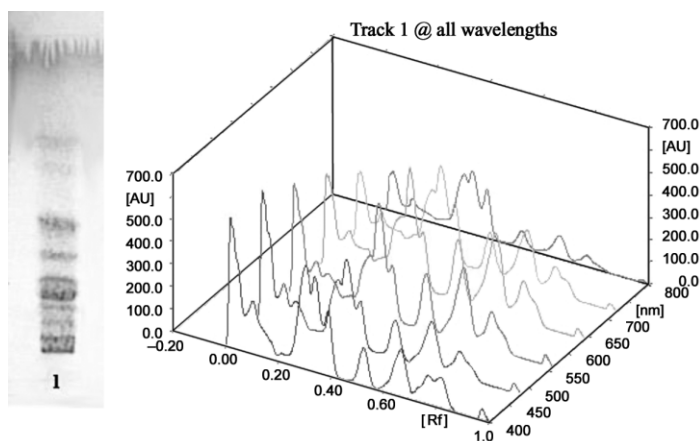


Fig. 1. General HPTLC fingerprint profile of *Anogeissus latifolia* stem bark extract after derivatization. Stationary phase: silica gel (Merck 60 F 254), mobile phase: chloroform/methanol/formic acid (9:1:0.1).

CONCLUSIONS

The use of *A. latifolia* in Indian traditional systems of medicine for various skin diseases, such as sores, boils and itching has been justified by this work, as it showed a woundhealing potential and commendable activity against several microorganisms. These findings could justify, at least partially, the inclusion of this plant in the management of wound healing in folk medicine. Since the role of free radicals and antioxidants in wound healing are very clearly defined, wound healing potential *A. latifolia* may be partly due to the potent antioxidant activity of the plant. Further experiments are needed to test the effect of this plant in the treatment of chronic wounds.

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S A Ž E T A K

Liječenje površinskih rana štakora biljkom *Anogeissus latifolia*

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Ispitivan je potencijal liječenja alkoholnog ekstrakta kore biljke *Anogeissus latifolia* (ALE) na površinskim ranama štakora. U svrhu standardizacije snimljen je HPTLC kromatogram ekstrakta. Kako bi se odredio učinak na zacjeljivanje ozljeda praćeni su različiti parametri: vrijeme potrebno za epitelizaciju, površina ožiljka, sila naprežanja, kontrakcija rane i određivanje hidroksiprolina. Dobiveni rezultati ukazuju da *A. latifolia* ubrzava proces zacjeljivanja rana jer smanjuje površinu rane i povećava silu naprežanja. Mast nitrofurazona rabljena je kao pozitivna kontrola. Unutar 15 dana primjene ekstrakta zapažena je potpuna epitelizacija rane. Mjerenja zacijeljene površine i količine hidroksiprolina bila su u skladu. Antibakterijsko djelovanje ALE proučavano je na Gram-pozitivnim (*Staphylococcus aureus*) i Gram-negativnim bakterijama (*Escherichia coli*, *Pseudomonas aeruginosa* i *Klebsiella pneumoniae*) i uspoređeno s djelovanjem eritromicina i tetraciklina. Primijećeno je umjereno djelovanje protiv svih ispitivanih bakterija. Istraživanja su pokazala znanstvenu opravdanost tradicionalne primjene biljke *Anogeissus latifolia* u liječenju različitih bolesti kože kao što su rane, gnojni čirevi i svrbež.

Ključne riječi: *Anogeissus latifolia* (Combretaceae), liječenje rana, antibakterijsko djelovanje, HPTLC

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