*Original Research Article*

# Evaluation of infected and non-infected wounds healing activity of *Eriosemarobustum* hydroethanolic leaves extract ointments in streptozotocin induced diabetic rats




## ABSTRACT

**Aims:** Untreated diabetic wounds provide an optimal environment for bacterial growth, which, over time, can develop resistance to common antibiotics and ultimately result in amputation. Therefore, it is necessary to search for new sources of antimicrobial molecules with wound healing activity owing to the presence of different secondary metabolites in medicinal plants.
**Aims: T**his study was to evaluate the *in vivo* antibacterial and diabetic wound healing capabilities of 70° hydroethanolic extract of *Eriosemarobustum* leaves on non-infected and infected diabetic wound.

**Methodology:**To do this, obese albino *Wistar* male rats (200–280 g) were divided into eleven groups and were made diabetic by intraperitoneal injection with a low dose of streptozotocin at 45 mg/kg of body weight. An excision wound with a surface area of 314 $mm^2$ was created on the dorsal area of each animal, except in the uninjured diabetic (DNB) group. The 70° hydroethanolic extract was used to prepare 1%, 5%, and 10% ointments, with L-Mesitran serving as the reference ointment. Healing potential was assessed by measuring wound contraction rates and determining serum and tissue hydroxyproline, serum lactate deydrogenase (LDH) and total protein levels (TP). The antibacterial power evaluated *in vivo* of *Eriosemarobustum* leaves was also assessed by culturing the skin after healing.
**Results:**The results demonstrated a significantly faster healing rate in the non-infected groups (5%,10% and L-Mesitran) compared to the infected groups. The levels of tissue hydroxyproline and total proteins were significantly ($p < 0.05$) elevated in all treated groups compared to infected and negative controls, unlike serum hydroxyproline levels. LDH levels were significantly ($p < 0.05$) elevated in both negative control group compared to the treated groups. The culture of different skin samples on previously injured areas on the $20^{th}$ day of treatment showed no growth of *S. aureus* on completely healed areas and a low rate in the groups treated during the healing process.
**Conclusion:**70° Hydroethanolic leaves extract of *Eriosemarobustum*possess *in vivo* antibacterial activities and diabetic wound healing potential.


*Keywords: Diabetic wound, Eriosemarobustum, methicillin, antibacterial activity, resistance profile*.

## 1. INTRODUCTION

One of the complications of diabetes is the inability of wounds to heal properly [1], which poses a significant public health challenge [2]. Diabetic wound healing problems are estimated to impact approximately 25% of all diabetic patients [3]. Globally, the annual

incidence of diabetic wounds is 9.1 and 26.1 million and the prevalence Africa is 7.2% and 9.9% in Cameroon [4,5]. Diabetic wounds are characterized by impaired healing, prolonged inflammation, and reduced epithetisation kinetics [6]. So far, the exact pathogenesis of poor diabetic wound healing is not well understood. However, studies have shown alterations in the different phases of the healing process [7]. Diabetic wounds are refractory to healing owing to several factors, including hyperglycemia, which causes a range of local pathologies in the wound microenvironment, including chronic inflammation, dysregulated angiogenesis, oxidative stress, and end products, advanced glycation [6]. Diabetic foot ulcers account for 84% of all diabetes-related lower-extremity amputations. Therefore, it is important to elucidate the pathological processes that cause ulceration and affect wound healing in patients with advanced diabetics [6]. Cutaneous wound healing is a dynamic and highly regulated process of cellular, humoral, and molecular mechanisms that begins directly after the injury and can last for years [7]. It takes place in several stages: coagulation, inflammation, proliferation, and remodeling [8]. Wound healing process is often disrupted in individuals with diabetes, resulting in impaired wound healing and an increased risk of developing chronic non healing wounds [9].Currently, there are various synthetic drugs used to treat diabetic wounds, such as L-mesitran ointments and medihoney [10]. However, these treatments often lead to skin complications, allergies, and irritations, as well as high costs and the emergence of multi-resistant bacterial strains [11]. Therefore, turning to traditional medicine could be beneficial to overcome these limitations, especially since Cameroon has a rich and diverse flora, estimated at 8,260 plant species [12]. Plants represent a virtually unlimited source of new antimicrobial and several studies have shown the effectiveness of medicinal plants in treating diabetic wounds [13, 14, 15]. However, since not all medicinal plants have been studied, we chose to focus on *Eriosemarobustum*, a Cameroonian plant from the Fabaceae family. This plant was selected based on its traditional use of leaves in treating wounds and skin infections, as well as the lack of scientific research on its healing properties, especially for diabetic wounds. furthermore, Our recent work carried out on 90°, 70°, 30° hydroethanolic and aqueous extracts of E. robustum on multiresistant bacteria isolates of diabetic wounds demonstrated significant antibacterial activities and revealed the presence of several secondary metabolites on HPLC phytochemical study using 70° hydroethanolic extract [16]. Hence, the present study aimed to evaluate the wound healing and in vivo antibacterial potential of a 70° hydroethanolic extract of *Eriosemarobustum* leaves on multiresistant*Staphylococcus aureus* infected and non-infected wounds in diabetic *Wistar* rats.

## 2. MATERIAL AND METHODS

### 2.1. Material

#### 2.1.1. Plant material

The plant material used in this study was obtained from the leaves of *Eriosemarobustum*, collected in July 2022 in Balepo (geographic coordinates: 5° 43′ 08″ N, 10° 09′ 26″ E), located in the Sub-division of Babadjou, West Cameroon, and was identified in the national herbarium of Cameroon (Yaoundé), in comparison to the reference sample under the code 24535/SRFCam. This research was approved by the Regional Ethics Committee under authorization NO/373/2023/02/2023/CE/CRERSH-OU/VP.

#### 2.1.2. Experimental animals and Group distribution

Fifty-five (55) obese albino male Wistar rats, aged 10 to 12 weeks weighing between 240g-280g were used in this study. Breeding was performed at the animal facility of the Department of Biochemistry, University of Dschang, Cameroon. They were housed in individual polypropylene cages at 23±1°C with a 12h: 12h, nycthemeral cycle. The animals were fed a high-fat diet and provided water *ad libitum*. The food was removed 12 h before the start of the experiment.

#### 2.1.3. Extract Ointment Formulation Ingredients

Table 1 presents the raw materials used in the formulation of different *E. robustum*extract ointments [17].

**Table 1:** Different ingredients used in the formulation of extract ointments.

| Ingredients | Quantities |
|---|---|
| Alcoolcetostearyl | 5 |
| Lanoline | 5 |
| Solf Paraffin | 85 |
| Paraffin wax | 5 |

## 2.2. Methods

### 2.2.1. Formulation and characterization of extract ointments

*2.2.1.1. Preparation of ointments*

Different proportions of ingredients were weighed using an electric balance and placed in a water bath (PolyScience) set between 70 and 80°C. After complete melting, the mixture was transferred to a porcelain mortar, to which the extract was added gradually while stirring with a pestle to maintain a homogeneous mixture. After formulating the ointments at different extract concentrations (1%, 5%, and 10%), the pH was evaluated using a pH meter (PHS-3C) and was adjusted between 4 and 5, 5 and the final formulations were packaged and stored at 24°C. Sodium benzoate was used as the preservative.

### 2.2.2. Physico-chemical characterization of the different formulated ointments

This characterization made it possible to determine whether the different ointments formulated complied with the specifications or different requirements imposed by cosmetic standards [18].

*2.2.2.1. Organoleptic control*

The ointment was stored for 24 h, and the color, odor, and appearance were evaluated.

*2.2.2.2. Homogeneity*

It was checked by spreading it in a thin layer on a flat surface and using a spatula to check for the presence or absence of lumps or air bubbles.

*2.2.2.3. Centrifuge stability test*

The physical stability of the ointments was evaluated by centrifugation (mechanical stability) as described by. It was done by introducing 1.5 mL of ointment into Eppendorf tubes and subjecting it to centrifugation at 5000 rpm for five minutes, twice in a row (BECKMAN centrifuge, made in the USA, MicrofugeTM 12). The stability, phase separation, and appearance of each ointment after centrifugation were observed in each round [19].

*2.2.2.4.Accelerated stability test*

The different ointments were stored at 4°C in a refrigerator at 40°C in an oven (Quincy LAB INC, AF Model 30 Lab Oven) and room temperature for 4 weeks. During each week, the stability and organoleptic characteristics (color, texture, odor, and phase separation) of the ointments were observed [20].

*2.2.2.5. Chemical stability*

To evaluate the chemical stability, the pH of the different ointments stored at 4°C, 40°C, and room temperature was measured every week for one month.

### 2.2.3. Microbiological control

To carry out this test, 0.1 g of each ointment sample was cultured on four selective media (mannitol salt agar, cetrimide, Mc Conkey, Sabouraud Dextrose Agar) by the dial streak seeding method in order to isolate possible contaminating germs. The medium was prepared according to the manufacturer's instructions. These different media were sterilized in an autoclave at 121 °C for 15 min, and the ointments were cultured in Petri dishes.

Incubation in an incubator (HERAEUS) was carried out at 35°C for 24 h for the *Staphyloccocus aureus, Pseudomonase aeruginosa* and *Esherichia coli* and for 48 h for *Candida albicans* [21].

### 2.2.4. Toxicological controls of ointments based on plant extracts

*2.2.4.1. Draize eye irritation test*

Both eyes of each animal that were likely to participate in the test were first examined within 24 h before the start of the test. Animals showing signs of eye irritation, ocular defects, or corneal damage were excluded [22]. The coat was then removed from around the animal's eyes, and a single dose (100 mg) of ointment was instilled into the conjunctival sac of one of the two eyes of each animal. . The other eye, not undergoing treatment, served as a control, and no rinsing was carried out for at least 24 h after application of the ointment. Eye irritation was observed at 1, 24, and 48 h after instillation of the product [22]. Ocular lesions were evaluated according to their nature and severity, as well as their reversibility using scores [23]. Scores were assigned according to some criteria and calculated using the following formula:

IOI=∑RO

I.O.I: individual eye irritation index

∑RO: sum of scores attributed to ocular reactions

The maximum eye irritation index was calculated by taking the peak of the individual irritation indices recorded each time.

*2.2.4.2. Draize primary skin irritation test*

This test consisted of applying a single dose of the product to the skin of animals previously shaved on both sides and noting any manifestations that may occur. A few hours before the test, two areas of approximately 6 $cm^2$ were shaved on the dorsal level of each rat, avoiding any contamination using wipes soaked in ethanol (70°C). 0.5 g of the ointment to be tested was applied to one side, which served as the test area, while the second side, on which no product had been applied, served as a neutral control. The reactions linked to the application of the tested product were observed after 4 h of application, and then 24, 48, and 72 h after removing the adhesive strip [24]. The formation of edema and erythema on the treated skin was observed, and skin reactions were evaluated using skin irritation scores (Table 2) and calculated using the following formula:

PI= (∑ I.C) /n

PI: primary skin irritation index

∑I.C: sum of average skin irritation indices obtained at each period

The index results were then interpreted according to Table 2.

**Table 2:** Classification of products according to primary irritation index.

| PI Category | PI |
|---|---|
| Non-irritating | IP ≤ 0,5 |
| Slightlyirritating | 0,5< IP ≤2 |
| Irritant | 2< IP ≤5 |
| Très irritant | 5< IP ≤8 |

PI: primary irritation

### 2.2.5. Diabetes induction and distribution of groups

*2.2.5.1. Distribution of rats in groups*

The rats were divided into eleven (11) groups, with five rats in each group. Diabetes was induced in all rats in the 11 groups (G1%, G5%, G10%, POS, NEG, G1%NI, G5%NI, G10%NI, POSNI, NEGNI, and DNB) with streptozotocin injection. Excision wounds with a surface area of 314 $mm^2$ were created in the dorsal area of each animal, except for the uninjured diabetic (DNB) group. Groups G1%, G5%, G10%, POS, and NEG consisted of diabetic rats with wounds infected with multiresistant*S. aureus* isolat, having received 1%,

5%, and 10% ointment of 70° extract of *E. robustum* treatment, and POS (positive control) was the reference ointment(L-mesitran)and NEG, the group (negative control) having received no treatment. G1%NI, G5%NI, G10%NI, POSNI, and NEGNI groups consisted of diabetic rats with non-infected wounds, with G1%NI, G5%NI, G10%NI having respectively received 1%, 5% and 10% ointment of 70° extract of *E. robustum* treatment, meanwhile; POS (positive control) was the reference ointment(L-mesitran) and NEGNI, the group (negative control) having received no treatment.

*2.2.5.2. Diabetes induction*

Rats were induced with diabetes through an intraperitoneal injection of streptozotocin at a low dose (45 mg/kg body weight) dissolved in citrate buffer. Afterward, they were given a diet of 10% sucrose water, and their activity was closely monitored every 2 hours for 12 hours to check for signs of hypoactivity, unresponsiveness, or seizures. Four days following the STZ injection, blood samples were collected through tail vein puncture, and animals with blood sugar levels above 250 mg/dL were considered diabetic.

**2.2.6. Creation and infection of excision wounds**

The previous diabetic rats were anesthetized via intramuscular injection of ketamine at a dose of 40.08 mg/kg body weight. After being shaved on the upper back, the area was cleaned with 70° alcohol. Using a graduated ruler and an indelible marker, a 2 cm diameter or 314 mm² surface area pattern was created, and the skin was cut and pulled to create an excised wound. The wounds were then contaminated with a bacterial suspension of *Staphylococcus aureus* at $10^8$ CFU/ml in a sterile physiological solution of 0.9% NaCl using a 18-hour-old bacterial culture. The surface of the wounds was swabbed 24 hours post-infection and inoculated in Chapman and Muller Hinton media to assess infection effectiveness.

*2.2.6.1. Observation of wound healing*

The wound diameters of each animal were measured on days 4th, 8th, 12th, 16th and 20th days during treatment using a graduated ruler in two perpendicular directions. The wound contraction rate was calculated from the days of measurement of the injured areas, using the following formula [25].

Tc.= [(wound area on day 0-wound area on day X) / (wound area on day 0)] x 100

**2.2.7. Antibacterial activity *in vivo***

The *in vivo* antibacterial activity was assessed within the scarred skin region. On day 20, ketamine was used to anesthetize the animals, and a 5 gram biopsy of the scarred area was performed. The sample was then ground in a porcelain mortar containing a pinch of sterilized sand that had been heated at 100°C in an oven, along with 4 ml of physiological saline solution (containing 0.9% NaCl). The resulting homogenate was subsequently centrifuged at 3000 rpm for 15 minutes, and the supernatant was used to inoculate Chapman medium at 37°C for 24 hours to determine the growth of *S. aureus*. The counts were carried out according to the method described by Mouokeuet al.[26].

**2.2.8. Biochemical Analysis**

Blood samples were obtained from all animals in each group by cardiac puncture with ketamine overdose on day 20, and collected in tubes without anticoagulants. The samples were left to rest for an hour on ice and then centrifuged at 3500 rpm for 10 minutes to obtain serum, which was used for the determination of blood hydroxyproline and lactate dehydrogenase (China, Dully; kit lot 20220602), as well as total proteins (China, Dully kit lot 20220519). The levels of hydroxyproline in tissue were measured in both healed and unhealed skin samples, and all tests were conducted according to the manufacturer's instructions to ensure the accuracy of the results. All experiments were repeated three times.

**2.2.9. Statistical analysis**

The outcomes achieved *in vitro* during this research are presented as the mean ± standard deviation (SD) after conducting three repetitions. *In vivo* tests, the results were expressed as the mean ± standard deviation (SD), and an analysis of variance (ANOVA) was conducted. The discrepancies between the means of the various *in vitro* and *in vivo*tests were assessed using post-hoc Waller-Duncan multiple range tests with SPSS 26.0. A p-value of less than 0.05 was deemed statistically significant.

## 3. RESULTS

### 3.1. Quality control of *E. robustum* extracts ointments

#### 3.1.1. Organoleptic evaluation

The various ointments (illustrated in Figure 1) displayed uniformity when viewed without magnification, emitting an aroma of attenuated lanolin and exhibiting a beige hue for the 1% ointment, while the remainder displayed shades of light brown to dark brown, depending on the extract concentration. These ointments possessed a semi-solid texture, moderately viscous and softening instantly upon contact with the skin and at temperatures above 30°C, and feeling smooth and free of bubbles or lumps throughout a 28 day storage period. The organoleptic properties of the 1%, 5%, and 10% ointments remained consistent over time, with the exception of the 1% ointment's color, which lightened to brown on day 21.

#### 3.1.2. Stability test

Regardless of the concentration, the different ointments based on *E. robustum* leaves extract were all stable at room temperature (22°C - 25°C) on days 7, 14, 21, and 28 because of the absence of liquefaction after centrifugation of 10 g of each ointment at 5000 rpm for five minutes and no changes after storage at 4°C in a refrigerator at 40°C in an oven.

#### 3.1.3. Determination of pH

All the ointments at 37°C exhibited pH values that varied over time, therefore, after 24 hours of preparation of the ointments with 1%, 5% and 10% extracts, their pH had values of 6.75 respectively; 5.7 and 5.3. On day 7, they respectively showed pH values of 5.5, 4.9 and 4.4. On the other hand, 21 days later, the respective values of 5.2, 4.7 and 4.4 were obtained and remained constant during days 22, 25, and 28.

### 3.2. Microbiological Test

The examination of the different ointments on specific growth media revealed the complete absence of *S. aureus*, *E. coli*, *P. aeruginosa*, and *C. albicans* growth in the media.

### 3.3. Irritant effect of ointments made from hydro-ethanolic extracts of *E. robustum* leaves on the skin and eyes

The irritative effects of the tested ointments formulated with 70°C hydroethanolic extracts of *E. robustum*leaves were evaluated on the skin and eyes of *Wistar* rats, and it showed no irritation n. Concerning the eye irritant effects of the different extract ointments, no irritation was observed at the eyeball level 48 hours after application, with clinical evaluations of the conjunctiva for redness, iris for assessing pupil reactivity, cornea for opacity, ulceration, and granulation, and chemosis for tearing and swelling of the eyelids.

**Figure 1:** Ointment based on *E. robustum* leaves extract.

### 3.4. Healing activity of extract ointments on diabetic excision wounds

The healing activity of the different extract ointments was evaluated on diabetic wounds infected and non-infected with *S. aureus* by calculating the surface area and the rate of wound contraction during days 4, 8, 12, 16, and 20 during treatment.

#### 3.4.1. Effect of ointments with 70° hydro-ethanolic extracts of *E. robustum*leaveson wound contraction rate

The effectiveness of 1%, 5%, and 10% hydroethanolic extracts of *E. robustum*leaves on infected and non-infected diabetic wounds (NI) was evaluated in terms of wound contraction rates, which varied with treatment duration in all groups (Table 3). Non-infected diabetic wounds displayed a significantly ($p < 0.05$) higher contraction rate compared to infected diabetic wounds. Both large groups of diabetic wounds treated with different extract ointments showed an increase in contraction percentage, which was proportional to the extract concentration of the ointments. On the last day of treatment (day 20), the negative control group with infected diabetic wounds (NEG) had the lowest contraction rate (57.62%) and was significantly different from the negative control group with non-infected diabetic wounds (NEGNI), which showed a contraction percentage of 76.94%. The 10% extract ointment achieved a 100% contraction rate in both the infected and non-infected diabetic wound groups, while the reference ointment L-mesitran achieved this rate only in the diabetic group with non-infected wounds. The diabetic group with non-infected wounds treated with the 5% extract ointment showed a contraction rate similar to that of the diabetic group with infected wounds treated with the reference ointment (L-mesitran). In contrast, the diabetic group with non-infected wounds treated with the 1% extract ointment (G1%NI) showed significant difference ($p < 0.05$) compared to the diabetic group with infected wounds treated with the 1% extract ointment (G1%).



**Table 3:** Effect of ointments made from 70° ethanolic extracts of *E. robustum* on diabetic wounds infected and non-infected (NI) with *S. aureus* as a function of time.

| Wound contraction rate depending on treatment duration |
|---|

| Traitements | Jour 4 | Jour 8 | Jour 12 | Jour 16 | Jour 20 |
|---|---|---|---|---|---|
| G1% | 17.86 ± 2.28$^{bc}$ | 34.78 ± 1.93$^{b}$ | 50.92 ± 4.72$^{bc}$ | 69.19 ± 0.45$^{c}$ | 83.99 ± 0.65$^{c}$ |
| G5% | 21.21 ± 2.56$^{bc}$ | 35.87 ± 6.53$^{b}$ | 57.62 ± 5.30$^{cd}$ | 74.37 ± 9.13$^{c}$ | 94.90 ± 0.92$^{d}$ |
| G10% | 23.40 ± 3.57$^{bc}$ | 43.59 ± 6.84$^{c}$ | 66.62 ± 7.42$^{def}$ | 92.50 ± 0.41$^{e}$ | 96.59 ± 1.8$^{de}$ |
| L-Mesitran | 22.52 ± 3.97$^{bcd}$ | 45.17 ± 4.31$^{c}$ | 66.31 ± 2.82$^{def}$ | 89.43 ± 0.26$^{e}$ | 94.49 ± 1.06$^{d}$ |
| NEG | 12.062 ± 4.625$^{a}$ | 21.185 ± 4.37$^{a}$ | 35.87 ± 6.53$^{a}$ | 50.82 ± 0.88$^{a}$ | 56.59 ± 1.56$^{a}$ |
| G1%NI | 27.75 ± 0.00$^{d}$ | 37.937 ± 3.87$^{e}$ | 54.12 ± 81.90 ± 1.7$^{d}$ | 0.17$^{de}$ | 97.59 ± 100 ± 0.00$^{e}$ |
| G5%NI | 17.85 ± 2.92$^{bc}$ | 35.59 ± 0.80$^{b}$ | 8.71$^{c}$ | 89.11 ± 0.26$^{e}$ | 100 ± 0.00$^{e}$ |
| G10%NI | 22.54 ± 2.87$^{bcd}$ | 43.71 ± 3.06$^{c}$ | 64.00 ± 0.00$^{de}$ | 94.55 ± 1.06$^{e}$ | 76.94 ± 1.42$^{b}$ |
| L-Mesitran | 17.18 ± 2.23$^{a,b}$ | 48.65 ± 3.42$^{c}$ | 74.40 ±3.54$^{f}$ | 69.62 ± 4.49$^{ef}$ | 91.90 ± 1.29$^{e}$ |
| NI | | 43.75 ± 0.00$^{c}$ | 69.62 ± 4.49$^{ef}$ | 43.62 ± 6.12$^{ab}$ | 59.95 ± 2.95$^{b}$ |
| NEGNI | | 23.40 ± 3.57$^{a}$ | | | |

*Each value represents the mean value ± standard error; and in each column, the values assigned to different letters in the same column (a-g) are significantly different at the 5% probability threshold. NEGNI: non-infected negative control, NI: non-infected, NEG: infected negative control, POS: positive control (L-mesitran), POSNI: non-infected positive control.*

### 3.4.2. Effect of ointments with 70° hydro-ethanolic extracts of *E. robustum* leaves on wound surface

Figure 2 shows the effect of ointments on the surface of infected and non-infected diabetic wounds. While Figures 3 and 4 present the macroscopic aspects of non-infected and *S.aureus*infected wounds in diabetic rats treated with *E. robustum* extract ointments, showing that the wound surface or area decrease with Time.

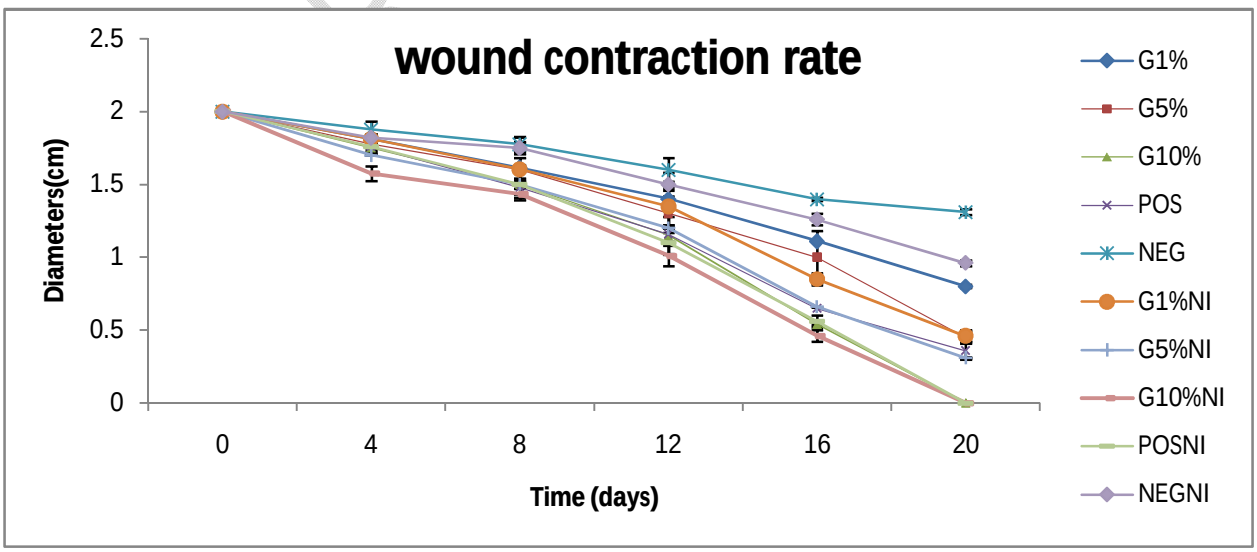


**Figure 2:** The effect of *E. robustum* leaves extract on the surface of diabetic wounds.

*Each value represents the mean value ± standard error; and in each column, the values assigned to different letters in the same column (a-g) are significantly different at the 5% probability threshold. NEGNI: non-infected negative control, NI: Non-infected, NEG: negative control, DNB: uninjured diabetic (neutral control), POS: positive control, POSNI: non-infected positive control.*

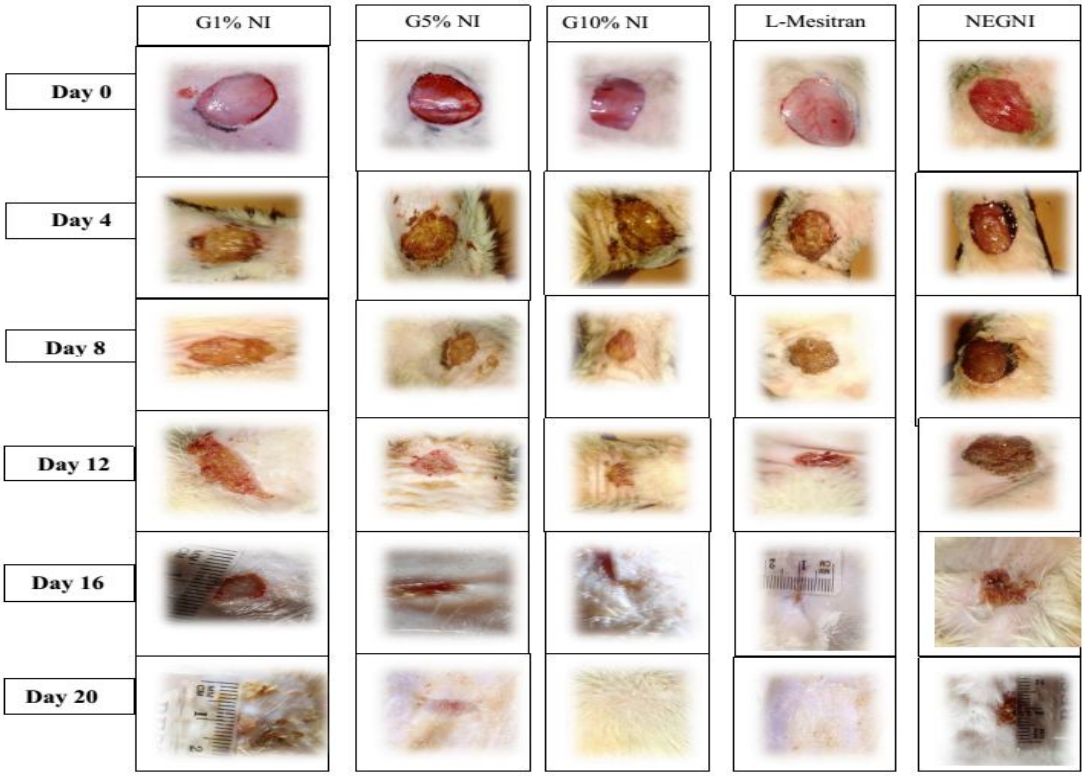


**Figure 3:** Macroscopic appearances of non-infected wounds in diabetic rats treated with *E. robustum* extract ointments.

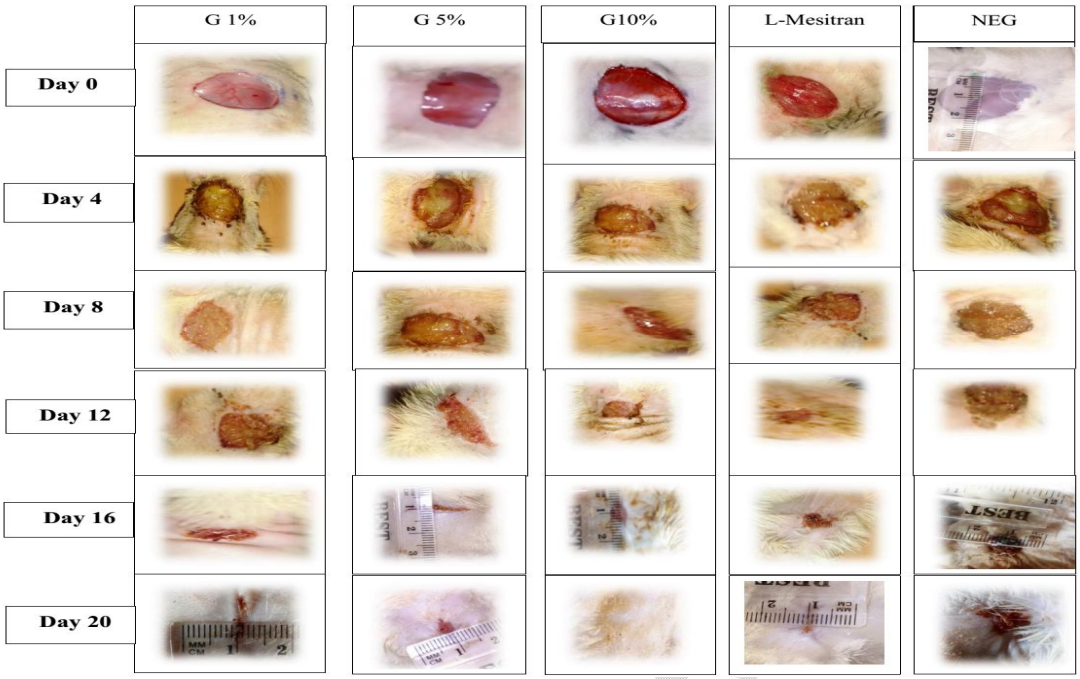


**Figure 4:** Macroscopic appearances of wounds infected by *S. aureus* in diabetic rats treated with *E. robustum* extract ointments.

### 3.5. Antibacterial activity of *E. robustum* extracts ointments on wounds infected by *S. aureus* in diabetic rats

The results of the study on the antibacterial activity of various ointments derived from the leaves of *E. robustum* are presented in Table 4. After 24 hours of infection, no significant difference was observed in the number of colony-forming units (CFU) of *S. aureus* at the wound infection site among different groups. However, on day 20 of treatment, the number of CFU of *S. aureus* at the wound infection site in all groups decreased significantly ($p < 0.05$). The groups treated with 5% and 10% extract ointment and L-mesitran ointment had no bacterial growth at the site of infection. In contrast, the untreated group (NEG) and the group treated with 1% extract ointment showed bacterial growth at the site of infection which was significantly lower ($p < 0.05$) than that of the untreated group (NEG).

**Table 4:** Antibacterial activity of different ointments of *E. robustum* leaf extract.

| Groups / Treatments | Day0 | Day 20 |
|---|---|---|
| G1% | $785000 \pm 28867.51^{a}$ | $1925 \pm 275.37^{b}$ |
| G5% | $772500 \pm 22173.55^{a}$ | $0 \pm 0^{a}$ |
| G10% | $795000 \pm 12909.94^{a}$ | $0 \pm 0^{a}$ |
| POS | $780000 \pm 141452.13^{a}$ | $0 \pm 0^{a}$ |
| NEG | $775000 \pm 53774.21^{a}$ | $65715 \pm 4741.26^{c}$ |

*Each value represents the mean value ± standard error; and in each column, the values assigned to different letters in the same column (a-g) are significantly different at the 5% probability threshold. NEG: negative control, POS: positive control.*

### 3.6. Effect of *E. robustum* ointments on serum LDH and total protein levels of diabetic rats with infected and non-infected wounds

The results for total protein and LDH levels are presented in Table 5. All non-infected groups had total protein content higher than infected groups with the highest been 5.59g/dL for G10%NI. Meanwhile, the protein content in infected groups was the highest in

group G10% with a value of 5.55g/dL which is significantly higher ($p < 0.05$) than G5% and G1%. POS and POSNI have shown no significant difference in protein content with G10% and G10%NI.

Regarding LDH levels, the negative control group of animals with infected wounds showed the highest level, which was significantly different from those of the negative controls with non-infected wounds ($p < 0.05$). In general, groups containing animals with infected diabetic wounds showed higher LDH levels than those without infections. The lowest rates were recorded in neutral controls, non-infected positive controls. Indicating that G10% and G10%NI were able to maintained LDH level in the normal range (normal rage LDH is below 400 UI/L) despite infection and non-infections stated and that the results are comparable to reference drug in POS and POSNI groups.



**Table 5:** Serum levels of total proteins and LDH of diabetic rats with infected and NI wounds.

| Groupes/ Traitements | Total proteins (g/dL) | LDH (UI/L) |
|---|---|---|
| G1%NI | $4.73 \pm 0.71^{c}$ | $506.66 \pm 33.5^{c}$ |
| G1% | $3.76 \pm 0.15^{b}$ | $589.99 \pm 25.81^{d}$ |
| G5%NI | $5.08 \pm 0.48^{c,d}$ | $400.00 \pm 12.17^{b}$ |
| G5% | $4.73 \pm 0.57^{c}$ | $491.66 \pm 14.39^{c}$ |
| G10%NI | $5.59 \pm 0.16^{d}$ | $343.33 \pm 8.6^{a}$ |
| G10% | $5.55 \pm 0.45^{d}$ | $348.33 \pm 16.66^{a}$ |
| POSNI | $5.50 \pm 0.16^{d}$ | $351.66 \pm 26.87^{a}$ |
| POS | $5.39 \pm 0.18^{d}$ | $399.45 \pm 81.82^{b}$ |
| NEGNI | $3.48 \pm 0.30^{b}$ | $901.66 \pm 10.00^{e}$ |
| NEG | $2.78 \pm 0.04^{a}$ | $1053.33 \pm 12.40^{f}$ |
| DNB | $3.98 \pm 0.05^{b}$ | $338.33 \pm 6.38^{a}$ |

*Each value represents the mean value ± standard error; and in each column, the values assigned to different letters in the same column (a-g) are significantly different at the 5% probability threshold. NEG: negative control, POS: positive control.*

### 3.7. Effect of *E.robustum* ointments on the hydroxyproline levels of diabetic rats with Infected and non-infected wounds

The levels of hydroxyproline in tissue and serum are presented in Table 6. The results indicate that there was no significant difference in tissue hydroxyproline levels among the G10%NI, POSNI, and DNB groups. However, there was a significant decrease in tissue hydroxyproline levels in the infected and non-infected negative control groups compared to the neutral control group ($p < 0.05$). It's worth noting that the tissue hydroxyproline level was not significantly different between the G5% group and the G5% NI group ($p > 0.05$) and they

show ni significant difference with Group DNB. Regarding serum hydroxyproline levels, G5%, G5%NI and POS where not significantly different but differ to G1%. POSNI was significantly different ($p < 0.05$)from G5%, G5%NI, POS, G1%, G1%NI. Serum hydroxyproline was higher in infected groups tested with extract ointments compare to the non-infected. There was no significant difference between the groups treated with plant extracts and the positive control groups.

**Table 6:** Tissue and serum hydroxyproline levels.

| Groups/ Treatements | Tissue Hydroxyproline (µg/mg) | Serum Hydroxyprolinen(µg/ml) |
|---|---|---|
| G1%NI | $0.28\pm0.07^{b,c}$ | $41.26\pm1.14^{d}$ |
| G1% | $0.20\pm0.05^{a,b}$ | $54.89\pm2.46^{e}$ |
| G5%NI | $0.31\pm0.06^{c}$ | $37.45\pm0.59^{c,d}$ |
| G5% | $0.30\pm0.07^{c}$ | $37.04\pm1.55^{c,d}$ |
| G10%NI | $0.40\pm0.00^{d}$ | $19.64\pm1.79^{a}$ |
| G10% | $0.35\pm0.07^{c,d}$ | $36.19\pm6.11^{c}$ |
| POSNI | $0.40\pm0.02^{d}$ | $26.32\pm0.55^{b}$ |
| POS | $0.32\pm0.02^{c,d}$ | $40.32\pm3.42^{c,d}$ |
| NEGNI | $0.19\pm0.04^{a,b}$ | $54.22\pm2.20^{e}$ |
| NEG | $0.16\pm0.041^{a}$ | $62.25\pm2.20^{f}$ |
| DNB | $0.40\pm0.01^{d}$ | $25.29\pm1.68^{b}$ |

*Each value represents the mean value ± standard error; and in each column, the values assigned to different letters in the same column (a-g) are significantly different at the 5% probability threshold. NEG: negative control, POS: positive control.*

## 4. DISCUSSION

### 4.1. Quality control of *E. robustum* extracts ointments

Stability testing of cosmetic products ensures that the product maintains its quality, including its physicochemical and microbiological properties, as well as its functionality and aesthetics when stored under appropriate conditions [27]. Notably, the human skin's physiological pH is between 4 and 6, so the pH of a topical formulation or cosmetic must be adjusted to the skin's pH to prevent skin irritation [28]. Although there were minimal variations in the pH of the different ointments with 1%, 5%, and 10% extracts during the first weeks, they reached stability from the 21st day, with respective pH values of 5.2, 4.7, and 4.4. These variations could be due to storage conditions such as temperature. The different ointments subjected to centrifugation for evaluation of physical stability did not cause phase inversion, indicating the homogeneity of the different phases. This can be attributed to the similarity in density between the oil and aqueous phases or to the strong interfacial interaction between the ingredients [29], and on the other hand, to the non-microbial proliferation because microbes can cause changes in the viscosity and texture of the ointment, affecting its ability to maintain homogeneity [30]. Toxicological tests have shown that ointments based on leaves extracts of *E. robustum* were non-irritating to the skin and eyes, suggesting that the ointments are safe to use.

### 4.2. Healing activity of extract ointments on diabetic excision wounds

Wound healing is a complex and dynamic process involving cell multiplication, re-epithelialization, cell migration, and extracellular matrix production [31]. The percentage of closure of the different wounds treated with *E. robustum* extract ointments in this study was significant ($p < 0.05$).The results demonstrated that the plant extract ointments showed a significant increase in wound healing compared to the negative control group. Furthermore,

these ointments showed a significant reduction in the number of colony-forming units (CFU) at the site of infection. These findings are supported by the studies of Awouafack et al. [32] in Cameroon, where the ethanolic extract of the stems of *E. robustum*has shown significant antibacterial activity against *S. aureus* (80 µg/ml). This antibacterial activity could be due to the presence of several secondary metabolites in the plant leaves such as phenolic compounds [16].

The potential healing properties of plant extracts may be due to the presence of phenols, flavonoids and total tannins. Flavonoids possess antioxidant and anti-inflammatory properties; can help reduce oxidative stress and inflammation, thereby promoting a favorable environment for healing and stimulating cell proliferation and collagen synthesis, which are essential for scar tissue structure and resistance [33]. Tannins also play a crucial role in accelerating healing by tightening tissues, promoting coagulation, and reducing inflammation [34]. Previous studies have shown than *E. robustum* is a good source of Tannins and flavonoids [16].

### 4.3. Biochemical Analysis

When a wound occurs, the body increases the production of proteins, including albumin and globulins, which are collectively referred to as total proteins, to maintain tissue integrity and promote cell regeneration [35]. In this study, we observed a higher total protein level in diabetic rats treated with plant extract ointments compared to controls. In fact, tissue proteins like collagen help strengthen and support cell tissue and are used as biochemical markers, indicating better healing quality treatment in wounds [36]. These findings support the work of Ekom et al. [37], who reported high plasma total protein content in rats treated with 1%, 5%, and 10% gel extracts of *Capsicum annum* and *Persia americana*, compared to controls. However, their study focused exclusively on non-diabetic wounds and used non-injured non-diabetic rats as neutral controls, enabling them to achieve a high total protein level in this control group. In contrast, the neutral control group (NEG) in our study had a very low total protein rate, which can be attributed to the complications associated with diabetes, such as kidney problems and dysfunction of certain organs [38]. The reason for the high content of total protein in the G5%,G10% and POS infected wounds founded in this study compared to DNB could be attributed to the fact that the ointments extract stimulate protein synthesis as the concentration of the extract ointments increase. And the low content of total protein in the infected negative control (NEG) could be due to the fact that inflammatory response of the body to infection may alter plasma total protein levels, unlike non-infected wounds which have a more balanced and regulated protein response.

LDH is a crucial marker in wound healing assessment as it allows the estimation of tissue damage extent and healing dynamics. Elevated serum LDH levels have also been reported as biomarkers for the diagnosis of various diseases in humans, including life-threatening bacterial infections [39]. In this study, we observed that the enzymatic activity of this marker was significantly higher in infected and injured diabetic patients than in simple uninjured or injured patients. This finding is consistent with previous studies [40, 41], which showed that LDH values were higher in the group of unhealed wounds. Other researchers have also reported that serum LDH levels significantly increase in the presence of infection [37]. This increase can be justified by the fact that bacterial toxins can target various cellular components, disrupting their function by damaging them. Additionally, bacteria produce certain enzymes such as proteases which degrade cellular proteins.

Tissue and serum hydroxyproline levels were significantly higher in non-infected wound diabetic rats treated with *E. robustum* extract ointments than in infected rats compared to the control groups. This result is consistent with the findings of Santram and Abhay [42] who obtained similar results in their study. They revealed that diabetic rats with non-infected wounds treated with *Martynia annua* extract ointments had high levels of tissue hydroxyproline, which was significantly higher than the control group. However, higher levels of tissue hydroxyproline was obtained in non-diabetic and diabetic rats wounds treated with

*Rhus coriaria* fruit extracts compared to the control group [43]. An increase in hydroxyproline in excision wounds suggests a rapid turnover of collagen and, consequently, accelerated wound healing. Collagen is the primary healing protein, and hydroxyproline is a vital marker because it is specific to collagen and indicates its quantity in healing tissues [44]. In our study, we observed that serum hydroxyproline levels were higher in the negative control groups than in the groups treated with plant extracts. Moreover, infected wound groups, in general, had higher serum hydroxyproline levels than non-infected groups. These results align with those of Amit et al.[45], who reported that increased serum hydroxyproline content could result from poor healing or several metabolic disorders. Diabetes and infections can cause metabolic alterations that affect collagen degradation, leading to elevated serum hydroxyproline levels.

## 5. CONCLUSION

*E. robustum*extract ointmentspossess *in vivo* antibacterial activities on multiresistant*S. aureus* isolates and infected diabetic wound healing potential. The 10% ointment of 70° extract of *E. robustum* normalized the biochemical parameters in non-infected and infected rats. Therefore *E. robustum*leaves could be considered as alternative to the treatment of infected diabetic wound, and further scientific researches need to be done in the plants, for discovery and development of new drugs against infected diabetic wounds.

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