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Wound healing activity of methanolic extract of leaves of *Achyranthes aspera* Linn using *in vivo* and *in vitro* model—a preliminary study

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ABSTRACT

Wound healing activity of methanolic extract of *Achyranthes aspera* Linn. was evaluated by excision and incision wound model (*in vivo*) in Sprague Dawley rats and by chorioallantoic membrane (CAM) model (*in vitro*) in 9-day-old embryonated chicken eggs. In excision wound, there was 82.23% wound contraction in control group on 21st day, whereas in *A. aspera* treated group, complete healing took place on 21st day. In the standard group, the wound persisted beyond 21st day. These findings were confirmed by histopathological examination. In incision wound, tensile strength of the healing tissue of *A. aspera* treated group was comparable with standard group and both were significantly higher compared to the control group. In CAM model, angiogenesis in *A. aspera* treated group @ 400 µg/disc was compared to the control group suggesting promising wound healing activity of methanolic extract of *A. aspera.*

Key words: *Achyranthes aspera*, Chorioallantoic membrane model, Excision wound, Incision wound

Wound healing is a complex phenomenon that results in the restoration of anatomic continuity and function, accomplished by several processes which involve different phases including inflammation, granulation, fibrogenesis, neovascularization, wound contraction and epithelization (Barua *et al.* 2009). The floral richness of the North East region cannot be neglected in context to its medicinal importance. Considering the rich diversity of this region, it is expected that screening and scientific evaluation of plant extracts for their analgesic activity may provide new drug molecule that can combat various side effects of the commercially available synthetic drugs, moreover reducing the cost of medication.

Achyranthes aspera L., locally known as Apang, is an annual, biennial, lower portion perennial erect under shrub or rather stiff herb growing upto 0.3 to 1.0 m in height. Yunani doctors and local *kabiraj* use stem, leaves and fruits as a remedy for piles, renal dropsy, pneumonia, cough, kidney stone, skin eruptions, snake bite, gonorrhea, dysentery etc. The plant has antibacterial, antitumor, anti-inflammatory, abortifacient activity and increases pituitary and uterine wet weights in ovarectomised rats and produces reproductive toxicity in male rats (Barua *et al.* 2009a). Although the local traditional healers have ethno-medical knowledge on the value of this plant, there have been no biological studies on the wound healing activity of this plant. The present study was undertaken to evaluate the wound healing activity of the methanolic extract of the leaves of this plant by excision, incision and chorioallantoic membrane (CAM) model.

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 February–June, 2008 identified by Taxonomist of NEIST, Jorhat, Assam and a voucher specimen (AAU/CVSCIPHT/01) was deposited.

Preparation of methanol extract: The leaves of the plant were cleaned, shade dried, powdered mechanically, weighed and stored in air tight container. About 250 g of powdered leaves was soaked in 1000 ml methanol for 72 h in beaker and mixture was stirred every 18 h using a sterile glass rod. Filtrate was obtained after passing through Whatmann filter paper no. 1 for 3 times and concentrated in Rotary evaporator at 50–60°C under reduced pressure leaving a dark brown

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residue which was stored in air tight container at 4°C till further use. Recovery was 6.89% (w/w).

Phytochemical screening: Phytochemical investigation of the extract was carried out for the presence of the phyto-constituents (Wagner *et al.* 1984).

Preparation of 5% *ointment of* Achyranthes aspera *(w/w):* 5 g methanolic extract of *A. aspera* was mixed with 95 g of vaseline to prepare 5% ointment (w/w). A commercial herb was used as standard drug.

Experimental Animals: Healthy Sprague Dawley rats of either sex approximately of same age, weighing between 150–250 g were used for the study. They were housed under controlled conditions of temperature (25±3°C), humidity (50±5%), 10-114 h of light-dark cycles and free access to food and water *ad lib.* Animals were housed individually in polypropylene cages containing sterile paddy husk bedding. The study was conducted after obtaining the approval of the Institutional Animal Ethics Committee. The animals were fasted for 12 h before tests to achieve better drug absorption through gastro-intestinal tract.

Determination of LD_{50}: The LDso of *A. aspera* was estimated by following up and down stair case method in mice as Per OECD TG425 guidelines. Doses were adjusted by a constant multiplication factor (4) for this experiment. The dose for each successive animal was adjusted depending on the previous outcome. The acute toxicity and gross effect of crude methanolic extract of *A. aspera* was studied in albino mice by using 1/2 LD_{50} dose. A total of 6 male albino mice were selected for each experiment. Animals were observed at hourly internal for 6 h and again after 24 h. The parameters for motor activity and gross effect were determined after administration of *A. aspera* orally @2.5 g/kg body weight.

Dosing schedule: 5% ointment of *A. aspera* was applied topically, twice daily from day 1 to the day of complete healing or the 21st postoperative day, whichever was earlier. Commercial drug was used as standard and animals with Vaseline application served as control.

In vivo model: Two models for *in vivo* wound healing activity were carried out in the present study viz. excision wound model and incision wound model. The animals were randomly allocated into 3 groups of 6 animals each. Group 1 was assigned as control, group 2 received the topical application of 5% ointment of A. *aspera* (w/w) and group 3 received the topical application of commercial drug.

Excision wound: The excision wounds were made by excising the full thickness circular skin (approximately 500 mm^2) on the back of the animal under ether anaesthesia (Morton and Malone 1972). Wound contraction was assessed by tracing the wound area on polythene paper first and subsequently transferred to 1 mm^2 graph sheet from which the wound surface area was evaluated on days 7, 14 and 21. The evaluated surface area was then employed to calculate the percentage of wound contraction, taking the initial size ofthe wound, 500 mm^2, as 100%, by using the formula [(Initial wound size—specific day wound size)/Initial wound size] × 100.

Histopathological study: On day 7, 14 and 21 animals from each of the groups were sacrificed and histopathological evaluation of granulation tissue was done as per the standard method (Lee and Luna 1968).

Incision wound: Two longitudinal para vertebral incisions of 6 cm length were made through the skin and cutaneous tissue at a distance of 1.5 cm from the midline on either side of the vertebral column on the depilated area of anaesthetized rats. The parted skin was sutured 1 cm apart. The ointment was applied till seventh day, on eight day sutures were removed and left without treatment. Skin breaking strength of the wounds was measured on the 10th day as per the standard method (Lee 1968).

In vitro model: Chorioallantoic membrane (CAM) model was used as *in vitro* model (Upadhyay *et al.* 2009). In this method, embryonated chicken eggs (9 days old) were selected and a small window (1 cm^2) was made in the shell. Through the window, a sterile disc of methyl cellulose impregnated with 200 or 400 µg/disc of methanolic extract of *A. aspera* was placed inside the triplicate sets of egg at the junction of 2 blood vessels. The window was resealed and the eggs were incubated at 37°C in a well humidified chamber for 72 h. The window was then opened and the growth of new capillary blood vessels were observed and finally compared with the control eggs containing sterile discs without any extract of the plant.

Statistical analysis: The results of various parameters were subjected to statistical analysis as per standard method (Snedecor and Cochran 1994).

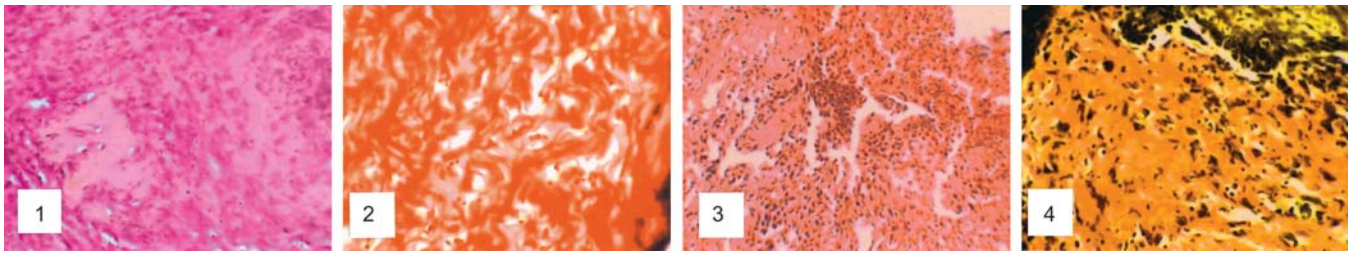
RESULTS AND DISCUSSION

Phytochemical screening of the methanolic extract of *A. aspera* showed the presence of alkaloid by Wagner's and Dragendroff's test, steroid by Salkowski's and Lieberman Burchardt's test and triterpenes by Salkowski's and Lieberman Burchardt's test. In other studies, various extracts of this plant revealed presence of 27-cyclohexyheptacosan-7-o1 and 16-hydroxy-26methylheptacosan-2-one, a long

Table 1. Effect of *Achyranthes aspera* on wound contraction in excision wound model in normal rat

Treatment	*Wound contraction (%)			
	Day 0	Day 7	Day 14	Day 21
Group 1 (Control)	0.00^{a} ±0.00^{a}	58.21^{c} ±2.019^{b}	69.48^{c} ±1.545^{c}	82.23^{c} ±1.352^{d}
Group 2 (*A. aspera*)	0.00^{a} ±0.00^{a}	81.27^{a} ±1.329^{b}	92.89^{a} ±1.488^{c}	100^{a} ±0.00^{d}
Group 3 (Standard)	0.00^{a} ±0.00^{a}	67.29^{b} ±1.821^{b}	81.72^{b} ±1.785^{c}	93.89^{b} ±1.083^{d}

*Mean in a column bearing same superscript and mean in a row bearing same subscript do not differ significantly (P<0.01), (n =6, mean ± SE).



Figs 1–4. Histopathological changes showing: **1**. granulation tissue formation in 14-day-old excision wound treated with *Achyranthes aspera* leave extract. Haematoxylin and Eosin stain × 10; **2**. collagen fibres in 14-day-old excision wound treated with *A. aspera* leave extract. van Gieson's stain × 40; **3**. necrosis and polymorphonuclear cell infiltration in 14-day-old excision wound of standard group. Haematoxylin and Eosin stain × 10; **4**. collagen fibres and reepithelialization in 21-day-old excision wound of control group. van Gieson's stain × 40.

chain alcohol and 17-pentatriacontanol, alkaloid, b-sitosterol and spinasterol (Barua *et al.* 2009a). In acute toxicity study, there was no change in motor activity and gross behaviour during 24 h of observation and the plant extract, was found to be safe up to 5g/kg body weight, p.o. Absence of toxicity of the plant observed in this study suggests that the plant extract is relatively safe for consumption and did not affect any of the parameters measured.

Excision wound: Per cent wound contraction in case of *A. aspera* treated group was significantly (P<0.01) higher compared to both control and standard groups during different days of observation (Table 1). Complete wound healing took place on 21st day itself in the *A. aspera* treated group, whereas in the standard and control group wound persisted beyond 21st day indicating better wound healing activity ofthe test plant. The results of the present study clearly demonstrated that the methanolic extract of *A. aspera* possessed a definite prohealing action in normal wound healing.

Wound healing involves a complex and superbly orchestrated interaction of cells, extracellular matrix and cytokines (Gupta *et al*. 2008). Granulation, collagen maturation and scar formation are some of the cascade of wound healing which run concurrently, but independent of each other (Udupa *et al.* 2006). The fibroblasts are responsible for the synthesis, deposition and remodelling of the extracellular matrix. The early re-epithelialization and faster wound closure in *A. aspera* treated wounds might also be associated with the increased keratinocyte proliferation and their migration to the wound surface (Blakytny and Jude 2006).

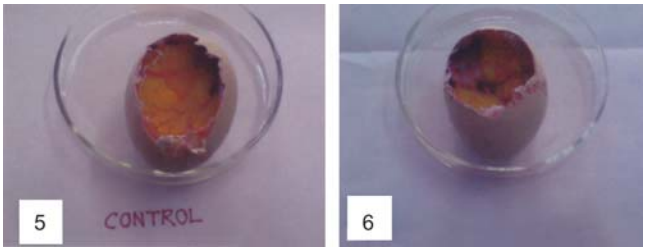
Histopathological evaluation: Histopathologically, on 14th day there was granulation tissue formation (Fig. 1) and collagen fibres were proliferating towards the surface of the wound treated with the *A. aspera* leaves extract (Fig. 2). There was necrosis and polymorphonuclear cell infiltration (Fig. 3) on 14-day-old excision wound of standard group. Re-epithelialization with proliferation of collagen fibres was present in 21-day-old excision wound of control group (Fig. 4). Collagen is a major extra cellular 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 (Upadhyay *et al*. 2009). The proliferation of collagen fibres on 14-day-old excision \wound in *A. aspera* treated group was more compared to the 21st day-old excision wound of the control group, indicating better wound healing activity of the test plant.

Incision wound model: In this model, tensile strength of the healing tissue of the *A. aspera* treated group was found to be 4.063±0.339 which was significantly (P<0.05) higher compared to control group (2.282±0.353). The tensile strength of the healing tissue of the standard (3.778 ±1.092) group was comparable with the *A. aspera* treated group indicating promising wound healing activity of the plant extract (Table 2). Acute wounds normally heal in a very orderly and efficient manner characterized by four distinct, but overlapping phases: haemostasis inflammation, proliferation and remodelling (Robert and Melissa 2004). The increased tensile strength in *A. aspera* treated wounds

Table 2. Effect of *Achyranthes aspera* 5% (w/w) ointment on tensile strength in rats

Treatment groups	Drugs	Mean ± SE
Group 1	Control	2.282 ±0.353
Group 2	*A. aspera*	4.063[a] ±0.339
Group 3	Standard	3.778[a] ±1.092

Treatments bearing same superscript do not differ significantly (P< 0.05).



Figs 5–6. Angiogenesis in 9 days old chick egg: **5**. control containing methyl cellulose disc without plant extract; **6**. treatment containing methyl cellulose disc impregnated with 400 µg methanolic extract of *Achyranthes aspera.*

may be due to increased proliferation and transformation of fibroblast cells into myofibroblasts (Gupta *et al*. 2007). 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. An increase in tensile strength of the healing tissue of *A. aspera* treated wound may be due to increase in collagen concentration and stabilization of fibres (Udupa *et al.* 2006).

In vitro *model:* In CAM model, the methanolic extract of *A. aspera* had shown angiogenic activity from slight to mark which was dose dependent. Increase in the number of blood vessels at a dose of 200 µg/ml was slight as compared to the control on the same day, whereas, a dose of 400 µg/ml caused a marked increase in the number of blood vessels (Figs 5 and 6). Angiogenesis is important in normal processes such as development of embryo, formation of corpus luteum and wound healing (Taylor and Folkman 1982). Angiogenesis during wound repair serves the dual function of providing the nutrients demanded by the healing tissues and contributing to structural repair through the formation of granulation tissue (Upadhyay *et al.* 2009). Methanolic extract of *A. aspera* increased angiogenesis from slight to marked, dose dependently as evidenced by the CAM model. A poly herbal formulation (PHF) prepared by aqueous lyophilized extracts of *Hippophae rhamnoides* and *Aloe vera* leaves and ethanol extract of *Curcuma longa* rhizome promoted angiogenesis as evidenced by CAM model and a faster wound contraction was also observed in PHF treated normal and diabetic rats (Gupta *et al.* 2008). Epidermal cells, fibroblasts, macrophages and vascular endothelial cells produce vascular endothelial growth factor (VEGF), fibroblast growth factor and TGFß which stimulate angiogenesis (Tonnesen *et al.* 2000, Battegay 1995). VEGF is an important pro-angiogenic cytokine and improves angiogenesis during wound healing by stimulating the migration and proliferation of endothelial cells through the extracellular matrix (Upadhyay *et al*. 2009a). Similarly the angiogenic activity in *A. aspera*, a treated group in the CAM model might be due to enhanced expression of these factors in regenerated tissue which needs further study.

In conclusion, it can be interpreted that topical application of Achyranthes *aspera* Linn. has a positive influence on different phases of wound healing, including wound contraction, fibroblastic deposition and angiogenesis. However, identification and elucidation of the active constituents in this plant may provide useful leads to the development of new and effective drugs against different types of wounds.

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