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Experimental evaluation of wound healing activity of *Croton macrostachyus* in rat

Abraham Fikru Mechesso[1*], Ayele Tadese[2], Reta Tesfaye[2], Wondemagegn Tamiru[3], Tadesse Eguale[4]

[1]School of Veterinary Medicine, Hawassa University, Hawassa, Ethiopia.
[2]Faculty of Veterinary Medicine, University of Gondar, Gondar, Ethiopia.
[3]Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, Addis Ababa University, Addis Ababa, Ethiopia.
[4]Akililu Lemma Institute of Pathobiology, Addis Ababa University, Addis Ababa, Ethiopia.

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Croton macrostachyus **leaves are used for treatment of wounds by traditional healers in Ethiopia. Despite the use of this plant in the treatment of wound, there is limited data to support its medicinal use. The present study provides scientific evaluation for the wound healing potential of methanolic extract of *C. macrostachyus* leaves in rats. The leaves of *Croton macrostachyus* were studied for the presence of some secondary metabolites and wound healing activity. Ointments were made by incorporating the methanolic extract in simple ointment base B.P. in the concentration of 5 and 10% (w/w). Standard Nitrofurazone was used for comparison. Wound healing activity was studied, using excision and incision wound models. In excision wound model, percentage wound contraction, period of epithelization and morphological changes on the healed wounds were studied while incision wound model was used to determine breaking strength. The results were expressed as mean ± standard error of mean (SEM) and comparisons among treatment groups were made using one-way analysis of variance. Phytochemical screening of the methanolic extracts of leaves of *C. macrostachyus* showed the presence of different metabolites such as flavonoids and saponins which are reported to have significant wound healing activity. The results of epithelization period, percentage of wound contraction and morphological evaluation of groups of animals in the test groups showed significant ($p < 0.05$) wound healing activity compared to those treated with simple ointment. Similarly, the difference in breaking strength was significant ($p < 0.05$) for both 5 and 10% (w/w) methanol extract of *C. macrostachyus* ointment treated groups. Morphological evaluation showed a relatively better healing and growth of hair around the wound area in the 10% methanol extract of *C. macrostachyus* ointment treated group. Methanolic extract of *C. macrostachyus* enhanced wound healing significantly, corroborating the folk medicinal use of this plant.**

Key words: *Croton macrostachyus*, excision, incision, *in vivo*, wound healing.

INTRODUCTION

Wound is a major problem in developing countries, often having severe complications and involving high costs of therapy (Shenoy et al., 2011). Wound healing is a complex process which requires the collaborative efforts of different tissues of varying cell lineage (Suntar et al., 2011). It involves platelet aggregation, blood clotting, formation of fibrin, alteration in the ground substances, angiogenesis, and re-epithelialization. Healing is not

complete until the disrupted surfaces are firmly knit by collagen (Guo and DiPietro, 2010). In spite of remarkable advances in pharmaceutical drug industry, the availability of drugs capable of stimulating the process of wound healing is still limited (Abraham et al., 2012). Conventional treatment for established wounds incorporates common principles that apply to the management of all wounds, including debridement of necrotic tissue, maintenance of a moist wound bed and control of infection. Unfortunately, there are no widely accepted, standardized protocols that define optimal standard treatment or the appropriate intensity of treatment delivery (Samson et al., 2004).

Medicinal plants are used extensively to enhance the healing of wounds (Suntar et al., 2011). *Croton macrostachyus* is a deciduous tree belonging to the family Euphorbiaceae. The leaves are large and green, turning to orange before falling. It is also characterized by creamy to yellow-white colored flowers with green (when young) to grey (at maturity) fruits. *C. macrostachyus* is commonly named as 'Bisana' in Ethiopia and is traditionally used for the treatment of wound (Giday et al., 2007; 2009; Teklehaymanot and Giday, 2007) malaria, rabies, and gonorrhea (Giday et al., 2007), *Tinea versicolor*, diarrhea, hepatitis, jaundice, and scabies (Teklehaymanot and Giday, 2007).

Studies revealed that *C. macrostachyus* has a wide range of activities which justifies its traditional use in the treatment of wound healing. The plant showed anti-microbial activity against pathogens which are common contaminants of wound, such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Kalayou et al., 2012; Taye et al., 2011). The plant is also reported to have significant anti-inflammatory and analgesic activities in animal models (Kamanyi et al., 2009). Even though majority of the Ethiopian population uses traditional medicine, only limited studies were conducted on the traditional medicinal plants compared to the multicultural diversity and the diverse flora of the country (Tekalign et al., 2010). Therefore, based on the aforementioned ethnobotanical and *in vitro* studies which suggest the importance of the plant in enhancing wound healing, the present study was conducted to evaluate the *in vivo* wound healing potential of crude extracts of *C. macrostachyus* leaves using an excision and incision wound models.

METHODOLOGY

Preparation of the extract and ointments

C. macrostachyus was collected from Gondar, North Ethiopia. Botanical authentication was obtained and voucher specimen was also deposited at the mini-herbarium of Institute of Pathobiology, Addis Ababa University. The leaves were garbled, dried under shade and grinded prior to storage. The grinded plant material was extracted with 80% methanol by maceration for 48 h with frequent agitation (Debella, 2002) and the resulting liquid was filtered with filter paper (Whatman No. 1, Whatman Ltd., England). Maceration was repeated three times and the filtrates of all portions were combined in one vessel. The methanol was removed by evaporation using Rota vapor at a temperature of 40°C. Simple ointment which is composed of cetostearyl alcohol, wool fat, white paraffin, and hard paraffin was prepared according to the standard guide line (Gaur et al., 2009). Finally, ointments containing 5 and 10% (w/w) methanol extract of *C. macrostachyus* leaves were made using the simple ointment as a vehicle.

Preliminary phytochemical screening

The methanolic extract was tested qualitatively for different phytoconstituents using various chemical test procedures according to the standard methods described by Debella (2002).

Test for alkaloids

Two grams of thoroughly grinded plant material was treated in a test tube with 5 ml of 1% HCl for 30 min in a water bath. The suspension was filtered through cotton into a test tube and 5 drops of Mayer's reagent was added. The formation of whitish opalescence was regarded positive for the presence of alkaloids.

Test for anthraquinones

Test for free anthraquinones: The hydroalcholic extract of the plant material (about 100 mg) was shaken vigorously with 10 ml of benzene. The extract was then filtered and the filtrate was treated with 5 ml of 10% ammonia solution. The mixture was shaken and observed for the presence of a pink, red or violet color in the ammonia phase.

Test for O-anthraquinone glycosides: To about 5 g of powdered plant material, 10 ml of 5% H_2SO_4 was added and boiled for 1 h. The mixture was filtered, cooled and extracted with 10 ml of benzene. To 5 ml portion of the filtrate, equal volume of 10% ammonia solution was added and shaken. The formation of a pink, red or violet color in the aqueous phase (ammonia phase) indicates the presence of O-anthraquinone glycosides

Test for phytosterols (Salkowski reaction)

One gram of powdered plant material was macerated with hexane, filtered and concentrated. The concentrated residue was dissolved in chloroform. Three to five drops of concentrated H_2SO_4 was added carefully, the production of a red or reddish brown or violet color was regarded as positive for the presence of steroidal compounds.

Test for plyphenols (Phenolic compounds)

To 2 ml of filtered solution of aqueous macerate of a plant material,

*Corresponding author. E-mail: abrahamfikru@yahoo.com, abrahamf@hu.edu.et. Tel: +251-912-082845. Fax: +251-46-220-5421.

3 drops of a mixture of 1 ml 1% $FeCl_3$ and 1 ml 1% $K_3Fe(CN)_6$ were added. The formation of a green blue color indicates the presence of polyphenols.

Test for saponins

Filtered solution of the extract (10 ml) was shaken in a large test tube; the formation of honeycomb froth that persisted for half an hour indicates the presence of saponins.

Test for tannins

To 2 ml of the extract few crystals of sodium nitrate and 2 to 3 drops of 0.1 N HCl were added and observed for brown coloration.

Test for flavonoids

Diluted ammonia (5 ml) was added to a portion of an aqueous filtrate of the extract. Concentrated H_2SO_4 (1 ml) was added and a yellow coloration that disappears on standing indicates the presence of flavonoids.

Experimental animals

Healthy albino rats (weight, 220 to 250 g) and mice (weight, 25 to 30 g) were procured from the Ethiopian Public Health Institute (EPHI). The animals were left for 8 days at room conditions for acclimatization. The animals were housed in polypropylene cages on standard pellet diet and water *ad libitum*. At the end of the experiment, animals were killed under halothane anesthesia. All experiments were carried out after the approval of the ethics committee at the Faculty of Veterinary Medicine, University of Gondar, Ethiopia and following the European guidelines for care and uses of laboratory animals (EEC Directive of 1986; 86/609/EEC).

Acute oral toxicity study

The acute toxicity study was carried out according to OECD guidelines – 425. Mice (25 to 30 g) were used to determine the safer dose. Distilled water was used as a vehicle to suspend the extract and administered orally at a dose of 2000 mg/kg. Animals were observed individually for changes in skin color, eyes and behavioral pattern.

During the experiment, the animals were weighed, food and water intake were monitored. Attention was also directed to observations of tremors, convulsions, diarrhoea, lethargy, sleep, and coma.

Observation was carried out at least once during the first 30 min after dosing, periodically during the first 24 h (with special attention given during the first 4 h), and daily thereafter for a total of 14 days (OECD 425, 2008).

Wound healing activity

The animals were grouped into four groups, namely, control (positive and negative) and two test groups with six animals in each group. The animals in the negative and positive control groups were treated with simple ointment base B.P and Nitrofurazone (Galentic Pharma, India) ointment, respectively. Whereas, the test groups were treated with ointment containing 5 and 10% (w/w) methanol extracts of leaves of *C. macrostachyus*.

Circular excision wound model

The hair on the dorsal thoracic area of the rats was shaved and anesthetized with Halothane. Ethanol (70%) was used to clean the shaved area and a circular wound of 350 mm^2 was created on the depilated dorsal thoracic region of each rat.

Measurement of wound area

The ointment was topically applied to the wounds once daily until complete wound closure. The measurements of the wound areas were taken on the day of wound infliction (350 mm^2) and once every 2 days using transparent paper and a permanent marker. The recorded wound areas were measured using graph paper and the measurement was used to calculate the percentage wound contraction, considering the initial size of wound as 100% (Shenoy et al., 2011).

$$\text{Wound contraction (\%)} = \frac{\text{Initial wound size} - \text{Specific day wound size}}{\text{Initial wound size}} \times 100$$

Measurement of epithelialization period

The epithelization period was determined by considering the number of days required for falling of the scab without any residual raw wound (Shenoy et al., 2011).

Morphological evaluation

Morphological evaluation was carried out to assess the change in skin surface, color, growth of hair, and presence or absence of unhealed wound among the test and control groups. Representative photographs were also taken during the wound healing period.

Determination of breaking strength

Linear incision wound model was used to assess the breaking strength of the healed wounds. The rats were anaesthetized with Halothane and shaved with shaving machine. Five centimeters long, linear-paravertebral skin incision was made on either side of the vertebral column at the distance of 1 cm from the midline. The incised skin was stitched with surgical thread and a curved needle at the intervals of 1 cm. The wounding day was considered as day 0. The 5 and 10% (w/w) ointments containing methanol extract of *C. macrostachyus* leaves, nitrofurazone and the simple ointment were applied topically once a day for a total of 9 days. The sutures were removed on the 9th day and breaking strength of each groups of animals were determined on the 10th day using continuous water flow technique. Briefly, anesthesized animals were secured on operation table in its natural position. Two forceps were firmly applied on the skin on either side of the incised and healed wound. The forceps on one side was hooked to a metal rod and fixed firmly to the operation table, while the other to a light plastic container with a string which passes over a pulley. Water was allowed to flow in to the plastic container at constant rate so as to build gradual pulling force necessary to disrupt the wound. As soon as the gaping of the wound was observed, the water flow was cut off. Further opening of the wound was avoided by removing the pulling force immediately, which was achieved by lifting up the plastic container. The volume of water in the plastic container was measured and converted to the corresponding weight assuming the density to be equal to ‘one’. The breaking strength was expressed as the

Table 1. Effect of topical application of methanol extract of *Croton macrostachyus* on percentage wound contraction of an excision wound.

Day	Wound area (mm^2) ± SEM (% Contraction)			
	Simple ointment	5% methanol extract	10% methanol extract	Nitrofutrazone
Day 2	288.67 ± 3.31 (17.53)	286.08 ± 13.07 (18.29)	286.67 ± 4.13 (18.1)	288.92 ± 4.59 (17.45)
Day 4	167.92 ± 14.25 (52.02)	158.17 ± 5.00 (54.81)	158.83 ± 4.78 (54.62)	120.04 ± 6.67** (65.7)
Day 6	77.67 ± 2.12 (77.81)	52.79 ± 1.89** (84.92)	23.29 ± 6.23*** (93.35)	49.54 ± 5.07** (85.87)
Day 8	45.50 ± 2.25 (87.02)	32.96 ± 2.14** (90.58)	19.67± 1.54*** (94.38)	17.96 ± 2.83*** (94.87)
Day 10	28.88 ± 1.25 (91.75)	16.50 ± 2.78*** (95.29)	7.33 ± 0.61*** (97.9)	3.83 ± 0.40*** (99.91)
Day 12	17.42 ± 1.90 (95.02)	7.58 ± 1.10*** (97.84)	1.17± 0 .42*** (99.67)	0.00 ± 0.00*** (100)
Day 14	9.38 ± 0.89 (97.32)	0.67 ± 0.42*** (99.81)	0.00 ± 0.00*** (100)	0.00 ± 0.00*** (100)

n = 6 animals in each group. ***P < 0.001; **P < 0.01; *P < 0.05.

Table 2. Effect of topical application of methanol extract of *Croton macrostachyus* on epithelization period and breaking strength.

Group	Epithelization period (days)	Breaking strength (g)
Nitrofurazone	11.67 ± 0.42***	547.33 ± 6.92***
5% methanol extract	14.17 ± 0.31*	484.33 ± 8.20*
10% methanol extract	12.67 ± 0.42***	500.83 ± 6.85***
Simple ointment	15.83 ± 0.31	445.17 ± 9.87

n = 6 animals in each group. ***P < 0.001; **P < 0.01; *P < 0.05.

maximum weight of water necessary to bring about breaking of the wound. Three such readings were recorded for a given incision wound and mean breaking strength for each animal was used to calculate the groups mean (Lee, 1968).

Statistical analysis

Percentage wound contraction was calculated as a percentage of the corresponding initial day (day 0) wound in mm^2. Data was processed using SPSS software Version 20.0. Results between treatment groups were compared using one-way ANOVA and the results were considered significantly different at P value < 0.05.

RESULTS

Preliminary phytochemical screening

Qualitative phytochemical analysis of methanolic extract of leaves of *C. macrostachyus* showed the presence of free anthraquinones, flavonoids, phytosterols, polyphenols, saponins and tannins; however, the extract was found to be negative for alkaloids.

Acute oral toxicity studies

The methanolic extract of *C. macrostachyus* was found to be safe up to 2000 mg/kg body weight by oral route. None of the animals showed behavioral, neurological and physical changes such as convulsion, coma, restlessness, lacrimation and diarrhea.

Excision wound study

The effects of methanolic extract of different doses of *C. macrostachyus* on percentage wound contraction and epithelization period are shown in Tables 1 and 2. The percentage of wound contraction of animals treated with ointment containing 5 and 10% (w/w) methanolic extract showed significant ($p < 0.05$) difference as of the 6th day after treatment as compared with the simple ointment treated group (Table 1). Similarly, the test groups required significantly shorter epithelization period (Table 2).

Gross morphological evaluation

Photographs of rats at 10th day post treatment with Nitrofurazone ointment, 10% extract ointment, 5% extract ointment and simple ointment are shown in Figure 1. The results showed progressive changes in percentage wound contraction in test groups as compared with the negative control (simple ointment treated) group. 10^{th} day photographs demonstrated that the 10% (w/w) extract of *C macrostachyus* ointment treated group demonstrated relatively better healing in which the color was close to the normal skin with smooth surface and relatively good

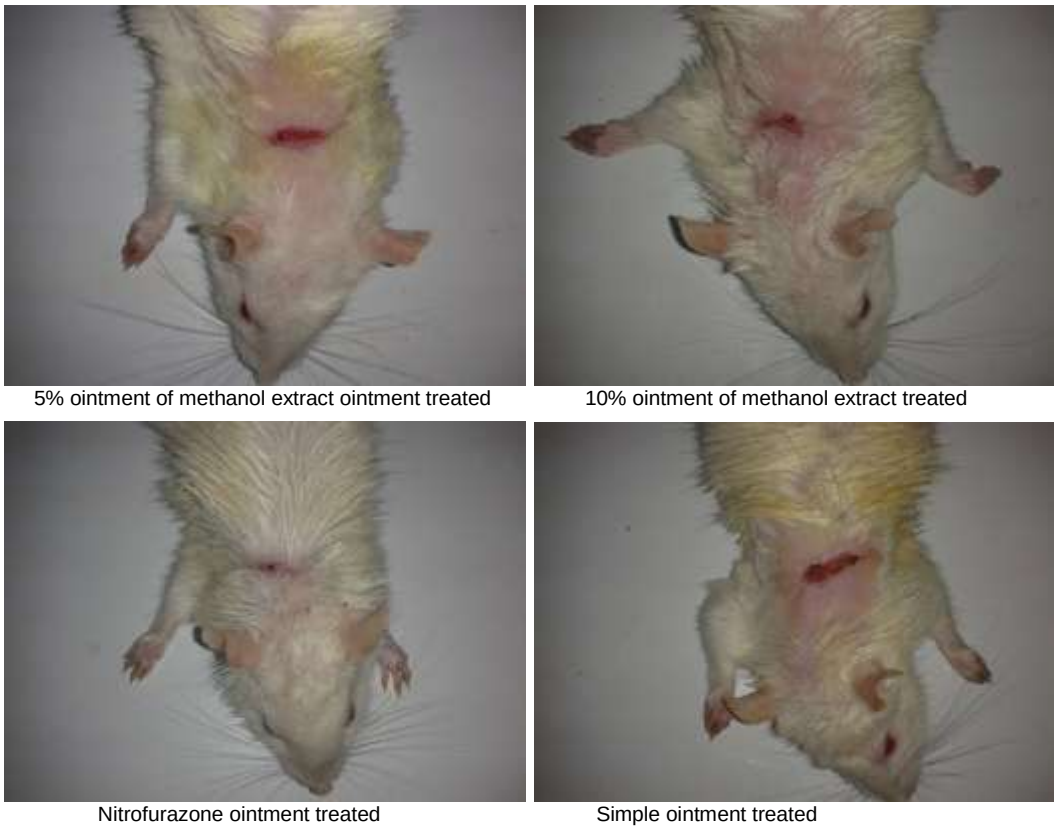

Figure 1. Photographs of rat dorsal wound at 10th day post treatment.

growth of hair. Whereas, rats treated with ointment containing 5% (w/w) of the extract showed the presence of unhealed wound with insignificant growth of hair.

Breaking strength

Rats treated with 5 and 10 % (w/w) ointments of the extract showed higher breaking strength, with the 10% ointment treated wounds being stronger than the 5% ointment treated wounds (Table 2).

DISCUSSION

Many studies indicated that plant products are potential agents for wound healing and largely preferred, because of the absence of unwanted side effects and their effectiveness (Joseph and Justin, 2011). In the excision wound model of this study, progressive reduction in wound area and enhanced period of epithelization were observed in both 5 and 10% ointment of methanolic extract of *C. macrostachyus* treated groups, while the fastest (12.67 days) and complete wound healing (100% wound contraction) were observed in the 10% (w/w) extract treated group, compared with the negative control group. The changes in the test groups might be attributed to the potential of the test extracts or its constituents to promote epithelization either by facilitating proliferation or by increasing the viability of epithelial cells (Mukherjee et al., 2013).

Wound has little breaking strength in the beginning, which increases rapidly during healing due to the synthesis and maturation of collagen (Wang et al., 2011). Collagen imparts strength and elasticity to healed skin. As the wound heals, collagen molecules are synthesized and laid down at the wound site. These molecules become cross-linked to form fibers (Pather et al., 2011). In this study, the strength of the repaired wound tissue might be the result of the remodeling of collagen and the formation of stable intra- and inter-molecular cross linking which is necessary for maturation of collagen as described by Pather et al. (2011) and Abraham et al. (2012) on different plant extracts. Accordingly, these results may suggest that the extracts could increase collagen synthesis and possibly aid in formation of cross linkages as the collagen matures.

In vitro and *in vivo* studies indicated the use of plant materials as topical antimicrobial agents to enhance wound healing (Akkol et al., 2011; Nayak et al., 2011).

Similarly, *C. machrostachyus* has been reported to show antimicrobial activity against those pathogens

Which commonly infect wounds such as *Streptococcus pyogens* (Taye et al., 2011) and *Pseudomonas aeruginosa* (Wagate et al., 2010). Usually, the first aim of the wound management is to keep the wound free of infections and complications. Such types of agents are always required to contribute to the rapid healing of wound (Akkol et al., 2011). Consequently, the antimicrobial activity of the 80% methanol extract of *C. machrostachyus* might be associated with the wound healing activity.

A study conducted by Teugwa et al. (2013) showed that methanolic extract of *C. machrostachyus* has an anti-oxidant activity. Earlier studies also indicated that antioxidant activity of plant extracts contributed significantly to wound healing activity (Pesin Suntar, 2010). Similarly, the antioxidant effect Vitamin C has been shown to contribute significantly during wound healing (Weeks and Perez, 2009). Therefore, the antioxidant activity of *C. machrostachyus* might contribute to its wound healing activity.

Secondary metabolites like anthraquinones, flavonoids, phytosterols, polyphenols, saponins and tannins were among the major phytoconstituents found in this plant. A number of secondary metabolites/active compounds isolated from plants have been demonstrated in animal models as active principles responsible for facilitating healing of wounds. Previous study on *Terminalia arjuna* showed that tannins enhance wound healing action by improving regeneration and organization of the new tissue (Chaudhari and Mengi, 2006). Flavonoids are known to reduce lipid peroxidation by preventing or slowing the onset of cell necrosis and by improving vascularity. Accordingly, a drug that inhibits lipid peroxidation is believed to increase viability of collagen fibrils by increasing strength of collagen fibres, increasing circulation, preventing cell damage and by promoting DNA synthesis (Pesin Suntar, 2010; Upadhyay et al., 2011). Flavonoids are also known for their astringent and antimicrobial property (Devipriya and Shyamala, 1999). Thus, wound-healing property of *C. machroystachus* may attribute it to the individual or additive effect of the phytoconstituents present.

In conclusion, the leaf extracts *C. macrostachyus* has remarkable wound healing activity and it may have a tremendous potential for treating wound. Further studies with purified constituents are needed to isolate active component (s) responsible for its wound healing activities and to understand the complete mechanism of wound healing activity.

Competing interests

The authors have not declared any of conflict interests.

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