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Evaluation of the wound healing activity of formulated ointments and water preparation from *Sida rhombifolia* leaf extract

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Abstract
Background: Several plants including *Sida rhombifolia* Linn. (Malvaceae) which are said to be used by traditional health practitioners in Tanzania for wound treatment have not yet been evaluated. The objective of this study was to investigate the ointment formulation of *S. rhombifolia* leaves for its potential wound healing activities.
Methods: Wound healing activity of *S. rhombifolia* leaves was investigated in mice using 50%, 33% and 25% formulated 80% ethanolic leaves extract ointment and water preparations. Excisional and incisional wound-induction models were used with 6 albino mice in each group. The wound diameter (for contraction assessment), duration of re-epithelisation in days, percentage tensile strength as well as the degree of collagenisation and fibrosis were investigated.
Results: *S. rhombifolia* leaves extract had significant mean percentage wound closure for all ointment formulations used and for the water preparation from day 7. A significant percentage tensile strength on day 10 for all formulations used was observed. The 50% ointment had a mean of 64.1±1.7 ($p=1.2^{-09}$), 33% ointment had a mean of 64.0±3.2 ($p=2.4^{-08}$) and the 25% ointment had a mean of 53.1±4.0 ($p=1.3^{-06}$). A remarkable fibrosis and collagenisation for the 50% ointment and the water preparation was observed.
Conclusion: The formulated ointments and the water preparations of *S. rhombifolia* leaves have a potential benefit in enhancing wound healing. A bioassay guided fractionation is recommended to allow identification of its active compound(s) with wound healing activity for drug development.

Keywords: wound, healing, activity, ointment, *Sida rhombifolia,* leaves, Tanzania

Introduction

Wounds represent a major health burden and a drain on healthcare resources globally. The majority of Africans still utilize traditional medicine for their health-care needs including the treatment of various forms of wounds (Agyare *et al.*, 2009). It has been documented that both traditional and modern medicine are being used together in wound treatment mostly for minor injuries as well as for male circumcision in Tanzania (Mboera *et al.*, 2009; Maregesi *et al.*, 2016a). In this regard, the use of traditional and alternative medicine for wound management has been made possible through people's improved perception towards the same as well as the incorporation of new concepts to expedite the wound healing process in traditional medicine. Furthermore, the documentation of several studies in wound care management using herbal and traditional medicine from different continents is increasing (Molan, 2006). These wound healing herbal extracts are said to fight infection, promote blood clotting and accelerate the healing process of wounds (Kumarasamyraja *et al.*, 2012).

Some of the plants that have been documented to be used by traditional health practitioners in Tanzania for management of wounds include *Azadirachta indica, Sida rhombifolia, Balanites aegyptiaca, Trichodesma zeylanicum, Melanthera scandens, Rytigynia celastroides* and *Hugonia castaneifolia* (Muanza *et al.*, 1994; Mgole *et al.*, 2007; Moshi *et al.*, 2010; Yadav & Panghal, 2010; Lupala *et al.*, 2014; Baylor, 2015). *S. rhombifolia* has many characteristics which suggest it to have wound healing activity which led to the choice of the plant for in vivo wound healing testing. First, the aqueous and methanolic root extracts of this plant showed effective free radical

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scavenging activity, reducing power, superoxide scavenging activity and moderate antibacterial activity in some bacterial species (Dhalwal *et al.*, 2007; Assam *et al.*, 2010; Narendhirakannan & Limmy, 2012; Woldeyes *et al.*, 2012). Secondly, hydro-alcoholic extract of *S. rhombifolia* leaves exhibits a significant anti-inflammatory activity which is a good indication of wound healing property (Logeswari *et al.*, 2013). Thus, our current study aims to explore the wound health activity of *S. rhombifolia* leaves.

Materials and methods

Study design

This was an experimental pre-clinical *in-vivo* study conducted at the Institute of Traditional Medicine (ITM), Muhimbili University of Health and Allied Sciences (MUHAS) in Dar es Salaam, Tanzania. With the aid of a field botanist, *S. rhombifolia* leaves were collected from Kwasemangube village in Korogwe, Tanzania. The leaves were harvested, shade dried, sorted and grounded. Voucher specimens were prepared and kept in the herbarium of University of Dar es salaam and of ITM-MUHAS.

Preparation

About 0.8kg of powdered leaves was placed in a glass percolator with 3 litres of 80% ethanol and allowed to stand at room temperature for 48 hours. The percolate was collected two times and the combined extract was concentrated by using a rotary evaporator at the temperature of 60°C. Approximately, 200 ml of the concentrated extract was evaporated to dryness using a freeze dryer to yield the dry powdered extract. Water preparations of *S. rhombifolia* was done by mixing 25 gm of ground fresh leaves with 50ml of water to get a paste which was applied on wound topically as test substance in order to access wound healing activity in the preparation form which was assumed to be equivalent to the one used by traditional healers. Adopting the method of Zahi *et al*. (2015), 80% ethanolic extract of the *S. rhombifolia* leaves was incorporated into simple ointment base to make 25%, 33% and 50% formulations by triturating in a ceramic mortar and pestle to obtain herbal ointments (Table 1).

Table 1: Ratios and composition of plant extracts and ointment in grams

Dosage	Extract weight	Ointment weight	Total	% Extract (W/W)
1000mg/Kg	7.5	7.5	15	50%
500mg/Kg	7.5	15	22.5	33%
250mg/Kg	2.5	10	12.5	25%

Experimental animals

The study used white albino mice of either sex (25–30 gm), aged 8 weeks, obtained from the animal house of ITM-MUHAS. The animals were acclimatized for 5 days and kept in separate cages in groups assigned randomly for wound healing monitoring. The animals were given access to light 12 hours a day and fed ad-libitum. Animal handling and care followed internationally accepted principles for humane laboratory animal use. Ethanol (70%) was used as antiseptic for the shaved region before making the wound. The mice were anaesthetized with ether and hairs were removed by shaving the dorsal back of the mice using a razor blade.

An excision wound of 8mm diameter was made by removing a full thickness piece of the skin from a predetermined shaved area using a biopsy punch which caused two circular wounds of equal size on both sides. The wounds were left undressed to the open environment and no local or systemic anti-microbial agents was used (Subhashini & Arunachalam, 2011; Rashed *et al.*, 2003). In the second post-wounding day, six groups of mice were created by randomization of animals. The three formulated herbal ointment (50%, 33% and 25%), positive (Povidone Iodine), the water

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preparation and negative (simple ointment) control were topically applied on the excised wounds at two days intervals to the four treatment groups.

From the 3rd post-wounding day, wounds were traced using a divider at four days interval. The animals were restrained in proper position during tracing and the tracings were then transferred to 1mm^2 graph paper and the size of the wound diameter scored. Wound diameters were read and the percentages of wound contraction were calculated taking the initial size as 100%. Wound closure (%) = $(A_o - A_n) \times 100\%/A_o$ where A_o is the initial wound diameter, and A_n is the wound diameter at the time interval of 4 days. Where n= number of days 3rd, 7th, 11th and 15th.

Re-epithelisation and histopathological assessment

The healing duration was considered as the number of days the wound disappeared macroscopically without any residue from each animal (Kommu, 2013). The number of days re-epithelisation occurred from each group were recorded in a separate sheet and their averages were determined. After wounds had healed, animals in each group were sacrificed and the wound parts were excised together with the surrounding skin. They were then fixed in 10% neutral well-buffered formalin. Microscopic examination of the wound bed material was performed on haematoxylin and eosin stained 5µm thick paraffin sections as previously described (Geethalakshmi *et al.*, 2013; Maregesi *et al.*, 2016b). The sections were assessed under the light microscope and graded semi-quantitatively in respect of the proliferation of fibroblast cells and collagenisation (Shukla *et al.*, 1999; Bhat *et al.*, 2016). The semi-quantitative grading was done using a scale up: – (equivocal/minimal/mild), + (weak), ++ (moderate) and +++ (remarkable) as previously described (Pes *et al.*, 2010).

A one cm longitudinal incision was made on the midline of the vertebral column of each mouse after shaving. A surgical thread (No. 2/0) and a curved needle (1/2 circle 50mm) were used for the stitching 0.5mm from the edge of the wound. The wounds were then left undressed to the open environment and no local or systemic anti-microbial agents was used as previously described (Rashed *et al.*, 2003; Subhashini & Arunachalam, 2011). On the second day post-wounding, five groups of mice were formed by randomization process. Each group received one of the following; 50% formulated herbal ointment, 33% formulated herbal ointment, 25% formulated herbal ointment, Povidone Iodine (positive control) and simple ointment base (negative control) which were topically applied on the incised wound on daily basis. One group was left untreated identified as untreated group. The sutures were then removed on the 7th day and skin breaking strength was measured by continuous water flow method on day 10 (Shukla *et al.*, 1999).

Tensile strength determination

Skin breaking strength was measured by a designed tensiometer which consisted of a 3 x 6 × 12 inch wooden board platform with two posts fixed on a retort stand on each side of the wooden platform. A pulley was mounted on the clamp on another retort stand. An alligator clamp was tied on another post in such a way that the clamp could reach the middle of the board. Another alligator clamp was tied on a longer fishing line and a 0.5litres polyethylene bottle was tied on the other end passing through the pulley to make the polyethylene bottle suspended free in air at 90° as previously described (Mukherjee *et al.*, 2000).

Before testing, the mice were euthanized and each mouse was placed on the wooden board. The clamps were then carefully clamped on the skin on opposite sides of the wound at a distance of 0.5 cm from the wound. The longer piece of fishing line was placed on the pulley and finally to the polyethylene bottle and the position of the board was adjusted so that the bottle suspended in air. Water was added slowly in the polyethylene bottle until wound was reopened. The weight of both polyethylene bottle and added water were considered as the tensile strength of the wound. The percentage tensile strength was calculated using the formula described below:

%Tensile strength (TS) of test sample = (TS of test sample - TS s:o)*100/TS s:o

%Tensile strength (TS) of reference = (TS of reference-TS s:o)*100/TS s:o

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%Tensile strength (TS) of s:o = (TS s:o-TS l:u)*100/TS l:u

Where, s.o and l.u stand for simple ointment (vehicle) treated and left untreated groups respectively (Gebrehiwot *et al.*, 2015).

Data analysis

Microsoft Excel program was used to run data analysis using One way ANOVA followed by Tukey's method to assess the significant difference between test groups and that of controls. Differences between means were considered significant at $p \leq 0.05$.

Results

Wound contraction

The percentage of wound diameter contraction for animals treated with *S. rhombifolia* leaves extract was significant from day 7 to 15 in two formulations; the 50% as well as the 33% ointments when compared to the negative control. Furthermore, it was also observed that, the 50% ointment formulation showed significant mean percentage wound diameter contraction on day 7, 11, and day 15 as compared to the negative control. In addition, the 33% ointment formulation had significant mean percentage wound diameter contraction on day 7, 11, and day 15 as compared to the negative control. On the other hand, when compared to the negative control, the 25% *S. rhombifolia* ointment formulation showed a significant mean percentage wound diameter contraction of 97.9±2.1 (Table 2) on day 15. The water preparation also, exhibited significant wound contraction on day 7, 11 and 15 when compared to the negative control. No any tested material showed significant wound diameter contraction comparable to that of standard drug (Povidone iodine) used. The mean percentage wound diameter contraction for the untreated group of mice was significantly different from the positive control group for all examined days ($p<0.05$, Table 2).

Table 2: Effect of different *S. rhombifolia* 80% ethanolic leaves extract formulations and water preparation on wounds diameters

	Negative control	25%	33%	50%	Water preparation	Positive control
Day3	4.2±1.3	6.3±1.6	6.3±2.3	8.3±2.1	7.3±1.9	17.7±1.9*
Day7	33.3±3.1	40.6±1.4	44.8±2.5*	45.8±2.1*	43.8±2.3*	63.5±1.9*
Day11	63.8±3.1	73.6±3.0	78.1±2.7*	79.2±2.6*	79.2±1.3*	92.7±3.4*
Day15	81.3±2.0	97.9±2.1*	100±0*	100±0*	97.9±2.1*	100±0*

The values are the mean percentage contraction of wound diameters calculated using ANOVA followed by Tukey's method for comparison. *Means $p<0.05$ and (Significant against negative control)

Re-epithelisation

As shown in Table 3, neither the ointment formulations nor the water preparation of *Sida rhombifolia* leaves extract showed significant mean re-epithelisation days over negative control.

Table 3: Effect of different formulated herbal ointments on wounds epithelisation

Formulation	Negative control	*Sida rhombifolia*	Positive control
50%	18.8±0.3	17.2±0.3	16.7±0.4*
33%	18.8±0.3	17.8±0.3	16.7±0.4*
25%	18.8±0.3	18.0±0.4	16.7±0.4*
Water preparation	18.8±0.3	17.3±0.4	16.7±0.4*

Values are means percentages of epithelisation days calculated using ANOVA followed by Tukey's method for comparison. *Means $p<0.05$ (Significant against negative control)

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Tensile strength results and histopathological results

All formulated ointments used had significant mean percentage tensile as shown in Table 4.

Table 4: Effect of herbal formulations on tensile strength of wounds

Dose/group	Negative control	*Sida rhombifolia*	Positive control
50%	10.7±1.2	64.1±1.7*	98.0±3.8*
33%	10.7±1.2	64.0±3.2*	98.0±3.8*
25%	10.7±1.2	53.1±4.0*	98.0±3.8*

The values are the mean percentage tensile strength calculated using ANOVA followed by Tukey's method for comparison. *Means $p<0.05$ (Significant against vehicle control.)

The 50% *Sida rhombifolia* leaves ointment formulation as well as the water preparation were found to have remarkable fibroblast cells proliferation and collagenisation (Table 5; Figure 1). However, there was also associated moderate inflammation in all the water preparations.

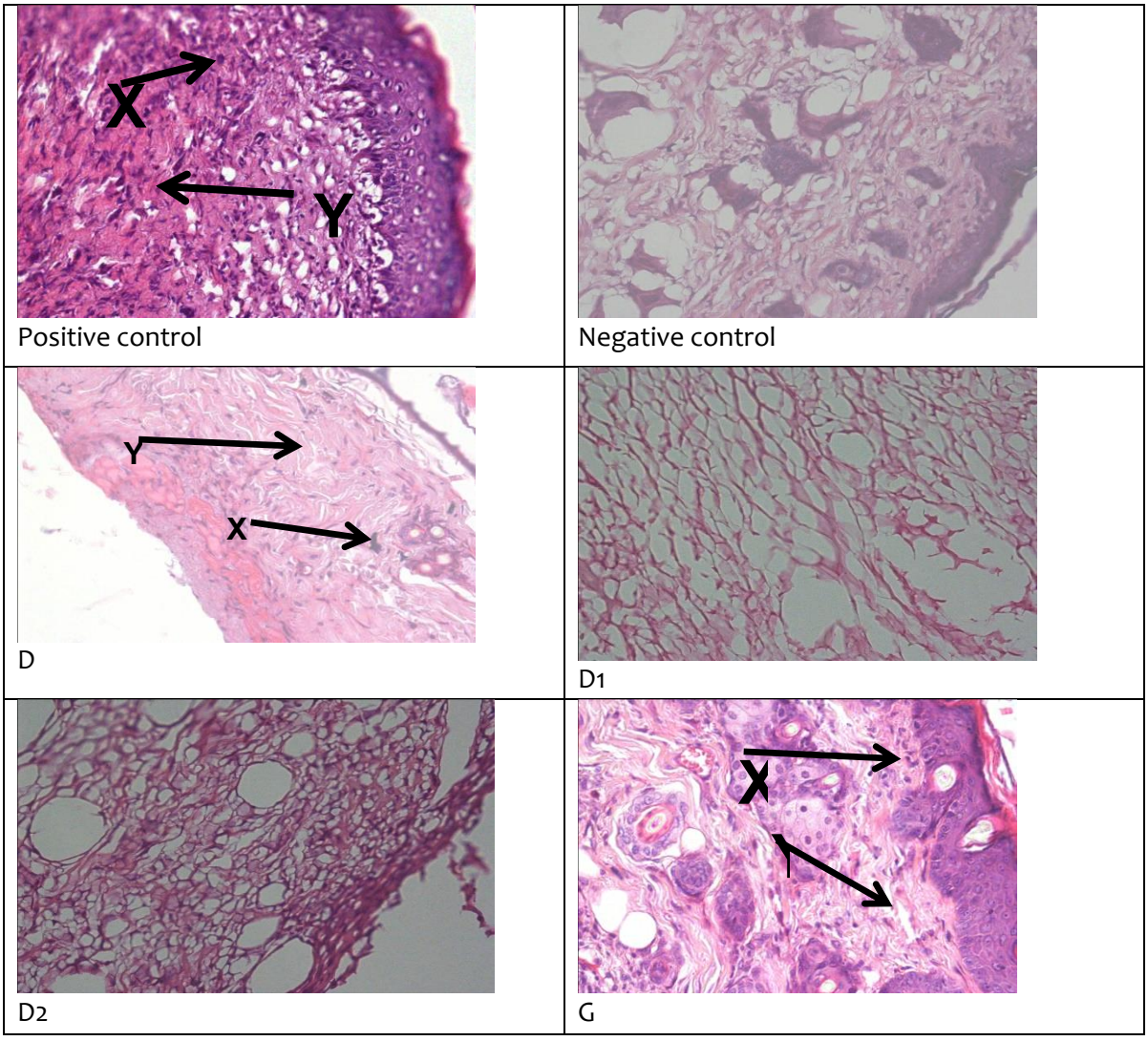

Figure 1: H&E stained microscopic mice skin sections of tissues from the healed area of the wounds from different groups, treated with different herbal formulations at x40 magnification (The X arrow shows fibrosis (Blue) and the Y arrow shows deposited collagen (Pink). (D) Treated with 50%, (D1) Treated with 33%, (D2) Treated with 25% and (G) Treated with water preparation)

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Table 5: Histological assessment results

Test group	Fibroblast cells	Collagen fibre
Positive Control	++	++
50%	+++	+++
33%	++	++
25%	+	+
Water preparation	+++	+++
Negative control	++	++

Key: + =weak; ++ =moderate; +++ =remarkable

Discussion

In the present study three parameters for wound healing activity were investigated, percentage wound contraction, tensile strength of skin and the period of re-epithelisation. According to these results, *S. rhombifolia* leaf formulations at different percentages tested, significantly enhanced the rate of wound healing as indicated by higher mean percentage of wound diameter contraction and higher tensile strength of the wound tissues as compared to negative controls. The ointment formulation at 50% showed significant wound contraction from day 7 post wounding which was closer to that of the standard drug (Povidone iodine). This formulation increased the collagen content of the skin which ultimately contributed to increased wound tensile as also shown in the results. The collagenisation was complimented by the remarkable fibrosis which was also associated with this formulation.

It should also be noted that previous reports have indicated that the wound healing effects of *S. rhombifolia* could partly be attributed to their antibacterial and antioxidant properties as evidenced in their ability to inhibit bacteria growth and protect fibroblast cells against oxidant injury (Dhalwal *et al*., 2007; Kiessoun *et al.,* 2012; Logeswari *et al.*, 2013). The wound healing properties of *S. rhombifolia* may also be contributed to by the phytochemical constituents present in the plant. This plant species has been reported to have tannins, polyphenols, alkaloids, glycosides, flavonoids and saponins all of which play different roles in wound healing (Sapna *et al.*, 2016).

A numbers of compounds isolated from *S. rhombifolia* leaves have been demonstrated *in vitro* as active principles responsible for facilitating healing of wounds such as antibacterial, antifungal, ant-inflammatory and antioxidant activities (Dhalwal *et al*., 2007; Assam *et al*., 2010; Poojari, 2011; Narendhirakannan & Limmy, 2012; Woldeyes *et al.,* 2012). It has further been previously reported that, *S. acuta* and *S. cordifolia* which belong to the same family as *S. rhombifolia* showed antimicrobial activity and significant wound healing activity in incisional and excisional models (Ekpo & Etim, 2009; Akilandeswari *et al.*, 2010). These reports corroborate the results observed in the current study, since all the species are under the same Genus, they are likely to contain relatively similar compounds responsible for wound healing activity. The remarkable fibrosis and collagenisation for water preparation suggest that the plants may have compounds which promote healing and repair. This also suggests that probably the wound healing compounds from these plants may be more water soluble.

The findings in this study suggest that the 80% ethanolic extract and the water preparation of *S. rhombifolia* leaves have a potential benefit in enhancing the wound healing process. This observation corroborates its use in traditional medicine in the treatment of wounds. The observed efficacy may possibly be attributed to the presence of different compounds in the extracts which are known to contribute in the wound healing properties of this plant. It is therefore recommended that, more fractionation and isolation studies be done to identify active compound(s) in *Sida rhombifolia* leaves which are responsible for the wound healing activity.

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