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In vivo Efficacy of *Diospyros abyssinica* Leaf 80% Methanolic Extract from Tooro Botanical Gardens on Wounds using Mice

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ABSTRACT

Various communities in Uganda have historically utilized the leaves of *Diospyros abyssinica* to treat wounds, although, regarding its capability to treat wounds, there were no published scientific studies. To get around the drawbacks of traditional medications, research into natural therapies for the treatment of wounds is necessary. The study's objective was to assess the *in vivo* effectiveness of a *Diospyros abyssinica* leaf extract from the Tooro botanical gardens on wounds in mice. The leaves of *Diospyros abyssinica* were cleaned, dried in the cooling shade, and milled into fine dust. After that, they were macerated in eighty percent methanol for three days. The percentage of the wound's original area that contracted was used to assess the healing process in incised wounds. Afterward, animals were divided into 5 groups, caged and treated in their respective groups topically. Group 1 was treated with 25mg/mL of concentrated extract, group 2 with 50mg/mL of *Diospyros abyssinica* concentrated methanol extract, group 3 was treated with 100mg/mL of *Diospyros abyssinica* methanol extract, group 4 with Vaseline (negative control), group 5 with sulfadiazine 0.1% cream (positive control) until the wounds are fully healed. During wound treatment, 100mg/ml of 80% methanolic crude extract *Diospyros abyssinica* leaves showed significant mean wound area ($p<0.05$) during days 2 to 6 and on days 10 to 14 evaluations, followed by 50mg/mL and 25mg/mL. The study's findings supported previous claims by demonstrating that an 80% methanolic extract of *Diospyros abyssinica* leaves exhibits dose-related wound healing efficacy.

Keywords: Wound, Diospyros abyssinica, in vivo, Traditional medicine

INTRODUCTION

A wound is defined as a breach or break in the protective layer of the epidermis which compromises the metabolic and structural integrity of living tissues. A wound develops each time the cutaneous barrier's integrity is threatened. This might be as shallow as a small crack in the skin's epithelial integrity or as deep as subcutaneous tissue [1-3]. Traditional medicine is the sum total of knowledge, skills, and practices based on indigenous theories, beliefs, and experiences that are utilized to sustain health in various cultures. It encompasses the prevention, diagnosis, or treatment of health illnesses of the body and mind [4-6]. 80 percent of people in underdeveloped countries use traditional medicine, according to the World Health Organization (WHO). The use of complementary and alternative medicine (CAM), particularly herbal remedies, has increased throughout the industrialized world during the past few decades [7-9]. Herbal medications include herbs, herbal materials, herbal preparations, and completed herbal products that contain active substances that are plant parts or other plant components. The widespread usage of traditional medicine in Africa, which is primarily comprised of medicinal plants, has been related to cultural and economic factors. This is why the WHO encourages African countries to promote and incorporate traditional

medicinal practices into their health system. Phytochemicals, often referred to as secondary metabolites, are frequently found in plants and can either act alone, in combination, or in a synergistic manner to benefit health [10-13]. Contrary to pharmaceutical drugs, medicinal plants usually include a variety of chemicals that work in combination catalytically and synergistically to produce a combined effect that is higher than the sum of the activities of the individual components [14-16]. Therefore, a number of these traditional medicines have been associated with wound healing [17-19]. Any disruption of the skin caused by microbiological, physical, or chemical causes is referred to as a wound. Morbidity, disability, socioeconomic crisis, and mortality are all linked to it [20]. Wound management is the practice of preserving a warm, moist, nontoxic environment that supports organic wound healing. Traditional medicine uses mineral, animal, and plant-based medicines to treat, diagnose, and maintain health. It is a widely accepted, efficient, practical, and affordable kind of healthcare. Traditional herbs have proven analgesic, proliferative, antioxidant, anti-diabetic, anti-inflammatory, and antibacterial properties due to their phytoconstituents [21-23]. Thus, traditional medicinal herbs have the ability to heal wounds. For example, *abyssinica* is a widespread plant in Africa, spanning from Guinea to Eritrea, southwards to the Flora Zambesiaca, and Angola. The species is a huge tree with a crown that is sparsely branched and short. It is an evergreen plant, like other Diospyros. The leaves are oblong-elliptic, lustrous dark green, and alternate. Their edge is continuous and frequently wavy. The juvenile leaves are a bright scarlet color. Flowers are tiny, axillary, and found single or in clusters of a few flowers. Their color ranges from creamy white to golden. The plump, spherical fruit is contained in a lobed cup. When fully ripe, it turns a yellowish-green color before turning blue-black [24].

The antioxidant activity of ***D. abyssinica*** root bark has been investigated. Petroleum ether, dichloromethane, chloroform, 80 percent aqueous ethanol, and water (at 50°C and 100°C) were used to extract the substance. The root bark of ***D. abyssinica*** was found to be the richest source of extracted compounds, with antioxidants accounting for 36.7 percent of the plant's weight. The radical scavenging activity of ***D. abyssinica*** was highest in the 80 percent ethanol and methanol extracts. As a result, this plant appears to be high in antioxidants [25].

One of the most prevalent disorders, wounds frequently cause serious health problems and have expensive treatment expenses. The sequence of events must be progressed orderly and under control in order to establish the integrity of the injured tissue; otherwise, they may result in physical handicap or even death. There are substantial drawbacks, such as invasiveness and high expense, to current wound therapy methods including water distribution, trimming, antibiotics, enzymes that break down proteins, and tissue transplants. The evolution of tolerant strains, in addition to the dearth, high expense, and sluggish production pace of new antimicrobial agents, all contribute to an increase in the mortality and morbidity related to wounds. The rise of resistant bacterial strains, especially those that cause wounds, is a serious public calamity. Consequently, wound infection continues to be the leading contributing factor to the formation of nonhealing wounds and thus a significant burden for both patients and caregivers [26]. Furthermore, in Uganda, cuts, bites, or open wounds were the most common type of injury (38%) and the most common site of injury (44%) [27]. Herbalists and ethnics find little interest in Western medicine up to the present day, but rather find more interest and convenience in using traditional medicinal plants. It's believed that *Diospyros abyssinica* is used for wound healing in local communities in Uganda but no scientific study has been done on this to prove its effectiveness in healing wounds [28]. This study, therefore, was planned to determine the efficacy of *Diospyros abyssinica* 80% methanolic extract in wound healing using a mice model.

METHODOLOGY

Drugs and Chemicals Used

Vaseline, methanol absolute, distilled water, sulfadiazine cream 0.1% (as a positive control), Halothane, 70% alcohol, and Normal saline, electric Vernier caliper, were purchased and utilized.

Collection and preparation of plant material

Diospyros abyssinica leaves were collected from Tooro Botanical Gardens, Fort portal city, Kabarole district western Uganda. The latitude of Tooro Botanical Gardens is 0.66416°, and the longitude is 30.28474° with the GPS coordinates of 0.6668° N, 30.2854° E. A taxonomist from Mbarara University of Science and Technology, Uganda, identified *Diospyros abyssinica* and registered it with a number of reference species. And the experiment was conducted in Kampala international university pharmacognosy laboratory and Kampala international university animal house

Number of animals used

A total of 25 animals (mice) were used and divided into 5 groups of 5 animals. Each group was caged individually during the experiment.

Preparation of 80% Diospyros abyssinica methanolic extract

In order to improve the penetration of the extracting solvents, the leaves were cleansed with water to remove impurities and debris, shade dried at room temperature, and then coarsely pulverized with a mortar and pestle. Until extraction, plant powder was kept in a tightly closed container. *Diospyros abyssinica* leaves weighing 490g were finely ground and macerated in 2.25 liters of 80% methanol for three days while being regularly shaken. After three days of maceration, Whatman paper Number 1 was utilized for filtration and thick layers of 40 mesh gauze were used for extraction. The residue was repeatedly macerated for two days at a volume of 80% methanol to produce the greatest yield. The filtrate was deeply frozen overnight and then freeze dried in a lyophilizer at -50°C in order to remove the solvent. A rotary evaporator was employed to facilitate concentration. The proportion yield of the extract was computed and stored in a refrigerator at 4°C up until the formulation of the ointment and solvent fractionation.

Wound induction using the excision wound model

The mice were sedated with diethyl ether before being wounded. The region to be wounded was first cleaned with 70% alcohol, followed by a standard saline wash, and preceded by the removal of rat furs with shaving cream.
Using tweezers and a thin, sharpened pair of disinfected cutters, the skin was removed from each rat after a full-thickness incision measuring roughly 1.5 cm was made. Using a thin permanent marker pen, the estimated wound area was documented. Each group of mice was given its own cage after being randomly divided into 5 groups. In every case, the therapy was applied topically once daily. The date of injury was regarded as day 0. To treat the respective groups topically, group 1 was treated with 25mg/ml of concentrated extract, group 2 with 50mg/ml of *Diospyros abyssinica* concentrated 80% methanolic extract, group 3 was treated with 100mg/ml of *Diospyros abyssinica* 80%methanolic extract, group 4 with Vaseline (negative control), group 5 with sulfadiazine 0.1% cream (positive control) were used until the wounds are fully healed.

Table 1. Animal grouping and dosing

GROUPS	DESCRIPTION	TREATMENT
1	*Diospyros abyssinica* (low dose)	25mg/ml *Diospyros abyssinica*
2	*Diospyros abyssinica* (medium dose)	50mg/ml *Diospyros abyssinica*
3	*Diospyros abyssinica* (high dose)	100mg/ml *Diospyros abyssinica*
4	Vaseline (Negative control)	Vaseline
5	Silver sulfadiazine 0.1% cream Positive control)	Silver sulfadiazine 0.1% cream

Preparation of solutions

The 25mg/mL, 50mg/Ml, and 100mg/mL of *Diospyros abyssinica* methanol leaf extract were prepared by weighing 0.5 grams, 1gram and 2grams of the lyophilized extract respectively and later dissolving each of them in 20ml of distilled water. The diameter of the wounds was evaluated on all the days to determine the rate of wound healing and expressed as a percentage from day 0.

$$\%wound\ area\ contraction = \frac{(initial\ wound\ area - wound\ area\ on\ day\ n)}{initial\ wound\ area} \times 100$$

Percentage yield

The total mass of *Diospyros abyssinica* raw extract was calculated and the % output per mass of the sample was computed. The dried percentage yield was calculated using

$$percentage\ yield = \frac{weight\ of extract}{weight\ of\ sample} \times 100$$

LD50 determination

Acute toxicity (LD50) study

An acute toxicity study was carried out using Lorke's method [29]. In the first phase, nine mice were randomly divided into three groups of three mice per group and were administered 10, 500, and 1000 mg extract/kg body weight orally using a cannula, respectively. The mice were observed for signs of adverse effects and death for 24 hours and then weighed daily upto14 days. In the second phase of the study, the procedure was repeated using three mice randomly divided into three groups of one mouse each, given 1600, 2900, and 5000 mg extract/kg body weight, respectively. The mice were also observed for signs of toxicity, mortality, and weights taken for 14 days

Dermal toxicity determination

The acute cutaneous toxicity test of *Diospyros abyssinica* raw extract was performed in accordance with OECD proposed standard number 404. Three female mice with typical skin textures were chosen at random and housed in separate cages. The fur on the dorsal half of the body was shaved 24 hours before the inspection. For 24 hours, a 10% w/w extract composition was constantly applied to the shaved region. Throughout the exposure period, mice resided in separate housing. After the exposure time, the remaining test chemical was removed, and the mice will then undergo a 14-day period of daily skin reaction monitoring.

Statistical Data analysis

Data analysis was performed using the Social Science Statistical Software (SPSS) version 25 for Windows. For each group, the results were presented as mean ± standard mean error (SEM). Statistical variations among the groups will be examined using a one-way variance analysis (ANOVA) with Tukey as a posthoc test, with statistical significance assessed at $p<0.05$.

RESULTS.

The percentage yield of the crude extract

From 490 grams of fine powder of the leaves of *Diospyros abyssinica* 60g of crude extract was obtained. The 80% methanolic crude extracts of *Diospyros abyssinica* were weighed and the yields were calculated as a percent of the dried plant material. The yield was 12.24% of *Diospyros abyssinica.*

Percentage yield = $(60/490) \times 100$

$= 12.24\%$

LD50 determination test

On observation for the first 2-4 hours and after 24 hours there were no signs of toxicity or mortality in the first phase, but on the third-day mice numbered 1 died from group 3.

The minimum lethal dose of *Diospyros abyssinica* was determined using the formula below after the death of one mouse at a dose of 1000mg/ml

Minimum Lethal Dose $=\sqrt{(D_0 * D_{5000})}$

$= \sqrt{(1000 * 5000)}$

$= 2.2361$mg/kg

Acute Dermal Toxicity

There was no skin toxicity (inflammation, itchiness, or erythema) detected after 24 hours of topical treatment of 200mg/kg of 10% extract formulation. When the animals were examined for 48 hours and 14 consecutive days of cage-side observation, no indications, symptoms, or mortality were observed.

Phytochemical screening of the crude extract of *Diospyros abyssinica*

Table 2 shows the different phytochemicals of diospyros abyssinica

Phytoconstituent.	Description
Alkaloids	Positive
Flavonoids	Positive
Phenols	Positive
Tannins	Positive
Steroids	Positive
Glycosides	Positive
Anthraquinones	Positive
triterpenoids	Positive
Saponins	Positive

The wound healing effect of D. abyssinica
Table 3 shows the mean wound area of the study animals

Treatment groups	Wound area (mm^2)							
	Day 0	**Day 2**	**Day 4**	**Day 6**	**Day 8**	**Day 10**	**Day 12**	**Day 14**
25 mg/ml of *D. abyssinica*	227.96 ± 23.60	156.77 ± 30.23	103.98 ± 24.28	63.60 ± 17.89	45.29 ± 14.90	13.76 ± 3.00	5.06 ± 1.27	1.27 ± 1.37
50 mg/ml of *D. abyssinica*	207.60 ± 10.05	117.83 ± 6.78	84.91 ± 13.31	52.18 ± 7.81	30.00 ± 8.39	12.97 ± 1.82	5.22 ± 0.85	1.08 ± 0.39
100 mg/ml of *D. abyssinica*	115.08 ± 8.86	56.32 ± 5.63 [a]	38.74 ± 6.74 [a]	21.41 ± 3.52 [a]	11.44 ± 3.30	8.87 ± 3.02 [a]	3.22 ± 1.71 [a]	0.54 ± 0.54
Negative control.	101.19 ± 9.35	84.12 ± 13.07 [a]	43.46 ± 7.92 [a]	28.00 ± 5.12	22.48 ± 2.93	13.72 ± 2.99[c]	5.26 ± 1.52 [b, c]	3.33 ± 1.32[c]
Positive control	98.16 ± 14.84	67.79 ± 67.79 [a]	52.07 ± 4.33	32.93 ± 2.05	18.77 ± 1.40	9.91 ± 1.17	6.24 ± 1.22 [c]	3.24 ± 1.13

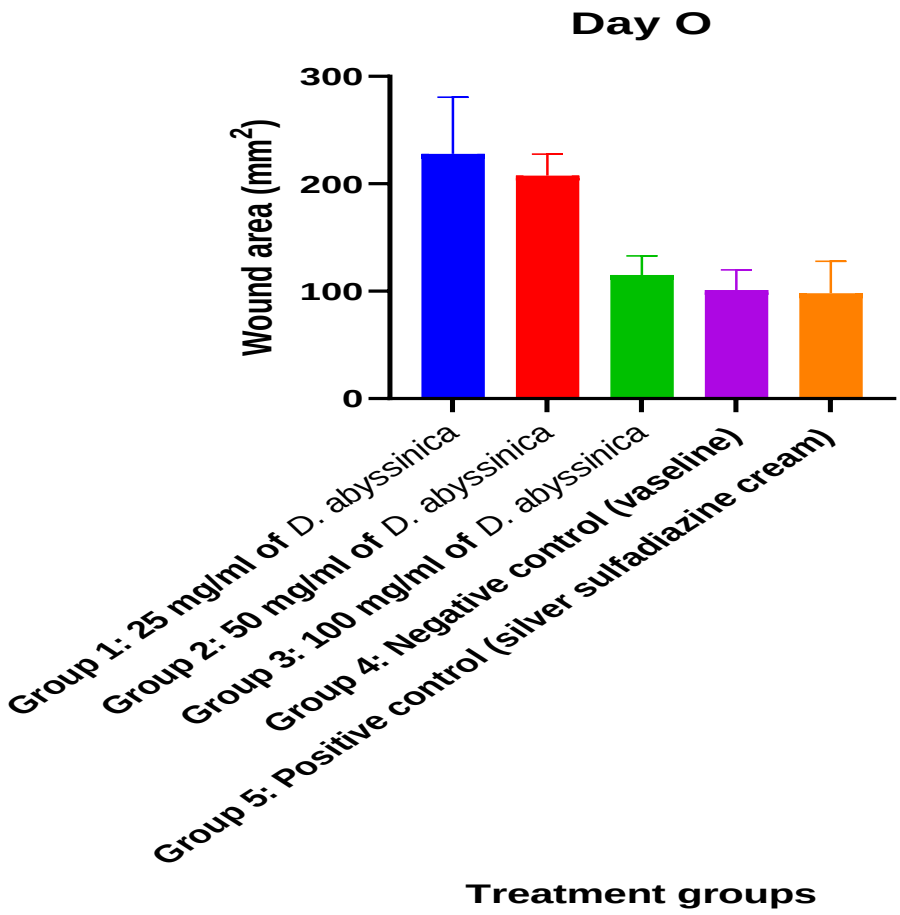

Figure 1: showing the mean wound area on day 0. Results are Mean ± SEM. n = 5.
Figure 1 shows the different wound areas of the individual treatment groups on day 0.

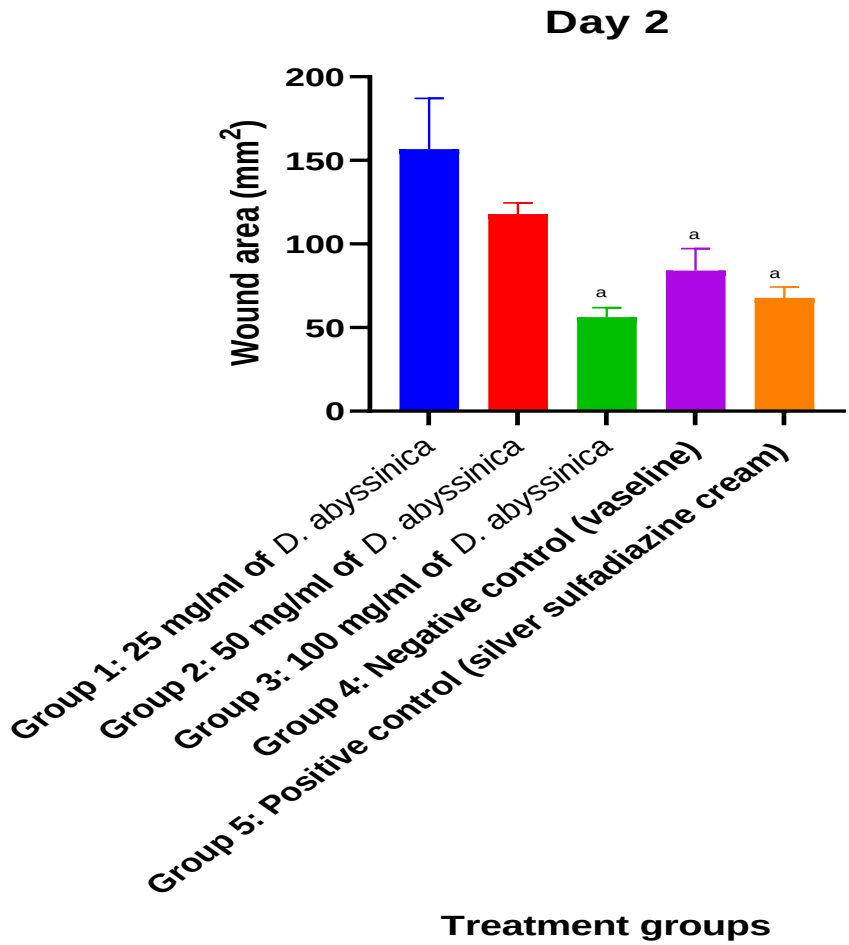

Figure 2: showing the mean wound area on day 2. Results are Mean ± SEM. n = 5. a = p < 0.05 vs group 1.
The study results in figure 2 show that the mean wound area on day 2 was highest in the 25 mg/ml group. The study also indicates that there was a significant (p = 0.0015), (p = 0.0257) and (p = 0.0050) difference in the mean wound area on day 2 in group 1 which received 25 mg/ml of *D. abyssinica* as compared to the 100 mg/ml of *D. abyssinica*, negative control and positive control groups respectively.

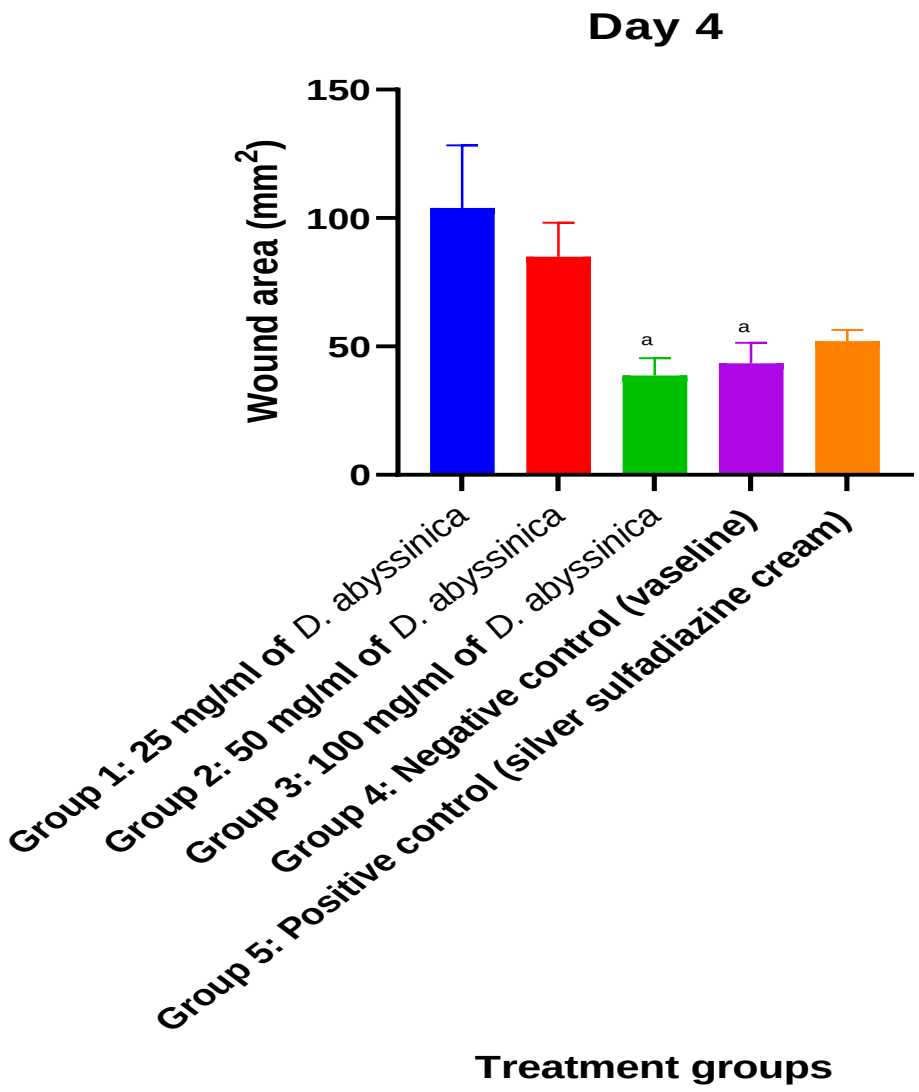

Figure 3: showing the mean wound area on day 4. Results are Mean ± SEM. n = 5. a = p < 0.05 vs group 1.
The study results in figure 3 show that the mean wound area on day 4 was highest in the 25 mg/ml group. The study also indicates that there was a significant (p = 0.0191) and (p = 0.0325) difference in the mean wound area on day4 in group 1 which received 25 mg/ml of *D. abyssinica* as compared to the 100 mg/ml of *D. abyssinica*, negative control groups respectively.

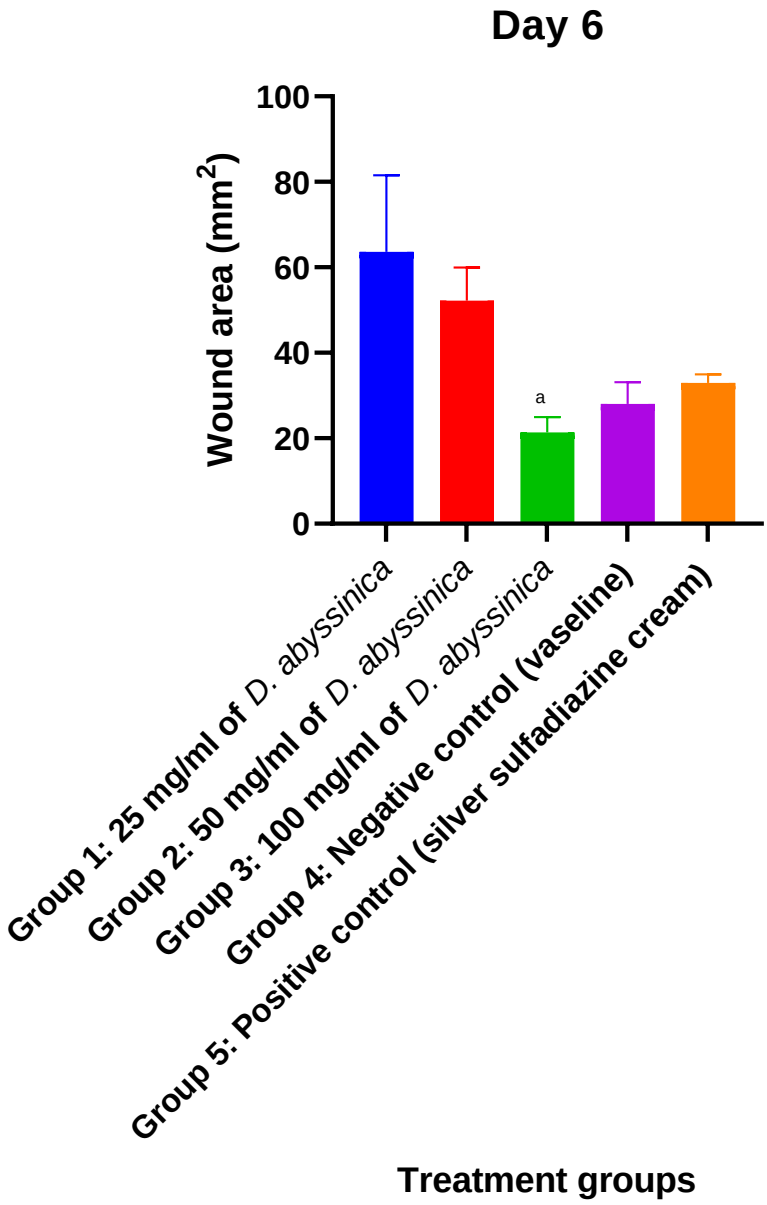

Figure 4: showing the mean wound area on day 6. Results are Mean ± SEM. n = 5. a = p < 0.05 vs group 1.
The study results in figure 4 show that the mean wound area on day 4 was highest in the 25 mg/ml group. The study also found a substantial (p = 0.0299) decrease in the average area of the wound on day 6 in the 100 mg/ml *D. abyssinica* group versus group 1 which received 25 mg/ml *D. abyssinica.*

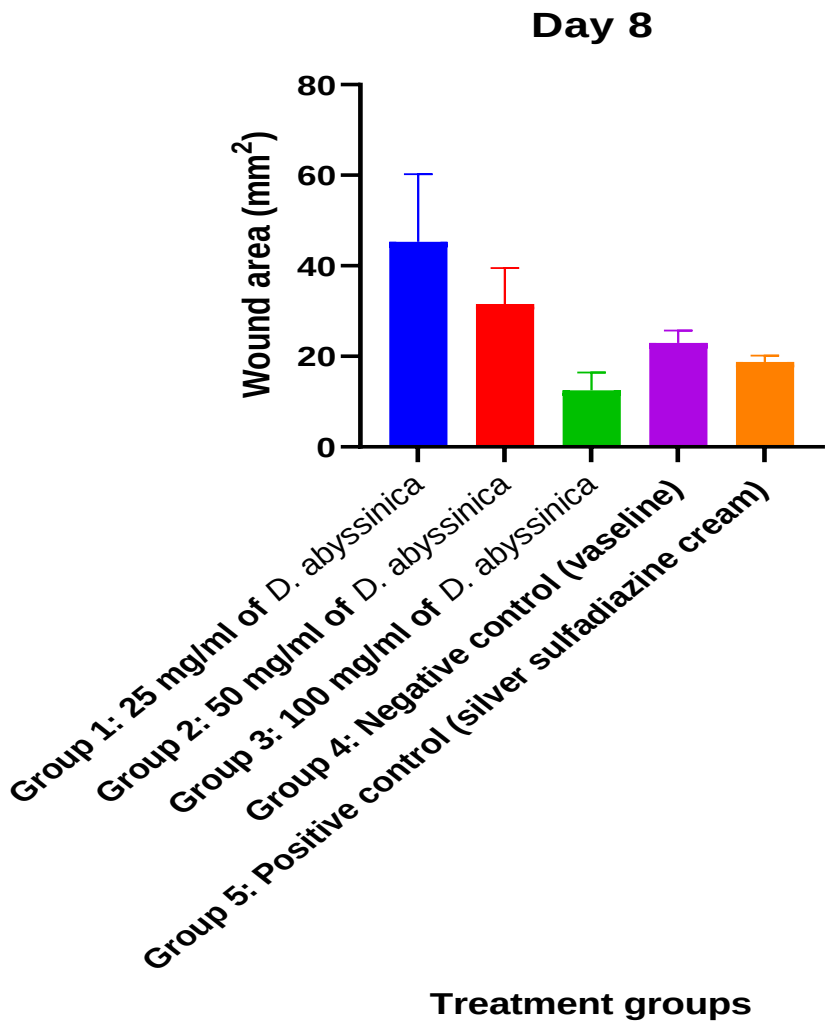

Figure 5: showing the mean wound area on day 8. Results are Mean ± SEM. n = 5.
The study results in figure 5 show that the mean wound area on day 8 was highest in the 25 mg/ml of *D. abyssinica* group. The study also indicates that there was no significant difference in the mean wound area on day 8 in all treatment groups.

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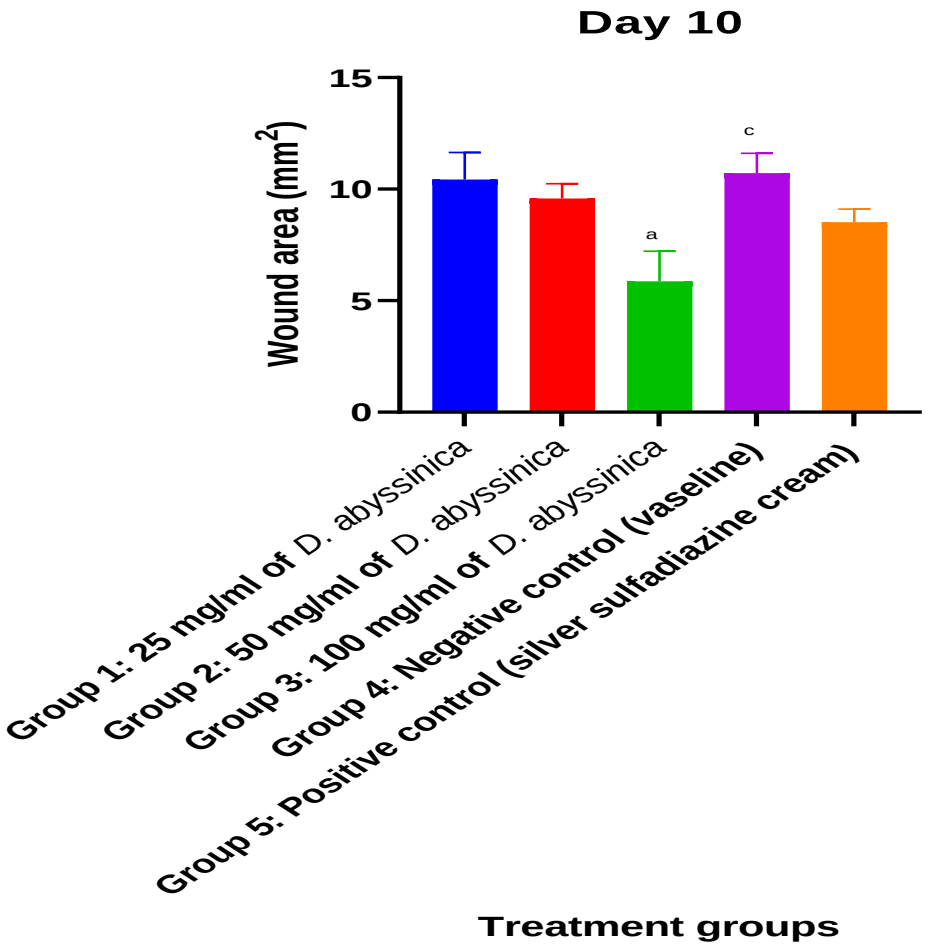

Figure 6: showing the mean wound area on day 10. Results are Mean ± SEM. n = 5. a = p < 0.05 vs group 1; c = p < 0.05 vs group 3.

Figure 6 shows that the average area of the wound on day 10 was greater in the negative control (vaseline) group than in the *D. abyssinica* 100 mg/ml group.

The study also found a substantial (p = 0.0286) difference in the average area of the wound on day 10 in group 1 treated with 25mg/ml of *D. abyssinica* versus group 3 treated with 100 mg/ml of D. abyssinica.

The study also found a substantial (p = 0.0181) difference in the average area of the wound on day 10 between groups 3 and 4, which received 100 mg/ml of *D. abyssinica* and vaseline, respectively.

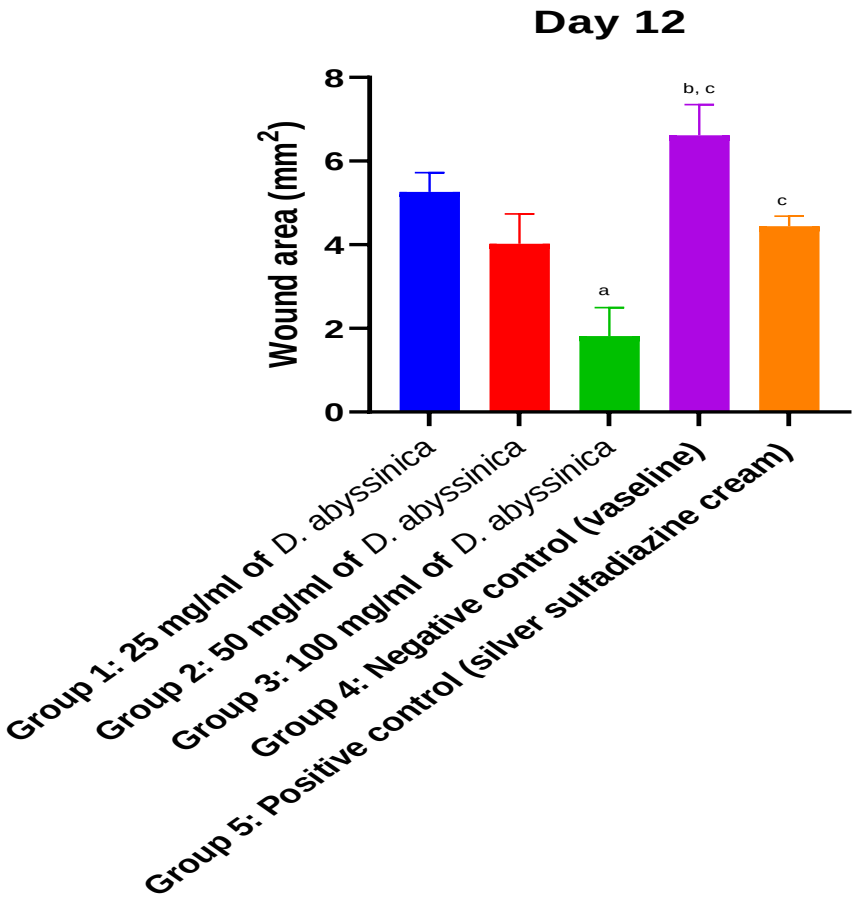

Figure 7 showing the mean wound area on day 12. Results are Mean ± SEM. n = 5. a = p < 0.05 vs group 1; b = p < 0.05 vs group 2; c = p < 0.05 vs group 3.

Figure 7 depicts the study results, which reveal that the mean wound area on day 12 was greatest in the negative control group.

The study also found a substantial (p = 0.0046) decrease in average area of wounds on day 12 in group 1 treated with 25mg/ml of *D. abyssinica* compared to group 3 treated with 100 mg/ml of *D. abyssinica*.

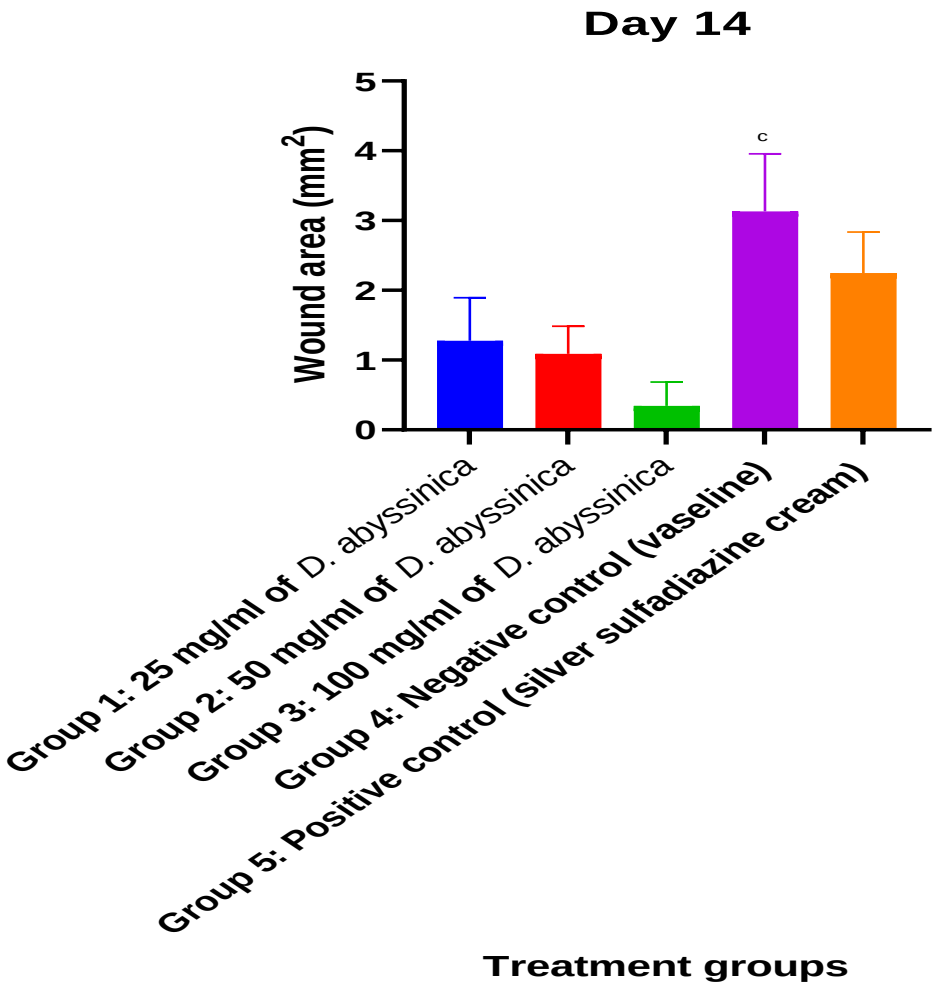

Figure 8: showing the mean wound area on day 14. Results are Mean ± SEM. n = 5. c = p < 0.05 vs group 3. Figure 8 shows that the mean wound area on day 14 was greatest in the negative control (Vaseline) group. The study also found a substantial (p = 0.0209) decrease in the average area of the wound on day 14 in group 4, the negative control, as compared to the 100 mg/ml of *D. abyssinica*.

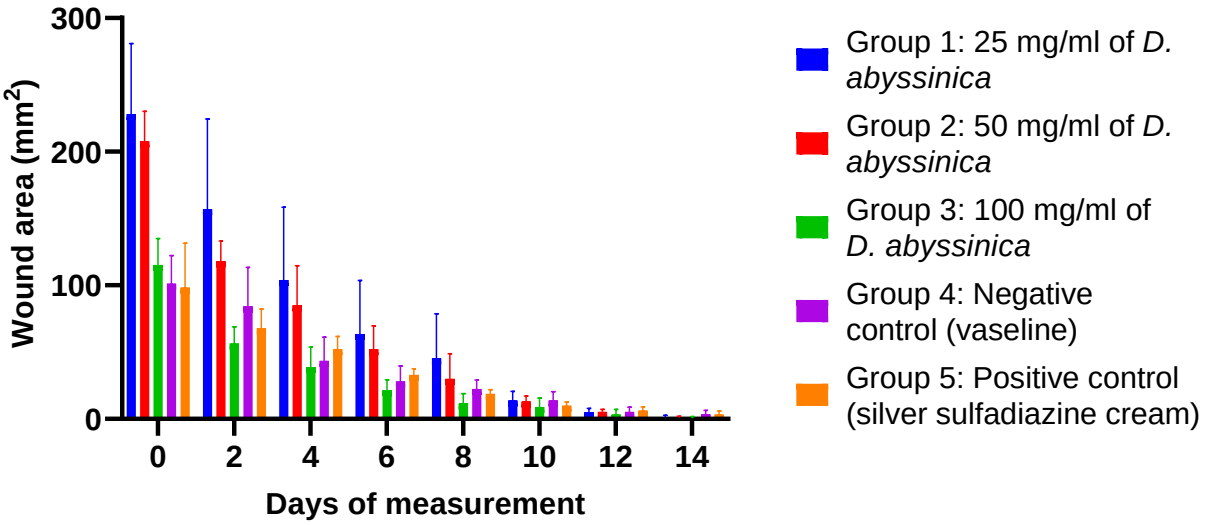

Figure 9: showing the mean wound area post induction. Results are Mean ± SEM. n = 5.
The figure 9 shows a decrease in mean wound area of the mice in groups 1-5 with increase in number of treatment days

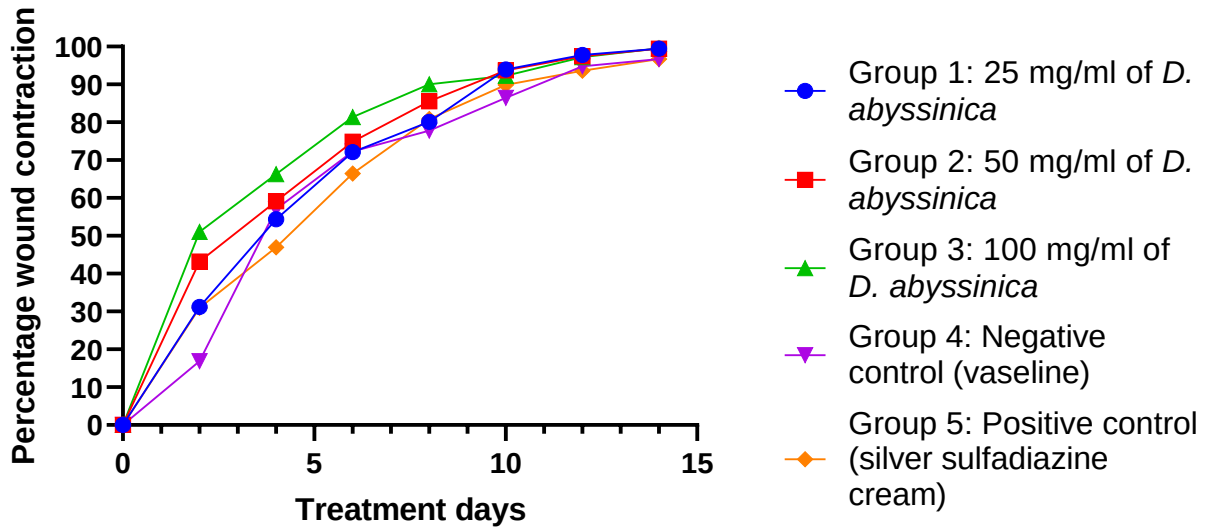

Figure 10: showing the percentage of wound contraction post-induction. Results are Mean ± SEM. n = 5
Figure 10.
For mice in group 1 that were treated with25mg/ml of *Diospyros abyssinica*
From day 0-8, the mice's proportion of wound contracture gradually increased from 0 to nearly 70%. Additionally, mice in group 1 that received treatment with 25mg/mL of *Diospyros abyssinica* saw a gradual increase in the

proportion of wound contraction from days 8 to 14 that eventually reached an almost consistent look.From day 0-2 there was a steep increase in percentage wound contraction, and from day 2-10 there was a gradual increase in percentage wound contraction, and later a slow increase in percentage wound contraction from day 10-14. For group 3: 1000mg/ml of *Diospyros abyssinica* treated mice. From day 0-2 the percentage wound contraction increased steeply, from day 2-8 the percentage wound contraction increased rapidly, and from day 8-14 the percentage wound contraction increased gradually. For group 4: Negative control (Vaseline). From day 0-2, the percentage of wound contraction increased gradually, from day 2-4, the percentage of wound contraction increased steeply, and from day 4-14 the percentage of wound contraction increased gradually. For group 5: silversulfadiazine0.1% cream (positive control). From day 0-10, the percentage of wound contraction increased gradually, and from day 10-14 the percentage of wound contraction increased slowly.

DISCUSSION

Numerous medicinal plants have historically helped wounds heal quickly and with little pain, discomfort, or scarring for the patient. The majority of these plants have been proven to be effective at healing wounds by science. Several communities in Uganda and throughout Africa have historically utilized the experimental plant *Diospyros abyssinica* to treat wounds. Prior to this investigation, its wound-healing abilities had never been evaluated scientifically in vivo [30]. This study was conducted to accurately assess the potential of this plant as an alternative treatment for wound healing *in vivo. In vivo* models are the most reliable prediction models for studying wound healing because they accurately represent the wound environment, including various cell types and environmental inputs [30]. Constriction is important during healing because it reduces the size of the wound and so shortens the healing time. Furthermore, contraction minimizes the amount of matrix outside the cell required to heal the defect and aids re-epithelization by shortening the distance traveled by migrating keratinocytes [26]. The presence of phytoconstituents such as unsaturated sterols, triterpenoids, alkaloids, flavonoids, saponins, tannins, and phenolic compounds, which are known to promote the wound healing process, may explain the increased rate of wound contraction and decrease in epithelialization period in mice treated with the extract. By preventing both cyclooxygenase and lipoxygenase activity, flavonoids lessen the production of inflammatory metabolites [4, 12]. Furthermore, flavonoids inhibit neutrophil degranulation, a direct technique of lowering the secretion of arachidonic acid from neutrophils along with other immune cells, which helps improve the extract's ability to heal wounds. These phytochemicals' astringent and antibacterial qualities may also be responsible for the constriction of wounds and accelerated rates of epithelialization. Tannins are thought to be powerful detoxifiers that also prevent bacterial development [10, 25].

Herbal extracts frequently contain a wide range of compounds, including those that are required for or function as signaling molecules, lubricants, proliferative process aids, wound contraction, cofactors, antioxidants, radical scavengers, anti-infectives, and minerals [15, 16, 31]. Extracts from plants *including C. quadrangularis*, *A. multiflorum*, and *Erythrina abyssinica* have been used to heal a variety of wounds. Thus, through repeated use, their effectiveness and safety have been shown. In order to be helpful in the therapy of wounds, herbal remedies should contain a number of general characteristics, such as controlling/managing secondary infections and inflammation as well as promoting tissue regeneration. An injury may cause damage to the epidermis, local vasculature, dermis, and maybe other underlying tissues. This frequently starts a number of wound-healing processes [31]. *Diospyros abyssinica* leaf methanolic extract showed effectiveness against common wound infections such as methicillin-resistant staphylococcus aureus, S. aureus, and P. aeruginosa in an in vitro investigation, which could support the results of this study [25].Group 3 animals that were treated with 100mg/ml of *Diospyros abyssininica* crude extract showed greater activity which had a meaningful ($p < 0.05$) difference in wound space decline juxtaposed with group 1 animals that were treated with 25mg/ml of *Diospyros abyssinica* extract, 50mg/ml *Diospyros abyssinica* extract, Vaseline treated groups and the positive control (silver sulfadiazine0.1% cream treated groups. When compared to the group 4: negative control treatment groups, the silver sulfadiazine-treated animals showed no notable difference in wound area shrinkage.

CONCLUSION

Generally, in the present study, 100mg/ml of *Diospyros abyssinica* extract showed better wound healing properties which were dose-related. As a result, the findings of this study give scientific evidence validating the therapeutic application of *Diospyros abyssinica* leaves for wound repair in Ugandan communities. Experimental *Diospyros abyssinica* 80% methanolic extract showed no symptoms of inflammation, redness, or swelling (dermal toxicity) after 24 hours and 14 days of observation, and the least hazardous dose was 2.2361 mg/kg, calculated after the death of one mouse at a dose of 1000mg extract/kg. Since the *Diospyros abyssinica* plant showed a greater wound healing potential at the concentration of 100mg/, more studies need to be carried out on the chronic toxicity of the plant to ensure adequate safety in the users because of the death of one mouse. Furthermore, the plant needs to be examined for other traditional claims like anti-snake venom.

Ethical considerations.

The institutional and international Research Ethical Committees and inter-guidelines about the use of animals for experimental research were followed so as to inflict no pain on the mice.

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