

Wound healing activity of methanolic extract of leaves of *Alternanthera brasiliana* Kuntz using *in vivo* and *in vitro* model

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Wound healing activity of methanolic extract of leaves of *Alternanthera brasiliana* Kuntz was studied 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 model, compared to the control group, per cent contraction of wound was significantly higher in *A. brasiliana* (5% w/w ointment) treated group. In incision wound model, tensile strength of the healing tissue after treatment with *A. brasiliana* was found to be significantly higher compared to the control group indicating better wound healing activity of the test plant. These findings were also confirmed by histopathological examination. The extract also promoted angiogenesis as evidenced by CAM model. The results suggested that methanolic extract of *A. brasiliana* possess significant wound healing potential in normal wound.

Keywords: *Alternanthera brasiliana*, Chorioallantoic membrane model, Excision wound, Incision wound, Wound healing.

Wound healing consists of an orderly progression of events that establish the integrity of damaged tissue. The healing involves different phases including inflammation, granulation, fibrogenesis, neo-vascularization, wound contraction and epithelialization¹. It is completed temporarily to termination involving apoptosis and matrix remodeling. The basic principles of wound healing-minimizing tissue damage, debriding nonviable tissue, maximizing tissue perfusion and oxygenation, proper nutrition and a moist wound healing environment have been recognized for many years.

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Use of herbal extract in place of crude herbs started with the aim to control quality and precise dosage for better results. 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.

Alternanthera brasiliana Kuntz belonging to the family Amaranthaceae is a herbaceous plant commonly known in Brazil as Penicillin or Brazilian Joy Weed is a neotropical native species, which grows easily on poor and deforested soil, is used against inflammation, cough and diarrhoea in Brazilian popular medicine². The extract of *A. brasiliana* exhibited antinociceptive effect in mice³, antimicrobial effect⁴ and anti-herpes simplex virus activity⁵. Aqueous or ethanolic extract of *A. brasiliana* are able to block human mitogen induced lymphocyte proliferation without any toxic effect⁶.

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. Hence, 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 CAM model.

Plant material—Leaves of *A. brasiliana* Kuntz were collected from the medicinal garden of the Department of Pharmacology & Toxicology, College of Veterinary Science, Khanapara during the month of Feb-June, 2008, identified by Taxonomist of NEIST, Jorhat, Assam and a voucher specimen (AAU/CVSC/PHT/ 02) was deposited.

Preparation of methanolic extract—Leaves of *A. brasiliana* were dried in shade and powdered. About 250g 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 Whatman filter paper no 1 for 3 times and concentrated in Rotary evaporator at 50°-60°C under reduced pressure leaving a dark brown residue which was stored in air tight container at 4°C till further use. Recovery was 6.12 %.

Phytochemical screening—Phytochemical screening of the extract was carried out by standard method⁶.

Preparation of 5% ointment of *A. brasiliana* (w/w)—Methanolic extract of *A. brasiliana* (5 g) was mixed with Vaseline (95 g) to get a 5% ointment (w/w).

Animals—Healthy Sprague Dawley rats of either sex approximately of same age, weighing 150-250 g were used for the study. They were housed under controlled conditions at $25^{\circ}\pm 3^{\circ}\text{C}$, RH $50\pm 5\%$ and kept under 10/14 h light/dark cycles with free access to food and water *ad libitum*. 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 14 h before tests to achieve better drug absorption through gastrointestinal tract.

Determination of LD_{50} —The LD_{50} of *A. brasiliana* was estimated by following up- and-down stair case method in mice⁷. Doses were adjusted by a constant multiplication factor (*viz.*, 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. brasiliana* was studied in albino mice by using $1/2 \text{ LD}_{50}$ dose. A total of six numbers of male albino mice were selected for each experiment. Animals were observed at hourly interval for 6 h and again after 24 h. The parameters for motor activity and gross effect were determined after administration of *A. brasiliana* orally at a dose level of 2.5 g/kg body weight.

Dosing schedule—Ointment (5%) of *A. brasiliana* was applied topically, twice daily from day 1 till day of complete healing or 21st post-operative day, whichever was earlier. Himax (Indian Herbs, Saharanpur, U.P) was used as standard drug. Animals with vaseline application served as control.

***In vivo* model**—Two models for *in vivo* wound healing activity were tried in the present study *viz.*, excision and incision wound models. The animals were randomly allocated into three groups of six animals each. Group I was assigned as control, Group II received topical application of 5% ointment of *A. brasiliana* (w/w) and Group III received the topical application of Himax ointment as standard Drug.

Excision wound model in normal rat—The excision wounds were made by excising the full thickness circular skin (approximately 500 mm^2) on the back of the animal under ether anesthesia⁹. 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 day 7, 14 and 21. The evaluated surface area was then employed to calculate the percentage of wound contraction (taking the initial size of the wound, 500 mm^2 , as 100%) by using the formula [(Initial wound size – specific day wound size)/ Initial wound size] $\times 100$.

Incision wound model in normal rat—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 depilated area of anaesthetized rats. The parted skin was sutured 1 cm apart. Skin breaking strength of the wounds was measured on day 10 as per the standard method¹⁰.

***In vitro* model**—Chorioallantoic membrane (CAM) model was used as *in vitro* model¹¹. 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 treated with 200 or 400 μg of methanolic extract of *A. brasiliana* was placed inside triplicate sets of egg at the junction of two 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.

Histopathological study—On day 7, 14 and 21, animals from each groups were sacrificed and histopathological evaluation of granulation tissue was done as per the standard method¹².

Statistical analysis—The results of various parameters were subjected to statistical analysis¹³.

Phytochemical screening of the methanolic extract of *A. brasiliana* revealed the presence of alkaloid by Wagner's and Dragendorff's test, steroid by Salkowski's and Lieberman Burchardt's test and triterpenes by Salkowski's and Lieberman Burchardt's test. In acute toxicity study, there was no change of 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.

In excision wound model, area of the wound decreased with time from day 0 till day 21 in all the groups. In the control group, the wound contraction was 82.23% on day 21, whereas in the *A. brasiliana* treated group, it was 100% on day 21. In the standard group, the wound contraction was 93.89%

on day 21 and the wound persisted beyond day 21 indicating better wound healing activity of the test group (Table 1).

Histopathologically, the *A. brasiliensis* treated group, had hemorrhage, edema and necrosis on day 7 (Fig. 1A), infiltration of some polymorphonuclear cells (Fig. 1B), showing 85.42% wound contraction on gross study. On day 14, the wound contraction was 96.61% and histopathologically the wound healing was completed with horizontally arranged fibrous connective tissue and abundance of collagen fibers (Fig. 1C, D). Surface of the granulation tissue was completely filled with epidermal cells covered with a thick keratin layer. In control group, though abundance of collagen fibers were present on day 14 (Fig. 1E), the wound surface was not completely covered with epidermal cells and the wound contraction was 82.23% on day 21.

Table 1- Effect of *A. brasiliensis* on wound contraction in excision wound model in normal rats
[Values are mean $\pm$ SE of 6 rats]

Group	Wound contraction (%)			
	Day 0	Day 7	Day 14	Day 21
I	0.00 ^a $\pm$ 0.00 ^a	58.21 ^c $\pm$ 2.019 ^b	69.48 ^c $\pm$ 1.545 ^c	82.23 ^c $\pm$ 1.352 ^d
II	0.00 ^a $\pm$ 0.00 ^a	85.42 ^a $\pm$ 1.52 ^b	96.61 ^a $\pm$ 1.140 ^c	100.00 ^a $\pm$ 0.00 ^c
III	0.00 ^a $\pm$ 0.00 ^a	67.29 ^b $\pm$ 1.821 ^b	81.72 ^b $\pm$ 1.785 ^c	93.89 ^b $\pm$ 1.083 ^d

Group I- control; II- (*A. brasiliensis*) Treated; and III-Standard
Same superscript in column and row indicates that the mean values do not differ significantly at $P < 0.01$

In the incision wound model, tensile strength of the healing tissue of the *A. brasiliensis* treated group (4.861 ± 0.664) was significantly higher compared to control group (2.282 ± 0.353). The tensile strength of the healing tissue of the standard (3.778 ± 1.092) group did not differ significantly with the *A. brasiliensis* treated group indicating the promising wound healing activity of the latter.

In CAM model, the methanolic extract of *A. brasiliensis* had shown angiogenetic activity from slight to marked which was dose dependent. Increase in the size of blood vessels at a dose of 200 μ g/ disc was slight as compared to the control on the same day, whereas at a dose of 400 μ g/ disc caused a marked increase in the size and number of blood vessels (Fig. 2).

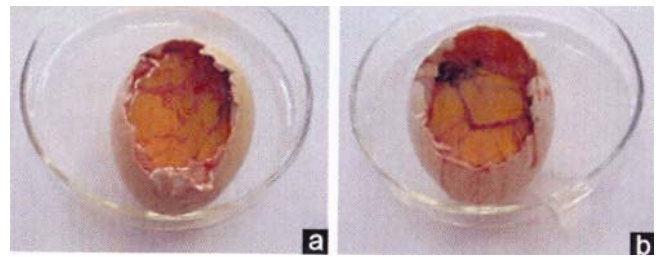


Fig. 2—Angiogenesis in 9 days old chick egg after treatment with methanolic extract of *Alternanthera brasiliensis*: (A)-Control containing methyl cellulose disc without plant extract and (B)-Treatment containing methyl cellulose disc impregnated with 400 μ g methanolic extract of *Alternanthera brasiliensis*

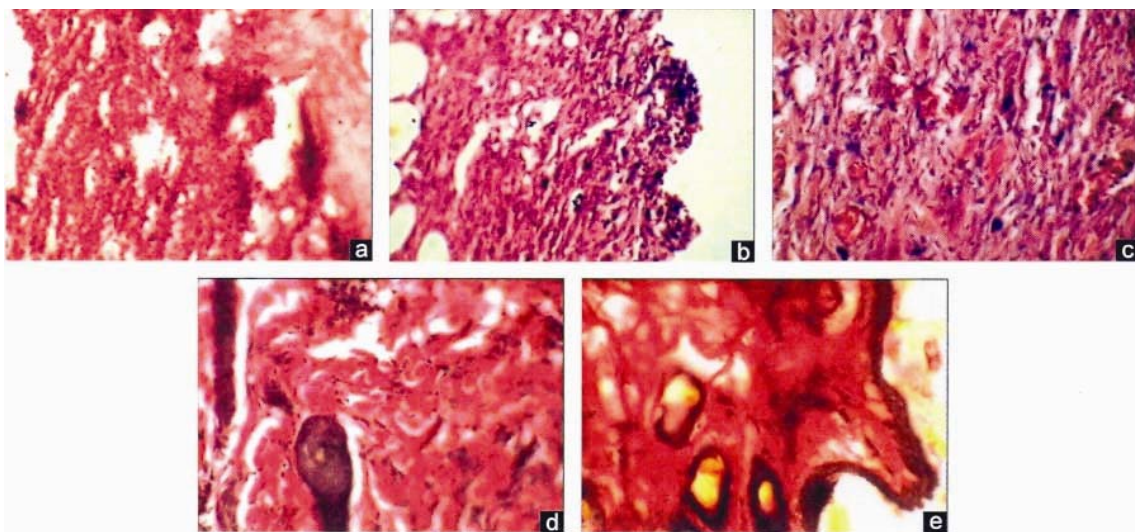


Fig. 1—Histopathological changes in 7 and 14 days old excision wound treated with *Alternanthera brasiliensis* leaf extract: and control group (a)- hemorrhage and edema (H&E stain, 40 $\times$) in 7 days old excision; (b)- polymorphonuclear cell infiltration (H&E stain, 40 $\times$) in 7 days old excision; (c)- capillary congestion, fibroblast proliferation and formation of collagen fibers (H&E stain, 40 $\times$) in 14 days old excision wound; (d)- collagen fibers (van Gieson's stain, 40 $\times$) in 14 days old excision; and (e)- control group showing collagen fibers (van Gieson's stain, 40 $\times$) in 14 days old excision wound

The present study clearly demonstrated that methanolic extract of *A. brasiliensis* possessed a definite prohealing action in normal healing as observed by significant increase in the rate of wound contraction, increase angiogenesis and tensile strength. An increase in tensile strength of the healing tissue in *A. brasiliensis* treated wound may be due to increase in collagen concentration and stabilization of fibres¹⁴. Wound contraction involves a complex and superbly orchestrated interaction of cells, extracellular matrix and cytokines. In the present study, extract treated wounds were found to contract much faster. Increased rate of wound contraction in *A. brasiliensis* treated wounds might be due to increase in proliferation and transformation of fibroblast cells into myofibroblasts. Granulation, collagen maturation and scar formation are some of the many phases of wound healing, which run concurrently, but independent of each other¹⁴. The use of a single model is inadequate and no reference standard exists that can collectively represent the various phases of wound healing. Therefore, three different models have been used in the present study to assess the effect of *A. brasiliensis* on the various phases of wound healing.

Angiogenesis is important in normal processes such as development of embryo, formation of corpus luteum and wound healing¹⁵. 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 tissue¹⁶. Several studies have previously demonstrated the wound healing activity of medicinal plants. A polyherbal 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, has also been observed in PHF treated normal and diabetic rats¹⁷. In another study, ethanolic extract of leaves of *Ocimum sanctum* Linn., had been investigated for normal wound healing and dexamethasone depressed wound healing using excision, incision and dead space wound models in albino rats and observed that the extract promoted wound healing significantly and able to overcome the wound healing suppressing action of dexamethasone¹⁴.

In conclusion, it can be interpreted that topical application of *Alternanthera brasiliensis* has a positive influence on different phases of wound healing, including wound contraction, fibroblastic deposition,

angiogenesis and therefore, has a beneficial role in wound healing. 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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