EVALUATION OF THE WOUND HEALING ACTIVITY OF METHANOLIC EXTRACT OF *AZADIRACHTA INDICA* (NEEM) AND *TINOSPORA CORDIFOLIA* (GUDUCHI) IN RATS

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Summary

The floral richness of the North East region cannot be neglected in context to its medicinal importance and it is expected that screening and scientific evaluation of plant extracts for their pharmacological activity may provide new drug molecule that can combat various side effects of the commercially available drugs. *Azadirachta indica* A. Juss; locally known as Neem, is reported to have significant anti-bacterial, immunomodulatory and anti-inflammatory activities, which are complementary to wound healing process. In India, various extracts of the plant *Tinospora cordifolia* (Sagunilota, Guduchi) are used for the treatment of diabetes, hepatitis as well as various types of skin diseases. Hence, the present study was aimed to evaluate the wound healing activity of the methanol extracts of leaves of these two plants using excision and incision wound models in Sprague Dawley rats. The healing effect produced by each of the plant extracts, was assessed by rate of wound contraction, skin breaking strength and histopathology of healing tissue. The methanol extract of leaves of *A. indica* and *T. cordifolia* significantly promoted the wound healing activity in both excision and incision wound models. In incision wound, tensile strength of the healing tissue of *A. indica* and *T. cordifolia* treated groups were found to be significantly higher ($P<0.05$) compared to the control group. These findings were confirmed by histopathological examination. The study thus revealed promising wound healing activity of methanolic extract of *A. indica* and *T. cordifolia* and provides a scientific rationale for the traditional use of these plants in the management of wounds.

Keywords: *Azadirachta indica*, Excision wound, Incision wound, *Tinospora cordifolia*.

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Introduction

Wound healing is a complex phenomenon that results in the restoration of disrupted anatomical continuity and disturbed functional status of the skin [1], accomplished by several processes which involve different phases including inflammation, granulation, fibro genesis, neo-vascularization, wound contraction and epithelization [2]. The basic principle of optimal wound healing is to minimize tissue damage and provide adequate tissue perfusion and oxygenation, proper nutrition and moist wound healing environment to restore the anatomical continuity and function of the affected part [3].

About 70-80% of the world's populations rely on non-conventional medicine mainly from herbal sources in their primary health care, according to a WHO report. It is specially the case in developing countries where the cost of consulting a western style doctor and the price of medication are beyond the means of most people. A medicinal plant is factually any plant which in one or more of its parts contains substances that can be used for therapeutic purposes or which are precursors for the synthesis of direct therapeutic agents. In spite of phenomenal development of the synthetic drug industry and antibiotics, medicinal plants still constitute an important part of pharmacopoeias in both the developed and developing countries. These plants are important elements of traditional medicine in virtually all cultures. The idea that certain plants had healing potential was known long before human being discovered the existence of pathogens. The plant extracts being more efficacious, free from undesirable side effects compared to their pure active principle revalidated the therapeutic benefits of herbs due to totality of constituents rather than the single molecule.

Azadirachta indica A. Juss (Meliaceae), commonly known as neem is perhaps the most useful traditional medicinal plant indigenous to India, which is cultivated widely in tropical areas of the world. Azadirachtin and other limonoids occurring in the seeds of neem tree show potent insecticidal effects against a wide variety of insect pests with low toxicity to humans [4]. Neem oil and the bark and leaf extracts have been therapeutically used as folk medicine to control leprosy, intestinal helminthiasis, respiratory disorders, constipation and also as a general health promoter [5]. The plant is reported to have anti-inflammatory and antipyretic [6], neuropsychological and antimicrobial [7], antimycotic [8], cardiovascular and immunomodulatory [9] and anti-hyperglycemic [10] activity. The plant is used for the treatment of rheumatism; chronic syphilitic sores and indolent ulcer [11]. Neem oil is used to control various skin infections [12]; bark, leaf, root, flower and fruit together cure blood morbidity, biliary afflictions, itching, skin ulcers, burning sensations and pthysis [13].

Traditionally, *Tinospora cordifolia* (Willd.) Hook. F. & Thomson (Menispermaceae) commonly known as rasayana plant, Sagunilota or Guduchi, is one of the commonly used medicinal plants in India for curing ailments ranging from common cold, skin diseases and dental infections to major disorders like diabetes, hypertension, jaundice, rheumatism, etc [14] and its rejuvenating property is well reported in Ayurvedic and other ancient literature. This plant is a glabrous, climbing shrub. Yunani doctors and local *kabiraj* use this plant as stomachic, antipyretic, analgesic, anti-inflammatory, anti-implantation, anti-diarrhoeal and in fracture of bones in animals. The plant has colony stimulating [15], immunopotentiating [16] and Kupffer cell activity modulating [17] effect. However, no comprehensive scientific study has been carried out on their wound healing activity so far.

Hence, the present study was undertaken to evaluate the wound healing activity of the methanolic extract of the leaves of *A. indica* and *T. cordifolia* by excision and incision wound model and also to compare their efficacy.

Materials and methods

Plant material

The leaves of the plants were collected from the medicinal garden of the Department of Pharmacology, College of Veterinary Science, Khanapara during the month of Feb - June, 2008, identified by Taxonomist of NEIST, Jorhat, Assam and the voucher specimen of *A. indica* (AAU/CVSc/PHT/ 03-04/04) and *T. cordifolia* (AAU/CVSc/PHT/ 03-04/ 06) were deposited.

Preparation of methanol extract

Fresh leaves of the plants were cleaned from extraneous materials, shade dried, powdered mechanically, weighed and stored in air tight container. About 250 g of powdered material was soaked in 1000 ml methanol for 72 hours in beaker and mixture was stirred every 18 hour using a sterile glass rod. Filtrate was obtained 3 times with the help of What man filter paper no 1 and the solvent was removed by rotary evaporator under reduced pressure at $<45^0$ C temperature leaving a dark brown residue which was stored in air tight container at 4^0 C until use. Recovery was 6.15 % for *A. indica* and 7.32% for *T. cordifolia*.

Phytochemical screening

The methanolic extract of the plants were tested for the presence of various active principles [18].

Determination of LD_{50}

The LD_{50} of the plants were estimated by the employment of up-and - down stair case method in mice [19]. Doses were adjusted by a constant multiplicative factor viz. 4, for this experiment. The dose for each successive animal was adjusted up and down depending on the previous outcome. The acute toxicity and gross effect of crude methanolic extract of *A. indica* and *T. cordifolia* were studied in albino mice by using 1/2 LD_{50} dose. A total of twelve numbers of male albino mice (six animals for each plant) were selected for the experiment. Animals were observed hourly for six hours and again after 24 hours. The parameters for motor activity and gross effect were determined after administration of the plant extract orally at a dose level of 2.5g /kg b. wt.

Preparation of 5% ointment of Azadirachta indica* or *Tinospora cordifolia (w/w)

Five gram of the methanolic extract of *A. indica* or *T. cordifolia* was mixed with 95 g of Vaseline to prepare 5% ointment (w/w). Himax (Indian Herbs Research & Supply Co. Ltd. Darra Shivpuri, Saharanpur) was used as standard drug.

Animals

Healthy Sprague Dawley male rats approximately of same age, weighing between 180-200 g and male albino mice weighing 18-22 g were used for the study. The animals were group housed in polypropylene cages under controlled conditions of temperature (21 ± 2^0C), humidity (50±5%), 12/12 hours of light-dark cycle and free access to standard food pellets and water was provided *ad libitum.* The study was conducted after obtaining the approval of the Institutional Animal Ethics Committee (No- 770/03/ac/CPCSEA/FVSc, AAU/ IAEC/06/21).

Dosing schedule

In excision wound model, five pert cent ointment of the plant extract was applied topically, twice daily from day 1 to the day of complete healing or the 28^{th} postoperative day, whichever was earlier. Himax was used as standard drug and the group with Vaseline application served as control. The animals were randomly allocated into four groups of six animals each. Group I served as vehicle treated control, group II received the topical application of 5% ointment of *A. indica* (w/w), group III received the topical application of 5% ointment of *T. cordifolia* (w/w) and group IV received the topical application of Himax.

Excision wound model

The excision wounds were made by excising the full thickness circular skin on the back of the animal under ether anesthesia [20]. Wound contraction was assessed by tracing the wound area on polythene paper first and subsequently transferred to $1mm^2$ graph sheet from which the wound surface area was measured on day 7, 14, 21 and 28 post wounding for calculation of percent wound contraction.

Incision wound model

The rats were anaesthetized by ether anesthesia and two longitudinal para vertebral incisions of 6 cm length were made through the skin and cutaneous muscle at a distance of 1.5 cm from the midline on either side of the vertebral column on the depilated area. The parted skin was sutured 1 cm apart. Five pert cent ointment of the plant extract was applied topically, twice daily from day 1 to day 7, post wounding. The sutures were removed on 8^{th} day and left untreated. The skin breaking strength of the healing tissue was measured on 10^{th} day [21].

Histopathological studies

For histological studies, granulation tissues collected on 7, 14, 21 and 28 days were fixed in 10% neutral formalin solution and dehydrated with a sequence of ethanol-xylene series of solution. The materials were processed by conventional paraffin embedding method. Microtome sections were prepared at 4-6 µ thickness, stained with H&E stain [22] and observed under microscope.

Statistical analysis

The statistical analysis was carried out as per standard method [23].

Results

Phytochemical screening of the methanolic extract of *A. indica* revealed the presence of glycosides, diterpenes, triterpenes, flavonoids, steroids and *T. cordifolia* revealed the presence of glycosides, alkaloids, diterpenes, triterpenes, phenolic compounds and steroids as phytoconstituents.

Methanolic extract of both the plant were found to be safe up to more than 5 gm/kg body weight, p.o. In acute toxicity study, there was no change of motor activity and gross behaviour at ½ LD_{50} i.e. 2.5 g/kg body weight p.o. during 24 h of observation after oral feeding of methanolic extract of *A. indica* or *T. cordifolia*. The low toxicity of the plant observed in this study suggests that the extracts are relatively safe for consumption and did not affect any of the parameters measured.

Effects of methanolic extract of *A. indica* and *T. cordifolia* on the wound healing in excision and incision wound model have been presented in Table 1 and Table 2, respectively and histopathological changes are shown in Fig 1-3.

Table 1. Wound healing activity of 5% (w/w) ointment of *A. indica* or *T. cordifolia* in excision wound model.

Sl no	Treatment	Area of wound during different days of observation				
		0	**7**	**14**	**21**	**28**
Group I	Control	1.61 ±0.00	1.32 ±0.03[b]	1.07 ±0.07[b]	0.71 ±0.08[c]	0.24 ± 0.06[a]
Group II	*A. indica*	1.61 ±0.00	1.14 ±0.04[a]	0.54 ±0.07[a]	0.07 ±0.03[b]	0.00 ±0.00
Group III	*T. cordifolia*	1.61 ±0.00	1.16 ± 0.08[a]	0.66 ±0.08[a]	0.37 ±0.11[a]	0.00 ±0.00
Group IV	Himax	1.61 ±0.00	1.17 ±0.01[a]	0.70 ±0.04[a]	0.25 ±0.041[a]	0.01 ±0.008[a]

(n=6, mean ± SE)
Treatment bearing same superscript don't differ significantly (P <0.01)

Table 2. Tensile strength of 5% (w/w) ointment of *A. indica* or *T. cordifolia* in incision wound model.

Sl no	Treatment	Tensile strength
Group I	Control	2.282±0.00 ±0.353
Group II	*A. indica*	5.487[b] ±1.074
Group III	*T. cordifolia*	3.081[a] ±0.245
Group IV	Himax	3.778[a] ± 1.092

(n=6, mean ± SE)
Treatment bearing same superscript don't differ significantly (P< 0.05).

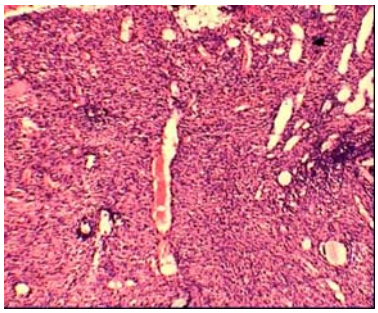

Fig 1. Histopathological changes showing abundant proliferation of connective tissue with angiogenesis in 21 day old excision wound treated with *Azadirachta indica* leaf extract. (H & E, 10X).

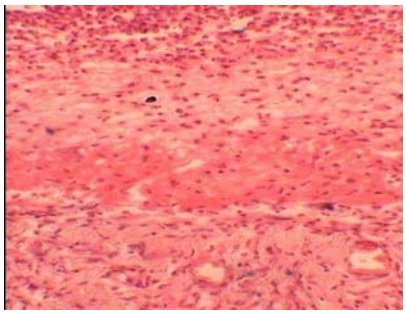

Fig 2. Histopathological changes showing Polymorph nuclear lymphocyte infiltration and proliferation of fibrous connective tissue with angiogenesis in 21 day old excision wound treated with T*inospora cordifolia* leaf extract. (H & E, 10X).

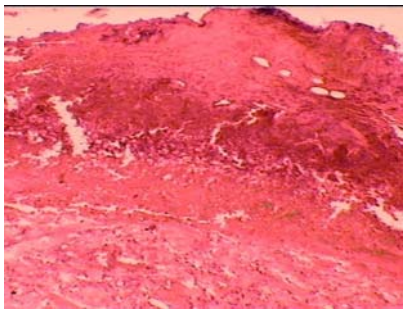

Fig 3. Histopathological changes showing bacterial infection and blood clot at the site of in 21 day old excision wound treated with the standard drug Himax. (H & E, 10X).

Discussion

In the present study, the wound healing activity of the methanolic extracts of leaves of *Azadirachta indica* and T*inospora cordifolia* was evaluated using excision and incision wound models in Sprague Dawley rats.

In the excision wound model, the mean percent of closure of wound increased significantly ($P<0.01$) from '0' day till 21st day which was 95.65% in case of *A. indica* and 77.02% in *T. cordifolia* treated group. In the standard group, percent contraction of wound area (94.68%) increased significantly ($P<0.01$) in comparison to the control group (75.15%) on 28th day post wounding (Table 1). Complete wound healing took place on 21st day itself in *A. indica* and *T. cordifolia* treated group, whereas in the standard and control group wound persisted beyond 21st day indicating better wound healing activity of the plants.

Wound healing involves a complex and superbly orchestrated interaction of cells, extracellular matrix and cytokines [24]. Granulation, collagen maturation and scar formation are some of the cascade of wound healing which run concurrently, but independent of each other [25]. The fibroblasts are responsible for the synthesis, deposition and remodeling of the extracellular matrix. The early reepithelialization and faster wound closure in *A. indica* and *T. cordifolia* treated wounds might also be associated with the increased keratinocyte proliferation and their migration to the wound surface [26].

In histopathological studies, there was abundant proliferation of connective tissues with angiogenesis in *A. indica* treated group on 21st day and in the group treated with *T. cordifolia* there was infiltration of polymorph nuclear lymphocytes (PMNL), proliferation of fibrous connective tissue and angiogenesis. A greater degree of epithelialization and fibroblastic deposition was observed in case of *Azadirachta indica* treated group, whereas, in the Himax treated group, blood clot was seen at the site in 21st day old excision wound (Fig. 1, 2 and 3). Collagen is a major extracellular matrix protein which confers strength and integrity to the tissue matrix and plays an important role in homeostasis and in epithelialization at the later phase of healing [27]. Healing is not complete until the disrupted surfaces are firmly knit by collagen [28]. Healing also involves platelet aggregation and blood clotting, formation of fibrin, an inflammatory response to injury, alteration in the ground substances, re-epithelialization and angiogenesis. The increased angiogenesis in the *A. indica* and *T. cordifolia* treated groups indicated better wound healing activity of the test plants.

In the incision wound model, significant increase ($P<0.05$) in tensile strength was observed in case of the methanolic extract of *A. indica*, *T. cordifolia* and Himax compared to the control group (Table 2). The increased tensile strength might be due to increased proliferation and transformation of fibroblast cells into myofibroblasts [29]. Myofibroblasts are believed to play key role in wound contraction by exerting tension on the surrounding extracellular matrix (ECM) and secreting ECM protein such as collagen to stabilize the contraction. Increase in tensile strength of the test and the standard treated group indicated enhanced collagen maturation by cross linking. The results of the present study clearly demonstrated that the methanolic extract of *A. indica* and *T. cordifolia* possess a definite prohealing action in normal wound healing.

Wound healing potential of *Azadirachta indica* was however, better than *Tinospora cordifolia* as evidenced by higher percentage of contraction of the wound and greater tensile strength of healing tissue in the former. Histopathologically also, it can be confirmed by absence of PMNL in 21 day old excision wound treated with *Azadirachta indica* extract, which was otherwise present in *Tinospora cordifolia* treated group.

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