

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: This 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 mm² 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 dehydrogenase (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 20th 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 affect 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]. The exact pathogenesis of poor diabetic wound healing is not well understood. However, studies have shown alterations in different phases of the healing process [7,46,47,48,49]. 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, end-products, and 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 injury and can last for years [7]. It occurs in several stages, including coagulation, inflammation, proliferation, and remodeling [8]. The wound healing process is often disrupted in individuals with diabetes, resulting in impaired wound healing and an increased risk of developing chronic nonhealing wounds [9]. Currently, various synthetic drugs are 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 emergence of multidrug-resistant bacterial strains [11]. As the human community is seeking for traditional healing, herbal medicine plays a vital role in the society. Therefore, turning to traditional medicine could be beneficial in overcoming these limitations, especially because Cameroon has a rich and diverse flora, estimated at 8,260 plant species [12]. Plants represent a virtually unlimited source of new antimicrobials, and several studies have demonstrated the effectiveness of medicinal plants in treating diabetic wounds [13, 14, 15]. However, because 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 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°, and 30° hydroethanolic and aqueous extracts of *E. robustum* on multiresistant bacterial isolates from diabetic wounds demonstrated significant antibacterial activities and revealed the presence of several secondary metabolites in an 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.

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 $23\pm 1^{\circ}\text{C}$ 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 the 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 gradually added 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 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 *Staphylococcus 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:

$$IOI = \sum RO$$

I.O.I: individual eye irritation index

$\sum RO$: 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 cm² 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:

$$PI = (\sum I.C) / n$$

PI: primary skin irritation index

$\sum I.C$: 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	$IP \leq 0,5$
Slightlyirritating	$0,5 < IP \leq 2$
Irritant	$2 < IP \leq 5$
Très irritant	$5 < IP \leq 8$

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 mm² 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 10⁸ 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].

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

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 \pm standard deviation (SD) after conducting three repetitions. *In vivo* tests, the results were expressed as the mean \pm 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. 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* leaves on 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					
Treatments	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	100 ± 0.00 ^e
L-Mesitran	22.52 ± 3.97 ^{bcd}	45.17 ± 4.31 ^c	66.31 ± 2.82 ^{def}	89.43 ± 0.26 ^e	96.59 ± 1.8 ^{de}
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	17.85 ± 2.92 ^{bc}	35.59 ± 0.80 ^b	54.12 ± 8.71 ^c	81.90 ± 1.7 ^d	94.49 ± 1.06 ^d
G5%NI	27.75 ± 0.00 ^d	43.71 ± 3.06 ^c	64.00 ± 0.00 ^{de}	89.11 ± 0.26 ^e	97.59 ± 0.17 ^{de}
G10%NI	37.937 ± 3.87 ^e	48.65 ± 3.42 ^c	74.40 ± 3.54 ^f	94.55 ± 1.06 ^e	100 ± 0.00 ^e
L-Mesitran NI	22.54 ± 2.87 ^{bcd}	43.75 ± 0.00 ^c	69.62 ± 4.49 ^{ef}	91.90 ± 1.29 ^e	100 ± 0.00 ^e
NEGNI	17.18 ± 2.23 ^{a,b}	23.40 ± 3.57 ^a	43.62 ± 6.12 ^{ab}	59.95 ± 2.95 ^b	76.94 ± 1.42 ^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. 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.

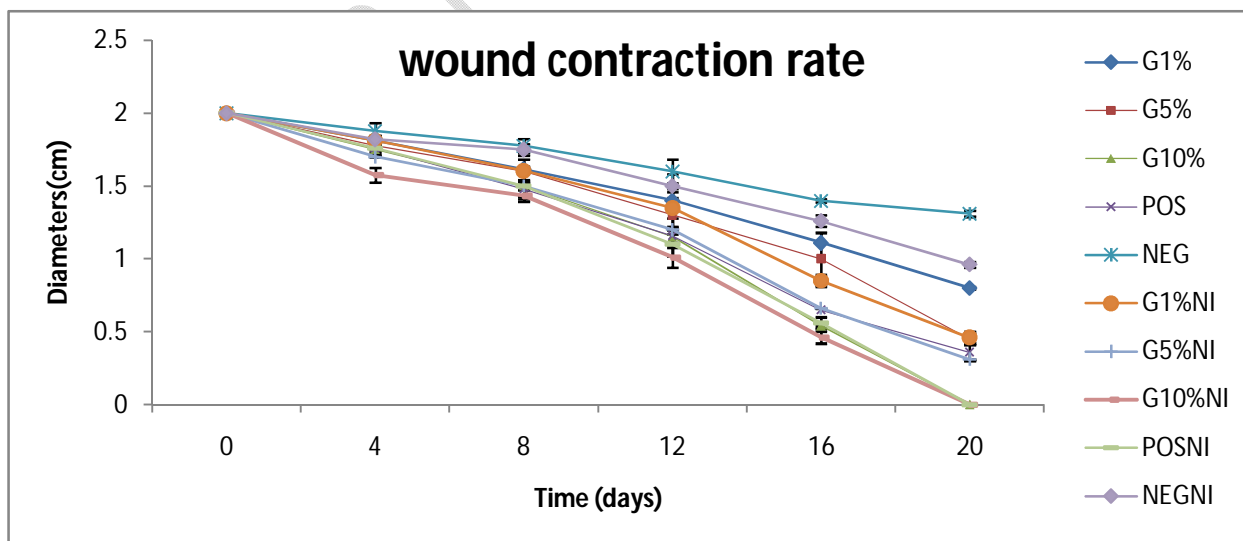


Figure 2: The effect of *E. robustum* leaves extract on the surface of diabetic wounds. Each value represents the mean value ± standard error; and in each column, the values assigned to different letters in the same column (a-g) are significantly different at the 5% probability threshold.

NEGNI: non-infected negative control, NI: Non-infected, NEG: negative control, DNB: uninjured diabetic (neutral control), POS: positive control, POSNI: non-infected positive control.

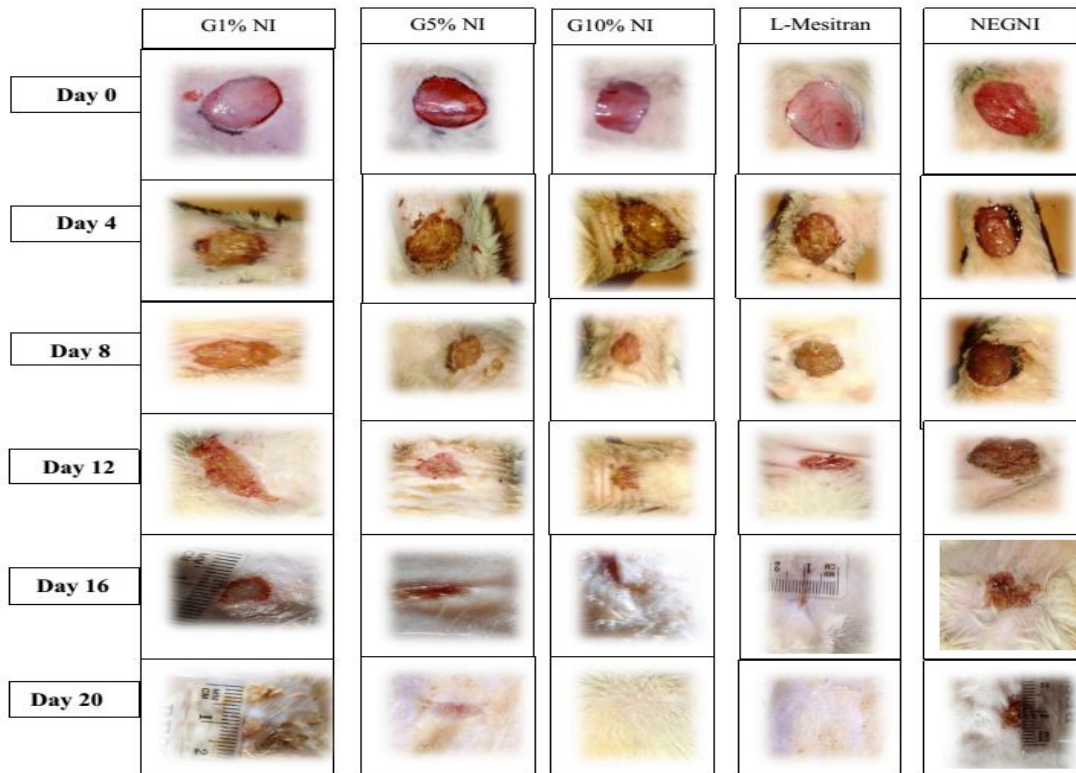


Figure 3: Macroscopic appearances of non-infected wounds in diabetic rats treated with *E. robustum* extract ointments.

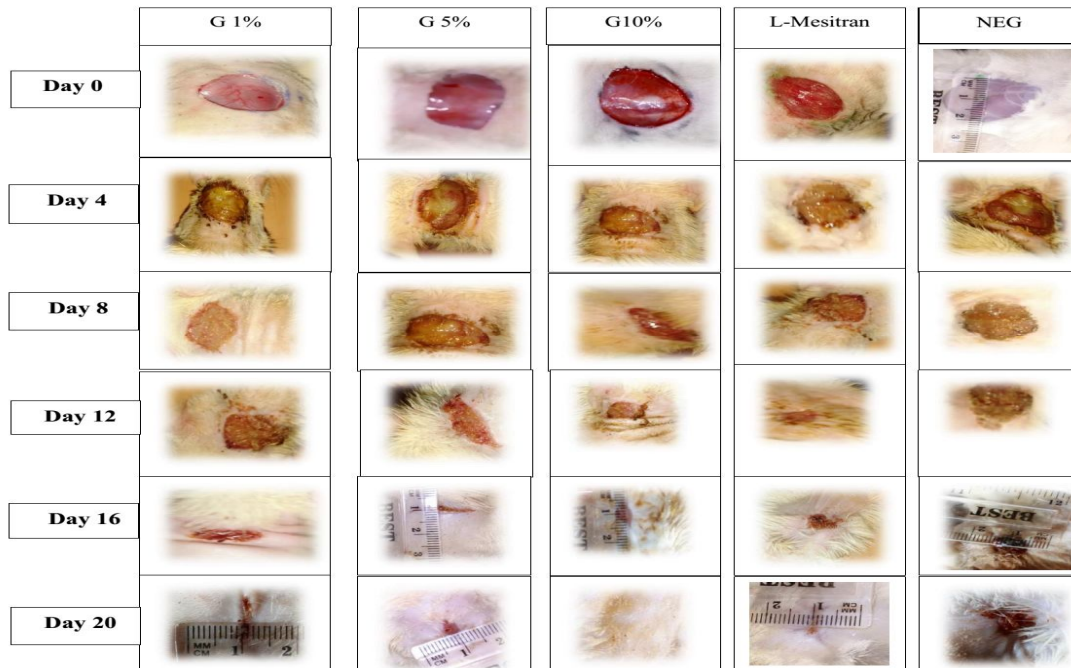


Figure 4: Macroscopic appearances of wounds infected by *S. aureus* in diabetic rats treated with *E. robustum* extract ointments.

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 \pm 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 the infected groups was the highest in group G10% with a value of 5.55g/dL which was significantly higher ($p < 0.05$) than G5% and G1%. POS and POSNI showed no significant differences in protein content compared to G10% and G10%NI. The negative control group of animals with infected wounds showed the highest LDH levels, which was significantly different from that of the negative controls with non-infected wounds ($p < 0.05$). In general, groups of animals with infected diabetic wounds showed higher LDH levels than those without infection. The lowest rates were recorded for the neutral and non-infected positive controls. This indicates that G10% and G10%NI were able to maintain LDH levels in the normal range (normal range LDH is below 400 UI/L) despite infection and non-infection, and that the results are comparable to the reference drug in the 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 ± 0.71 ^c	506.66 ± 33.5 ^c
G1%	3.76 ± 0.15 ^b	589.99 ± 25.81 ^d
G5%NI	5.08 ± 0.48 ^{c,d}	400.00 ± 12.17 ^b
G5%	4.73 ± 0.57 ^c	491.66 ± 14.39 ^c
G10%NI	5.59 ± 0.16 ^d	343.33 ± 8.6 ^a
G10%	5.55 ± 0.45 ^d	348.33 ± 16.66 ^a
POSNI	5.50 ± 0.16 ^d	351.66 ± 26.87 ^a
POS	5.39 ± 0.18 ^d	399.45 ± 81.82 ^b
NEGNI	3.48 ± 0.30 ^b	901.66 ± 10.00 ^e
NEG	2.78 ± 0.04 ^a	1053.33 ± 12.40 ^f
DNB	3.98 ± 0.05 ^b	338.33 ± 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 tissues and serum are presented in Table 6. The results indicated 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 no significant difference with Group DNB. Regarding serum hydroxyproline levels, G5%, G5%NI, and POS were not significantly different, but differed from G1%. POSNI was significantly different ($p < 0.05$) from G5%, G5%NI, POS, G1%, and G1%NI. Serum hydroxyproline was higher in the infected groups treated with extract ointments than in the non-infected groups. There was no significant difference between the groups treated with the plant extracts and the positive control group.

Table 6: Tissue and serum hydroxyproline levels.

Groups/ Treatements	Tissue Hydroxyproline (µg/mg)	Serum
		Hydroxyproline(µg/ml)
G1%NI	0.28±0.07 ^{b,c}	41.26 ±1.14 ^d
G1%	0.20±0.05 ^{a,b}	54.89±2.46 ^e
G5%NI	0.31±0.06 ^c	37.45 ±0.59 ^{c,d}
G5%	0.30±0.07 ^c	37.04±1.55 ^{c,d}
G10%NI	0.40 ±0.00 ^d	19.64±1.79 ^a
G10%	0.35±0.07 ^{c,d}	36.19 ±6.11 ^c
POSNI	0.40±0.02 ^d	26.32 ±0.55 ^b
POS	0.32±0.02 ^{c,d}	40.32 ±3.42 ^{c,d}
NEGNI	0.19±0.04 ^{a,b}	54.22 ± 2.20 ^e
NEG	0.16±0.041 ^a	62.25 ±2.20 ^f
DNB	0.40±0.01 ^d	25.29 ±1.68 ^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, functionality, and aesthetics when stored under appropriate conditions [27]. Notably, the physiological pH of human skin is between 4 and 6, so the pH of a topical formulation or cosmetic must be adjusted 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 pH values of 5.2, 4.7, and 4.4, respectively. These variations could be due to storage conditions, such as temperature. The different ointments subjected to centrifugation for the evaluation of physical stability did not cause phase inversion, indicating 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 *E. robustum* leaf extracts are 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 that involves cell multiplication, re-epithelialization, cell migration, and extracellular matrix production [31]. The percentage of closure of different wounds treated with *E. robustum* extract ointments in this study was significant ($p < 0.05$). The results demonstrated that the plant extract ointments significantly increased 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 *E. robustum* stems showed significant antibacterial activity against *S. aureus* (80 µg/ml). This antibacterial activity could be due to the presence of several secondary metabolites, such as phenolic compounds, in plant leaves [16].

The potential healing properties of plant extracts may be due to the presence of phenols, flavonoids, and tannins. Flavonoids possess antioxidant and anti-inflammatory properties and 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 that *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 than in the controls. In fact, tissue proteins such as collagen help strengthen and support cell tissues 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 high total protein levels in the control group. In contrast, the neutral control group (NEG) in our study had a very low total protein rate, which can be attributed to complications associated with diabetes, such as kidney problems and dysfunction of certain organs [38]. The reason for the high total

protein content in the G5%,G10%, and POS-infected wounds found in this study compared to DNB could be attributed to the fact that the ointment extract stimulates protein synthesis as the concentration of the extract ointments increases. The low total protein content in the infected negative control (NEG) could be because the 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 the extent of tissue damage 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 uninjured or injured patients. This finding is consistent with those of previous studies [40, 41], which showed that LDH values were higher in the group with unhealed wounds. Other researchers have also reported that serum LDH levels are significantly increased in the presence of infection [37]. This increase can be explained by the fact that bacterial toxins can target various cellular components and disrupt 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 were significantly higher than those in the control group. However, higher levels of tissue hydroxyproline were observed in non-diabetic and diabetic rat wounds treated with *Rhus coriaria* fruit extracts than in the control group [43]. An increase in hydroxyproline in excision wounds suggests 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 group than in the groups treated with the 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 levels could result from poor healing or several metabolic disorders. Diabetes and infection can cause metabolic alterations that affect collagen degradation, leading to elevated serum hydroxyproline levels.

5. CONCLUSION

E. robustum extract ointments possess *in vivo* antibacterial activities against multidrug-resistant *S. aureus* isolates and diabetic wound healing potential. The 10% ointment of the 70° extract of *E. robustum* normalized biochemical parameters in non-infected and infected rats. Therefore, *E. robustum* leaves could be considered as an alternative for the treatment of infected diabetic wounds, and further scientific research is needed to discover and develop new drugs for the treatment of infected diabetic wounds.

Ethical Approval:

This research was approved by the Regional Ethics Committee under authorization NO/373/2023/02/2023/CE/CRERSH-OU/VP.

Disclaimer (Artificial intelligence)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

REFERENCES

1. Gooyit M, Peng Z, Wolter W, Pi H, Ding D, Heseck D, Lee M. A chemical strategy to facilitate diabetic wound healing. *ACS chemical biology*.2014; 9(1): 105-110.doi:10.1021/cb4005468.
2. Walsh JW, Hoffstad OJ, Sullivan MO. Association of diabetic foot ulcer and death in a population-based cohort from the United Kingdom. *Diabet Med*. 2016;33(11):1493–1498.
3. Burgess J, Wyant W, Abujamra A, Jozic I. Science of diabetic wound healing. *Medicine*.2021; 57(10): 1072. Doi: <https://doi.org/10.3390>.
4. Armstrong DG, Boulton AJM, Bus SA. Diabetic foot ulcers and their recurrence. *New Engl J Med*. 2017; 376:2367-75.
5. Zhang P, Lu J, Jing Y, Tang S, Zhu D, Bi Y. Global epidemiology of diabetic foot ulceration: a systematic review and meta-analysis. *Annals of Medicine*. 2017; 49(2):106-116. DOI:10.1080/07853890.2016.1231932
6. Holl J, Kowalewski C, Zimek Z, Fiedor P, Kaminski A, Oldak T. Chronic diabetic wounds and their treatments with skin substitutes. *Cells*. 2021; 10(3): 655. doi:10.3390/cells 10030655.
7. Oguntibeju OO. Medicinal plants and their effects on diabetic wound healing. *Veterinary world journal*. 2019; 2 (5): 653-663. doi:10.14202/Vetworld.2019.653-663.
8. Buch P, Chai Y, Goluch E. Treating polymicrobial infections in chronic diabetic wounds. *ASM journals*. 2019; 32(2): 2-4. doi:10.1128/cmr.00091-18.
9. Zhang W, Chen L, Xiong Y, Panayi C, Abududilibain A, Hu Y. Antioxidant therapy and antioxidant-related bio nanomaterials in diabetic wound healing. *Frontiers*. 2021; 9:11. doi:10.3389/fbioe.2021.707479.
10. Koechler S. Honey in wound healing: a new medicine? Online thesis. Lorraine: University of Lorraine; 2015; article L 122.4. https://www.cfcopies.com/v2/leg_droi.php.
11. Andjic M, Bozin B, Dragicin N, Kocovic A, Jeremic J, Tomovic M. Formulation and Evaluation of Helichrysum italicum Essential Oil-Based Topical Formulations for Wound Healing in Diabetic Rats.*Pharmaceuticals*.2021; 14:2-3.doi:10.3390/ph14080813.
12. Tekeu JC. Report on the practice of Environmental Impact Studies (EIA) in Cameroon. Prepared for the United Nations Economic Commission for Africa; 2004.

13. Singh A, Pramod K, Rakesh KS. Antidiabetic and Wound Healing Activity of *Catharanthus roseus* L. in Streptozotocin Induced Diabetic Mice. *American Journal of Phytomedicine and Clinical Therapeutics*. 2014; 2(6):686-692.
14. Herowati R, Alip DS, Saputri TW, Gunawan PW. Antihyperglycemic and diabetic wound healing activity of *smallanthussonchifolius* leaves extract. Universitas Setia Budi, Faculty of Pharmacy, Jalan Letjen Sutoyo, 2018; Solo 57127, Indonesia MATEC Web of Conferences 197, 07001 <https://doi.org/10.1051/matecconf/201819707001> AASEC 2018
15. Dwita LP, Dewanti E, Fitra D, Ramadani IF, Mareta DS. *Rhodomyrtus Tomentosa* Extract Activity on Diabetic Wound. *Journal of Physics*. 2021; 1933:2020. doi:10.1088/1742-6596/1933/1/012002
16. Tsaffo GM, Djenguemtar J, Fodouop SPC, Merzouk H, Sokoudjou JB, Kamsu GT, Feudjio HBL, Kodjio N, Mebarki A, Gatsing D. Resistance Profile of Bacteria Isolated from Diabetic Wounds: Phytochemicals and Antibacterial Studies of *Eriosemarobustum* Leaf Extracts . *Trends in science*. 2024; 21(6): 7881. <https://doi.org/10.48048/tis.2024.7881>
17. British Pharmacopoeia (BP). Department of health and social security Scottish home and health department. Office of the British Pharmacopoeia Commission, UK, 1988; 2:713.
18. Tan PL, Rajagopal M, Chinnappan S, Selvaraja M, Leong MY, Tan LF, Yap VL. Formulation and physicochemical evaluation of Green Cosmeceutical Herbal face cream containing standardized Mangosteen Peel Extract. *Cosmetics*. 2022;9(3): 46. <https://doi.org/10.3390/cosmetics9030046>
19. Kumar SR, Akhtar MT, Ahmad S, Abas F, Ismail IS, Rukayadi Y, Shaari K. Utilization of the ethyl acetate fraction of *Zanthoxylum rhetsa* bark extract as an active ingredient in natural sunscreen formulations. *Industrial Crops and Products*. 2017; 96:165-172.
20. Netto MG, and Jose J. Development, characterization, and evaluation of sunscreen cream containing solid lipid nanoparticles of silymarin. *Journal of cosmetic dermatology*. 2018; 17(6): 1073-1083.
21. European Pharmacopoeia, 8th edition, 2013, EDQM, Strasbourg.
22. OECD. Guideline No. 405: *In vivo* eye irritation/serious eye damage test, 2021a; OECD guidelines for testing chemicals.
23. Benneouala C, Berchiche L, Mellaz N. Toxicity of cosmetic products: *In vivo* tests. Doctorate thesis in pharmacy, Mouloud Mammeri University, Algeria, 2021.
24. OECD. Test No 404: OECD guideline for testing of chemical Acute Dermal Irritation/corrosion, 2015.
25. Ameri A, Rajive BB, Vaidya JG, Apte K, Deokule SS. Anti-Staphylococcal and wound healing activities of *Ganoderma praelongum* and *Glycyrrhiza glabra* formulation in mice. *International journal of Applied Research in Natural Products*. 2013; 6(1):27-31.
26. Mouokeu RS, Ngonu ANR, Lungu KP, Koanga MM, Tiabou TA, Njateng GSS, Tamokou JDD. Antibacterial and dermal toxicological profile of ethyl acetate extract from *Crassocephalumbauchiense* (Hutch.) Milne-Redh (Asteraceae). *BMC Complementary and alternative Medicine*. 2011; 11 : 43.

27. Mostefa NM, Sadok AH, Sabri N, Hadji A. Determination of optimal cream formulation from long term stability investigation using a surface response modeling. *Int. J. Cosmet. Sci.* 2006; 28(3): 211-218.
28. Aremu O, Olayemi O, Ajala T, Isimi Y, Oladosu P, Ekere K, Judith J, Emeje M. Antibacterial evaluation of *Acacia nilotica* Lam (Mimosaceae) seed extract in dermatological preparations. *J. Res. Pharm.* 2020; 24: 170-181.
29. Mohsin S, Akhtar N, Mahmood T, Khan H, Mustafa. Formulation and stability of topical water in oil emulsion containing corn silk extract. *Tropical Journal of Pharmaceutical Research.* 2016; 15(6): 1115-1121.
30. Rosso L, Lobry LR, Bajard S, Flandrois JP. Convenient model to describe the combined effects of temperature and pH on microbial growth, *Applied and Environmental Microbiology.* 1995; 61(2):610-616.
31. Djoko E, Foutse Y, Stéphanie MK, Dimo T. Activité cicatrisante d'une pommade à base des feuilles de *Kalanchoecrenata* (Andr.) Haw chez le rat. (Wound scarring of a kalanchoe crenata leaves ointment). *International Journal of Research and Analytical Reviews.* 2019; 6(4):2348-1269.
32. Awouafack MD, McGaw LJ, Gottfried S, Mbouangouere R, Tane P, Spiteller M. Antimicrobial activity and cytotoxicity of the ethanol extract, fractions and eight compounds isolated from *Eriosemarobustum* (Fabaceae). *BMC Complement. Altern. Med.* 2013a; 13:289. Doi: 10.1186/1472-6882-13-289.
33. Carvalho MTB, Araújo-Filho HG, Barreto AS, Quintans-Júnior LJ, Quintans JSS, Barreto RSS. Wound healing properties of flavonoids: A systematic review highlighting the mechanisms of action. *Phytomedicine.* 2021; 90:153636. doi: 10.1016/j.phymed.2021.153636.
34. Su X, Liu X, Wang S, Li B, Pan T, Liu D, Wang F, Diao Y, Li K. Wound-healing promoting effect of total tannins from *Entada phaseoloides* (L.) Merr. in rats. *Burns.* 2017; 43(4):830-838. doi: 10.1016/j.burns.2016.10.010.
35. Xue W, Zhangping Y, Shengnan Z, Shiwei S, Wei C. The Effect of a Compound Protein on Wound Healing and Nutritional Status. *Evidence-Based Complementary and Alternative Medicine.* 2022; 2022:12. <https://doi.org/10.1155/2022/4231516>
36. Tang T, Yin L, Yang J, Shan G. Emodin, an anthraquinone derivative from *Rheum officinale* Baill, enhances cutaneous wound healing in rats. *European Journal of Pharmacology.* 2007; 567(3):177-185.
37. Ekom SE, Tamokou JDD, Victor K. Antibacterial and Therapeutic Potentials of the *Capsicum annuum* Extract against Infected Wound in a Rat Model with Its Mechanisms of Antibacterial Action. *BioMed Research International.* 2021; 2021:17 <https://doi.org/10.1155/2021/4303902>
38. Hathama RH, Aymen A. Influence of diabetes disease on concentration of total protein, albumin and globulins in saliva and serum: A comparative study. *Iraqi National Journal of Chemistry.* 2015; 15 (1):1-15.

39. Balasubramanian S, Kaarthigeyan K, Srinivas S, Rajeswari R. Serum ALT: LDH ratio in typhoid fever and acute viral hepatitis. *Indian Pediatr.* 2010; 47(4):339–41.
40. Trengove NJ, Langton SR, Stacey MC. Biochemical analysis of wound fluid from non-healing and healing chronic leg ulcers. *Wound Repair Regen.* 1996; 4:234–239.
41. James TJ, Hughes MA, Cherry GW, Taylor RP. Simple biochemical markers to assess chronic wounds. *Wound Repair Regen.* 2000; 8:264–269
42. Santram L and Abhay KS. Wound healing effect of flavonoid rich fraction and luteolin isolated from *Martynia annua* Linn. on streptozotocin induced diabetic rats. *Asian Pacific Journal of Tropical Medicine.* 2013; 6(4):253-9. doi: 10.1016/S1995-7645(13)60053-X.
43. Ahmad Za'al A, Sawsan A, Jumah MS. Effect of *Rhus coriaria* L. methanolic fruit extract on wound healing in diabetic and non-diabetic rats. *Journal of cosmetic dermatology.* 2021; 00:1–11. DOI: 10.1111/jocd.14668
44. Harding KG, Morris HL, Patel GK. Science, medicine, and the future: healing chronic wounds. *Br Med J.* 2002; 324:160-3.
45. Amit KS, Piush K, Hemant KN, Navdeep R, Rajnish S. Hydroxyproline: A Potential Biochemical Marker and Its Role in the Pathogenesis of Different Diseases. *Current Protein & Peptide Science.* 2016; 17(6): 596-602.
DOI: 10.2174/1389203717666151201192247
- 46 Fannang, Simone Veronique, Antoine Vayarai Manaoda, François Eya'ane Meva, Gisele Etame Loe, Benoit Deschamps Mbida Mvomo, Jacques Yinyang, and Jean pierre Ngene. 2022. "Assessment of the Wound Healing Activity of the Aqueous Extract of Leaves of *Diospyros Hoyleana* (F.) White (Ebenaceae)". *Journal of Advances in Medicine and Medical Research* 34 (16):40-51. <https://doi.org/10.9734/jammr/2022/v34i1631406>.
- 47 Tayade, S. D. and Silawat, N. (2021) "Phytochemical Screening and Wound Healing Activity of Different Leaf Extracts of *Rhynchosiarothis* in Rats", *Journal of Pharmaceutical Research International*, 33(46B), pp. 386–393. doi: 10.9734/jpri/2021/v33i46B32953.
- 48 Silambujanaki P, Chandra CB, Kumar KA, Chitra V. Wound healing activity of *Glycosmis arborea* leaf extract in rats. *Journal of ethnopharmacology.* 2011 Mar 8; 134(1):198-201.
- 49 Mukherjee PK, Verpoorte R, Suresh B. Evaluation of in-vivo wound healing activity of *Hypericum patulum* (Family: Hypericaceae) leaf extract on different wound model in rats. *Journal of ethnopharmacology.* 2000 Jul 15; 70(3):315-21.