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The Healing of Wounds Infected with *Staphylococcus aureus* is Accelerated by Leech Saliva Ointment

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ABSTRACT

This study aimed to explore the impact of pure leech saliva and its combination with Eucerin on wounds infected with *Staphylococcus aureus*. The experimentation involved inducing wounds on the dorsal area of the animals' chest. For the purpose of infecting the wounds, 100 µl of *Staphylococcus aureus* bacteria with a density of 0.5 McFarland were introduced to the wound site. 75 male Wistar rats were employed, grouped into 5 sets of 15 rats each, and further subdivided into 3 subgroups within each group: treated with nitrofurazone (positive control), leech saliva, leech saliva ointment, Eucerin ointment, and negative control (untreated). Subsequently, on days 7, 14, and 21, samples were collected from the wound sites to quantify bacterial presence and assess wound tissue recovery. Macroscopic observations revealed promising wound healing capabilities of both leech saliva ointment and pure leech saliva within a 14-day timeframe. Microbial analysis corroborated the antimicrobial efficacy exhibited by leech saliva and its ointment formulation. According to the findings, it is reasonable to infer that both leech saliva ointment and pure leech saliva exhibit commendable efficacy in facilitating wound healing and promoting epithelial tissue regeneration in the skin.

Introduction

A wound is defined as a disruption to the integrity of the skin, mucous surfaces, or tissue organs, resulting from various injurious agents.[1] The physiological processes governing wound healing encompass key stages: homeostasis and the commencement of inflammatory processes, subsequent proliferation, and ultimately, tissue regeneration.[2]

Various factors significantly impact wound treatment, encompassing elements such as age, obesity, smoking, underlying medical conditions, medications, wound surface moisture, nutritional status, wound infection, and the extent of the wound.[3] Upon skin injury, indigenous microorganisms residing on the skin surface infiltrate underlying tissues, culminating in wound contamination and subsequent infection. Notably, *Staphylococcus* assumes a pivotal role in precipitating bacterial infections within wounds. The persistence of biofilms containing these bacteria often impedes the healing of numerous chronic wounds. This mechanism can elucidate the ineffectiveness of antibiotics in addressing the treatment of chronic wounds.[4]

Leeches, aquatic worms with historical utilization in medical and traditional practices, offer insights. Among medicinal leeches, *Hirudo medicinalis* stands as the predominant variant.[5] Leech saliva comprises over 100 discernible active components. Hirudin, for instance, impedes blood coagulation through its interaction with thrombin. Hyaluronidase, on the other hand, facilitates the dispersion of therapeutic agents within tissues.[6] Moreover, leech saliva encompasses bedlin and eglin,

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both possessing notable anti-inflammatory properties. Choline functions as both an adhesion inhibitor and a platelet activator[7] Dastabilase, conversely, degrades fibrin. Serotonin and dopamine, prevalent within leech saliva, exhibit analgesic attributes. Additionally, acetylcholine assumes a role as a vasodilator.[8]

Staphylococcus aureus, a gram-positive pathogenic bacterium, perturbs host cell functions. The bacterium manifests and releases adhesive proteins (Eap), including extracellular adhesive protein. Notably, the interaction between Eap and the host's extracellular adhesive molecule hinders leukocyte attachment to endothelial cells, leading to the disruption of integrin-dependent leukocyte functions. Furthermore, Eap disrupts the functionality of the body's immune cells and prolongs the healing process of wounds infected with Staphylococcus aureus.[9]

Despite the presence of antimicrobial peptides like chlormistin, destabilase, thromamycin, thromazine, and neuromycin in leech saliva, and their evaluation against both gram-negative and gram-positive bacteria, these peptides have further fortified the antibacterial attributes inherent in leech saliva.[10–12] Leech therapy finds application in post-plastic surgery wounds and flaps,[13] as well as in reconstructive procedures for the jaw and facial regions.[14] Its utilization extends to treating conditions like varicose veins, hemorrhoids, and other ailments.[15] Notably, clinical investigations have also highlighted leech therapy's potential in cases of osteoarthritis, a distressing joint ailment, where the medicinal peptides within leech saliva could prove advantageous.[16] Furthermore, numerous uncontrolled clinical studies and pharmaceutical records have cited the utilization of leech therapy for the management of diabetic foot ulcers, as highlighted by Koeppen *et al.*[17] Given the inherent wound-healing attributes of leeches, coupled with the presence of antimicrobial agents in their saliva, leeches emerge as a viable contender for the utilization of biological materials in wound healing and addressing associated infections.

As per existing research, no study has been undertaken to explore the application of leech saliva on *S. aureus*-infected wounds, while concurrently comparing its efficacy with nitrofurazone ointment. Additionally, transforming this concept into practicality involves the development of a medicinal formulation, specifically a 5% leech saliva ointment. This study aimed to enhance our comprehension of the mechanisms underlying wound healing within the context of this therapeutic blend, thereby provided valuable insights into the treatment process for infected wounds.

Materials and Methods

Preparation of Leech Saliva

Leeches (*H. medicinalis*) were sourced from a reputable breeding center in Daruk leech breeding center (https://shop.darok.ir) and subsequently housed in a well-ventilated plastic container filled with chlorine-free water within a temperature-controlled environment. The water was regularly replaced every 48 hours. To extract leech saliva, parafilm was affixed to a 100 ml Erlenmeyer flask. Between the parafilm and the flask's outer layer, a solution containing 1 mmol (0.001 mol) of arginine (Merck Co.) and 0.15 mol of sodium chloride was introduced. The Erlenmeyer flask was secured to a metal tripod, while the leech was carefully attached to the parafilm, allowing it to feed on the solution and detach spontaneously. After separating the leech from the parafilm, it was placed in a beaker and submerged in ice for 10-15 minutes. Subsequently, the container was positioned near a flame. The leech was then reactivated, resulting in the collection of a dense liquid identified as leech saliva in the beaker. This saliva was carefully transferred to a microtube.[18] The collected saliva was subjected to a sterilization process in a -20 °C freezer for 72 hours, followed by total protein measurement using a Nano drop device by A280 method. The prepared leech saliva was quantified to contain 900 micrograms of protein per milliliter.

Preparation of Leech Saliva Ointment

Following the Minimum Inhibitory Concentration

Table 1. Classification of studied animals

Groups	Description	Number of animals for each treatment duration.		
		7 days	14 days	21 days
Control -	The animals sustaining injuries did not receive any form of treatment.	5	5	5
Control +	The injured animals were treated with 0.2% nitrofurazone ointment.	5	5	5
T1	The injured animals were treated with leech saliva.	5	5	5
T2	The injured animals were treated with a combination of 5% leech saliva and Eucerin.	5	5	5
T3	The injured animals were treated with Eucerin ointment.	5	5	5

(MIC) determination of leech saliva,[19] a 5% ointment was formulated by blending it with Eucerin (Sepidaj Company. Tehran, Iran). It was melted using indirect heat. The ointment formulation involved combining 95 ml of Eucerin with 5 ml of leech saliva.

Animal Groups and Treatments

For this investigation, a total of 75 adult male Wistar rats, with body weights ranging between 200 and 300 grams, were procured from (Semnan, Shahmirzad Laboratory Animal Research and Breeding Center). These rats were housed in specialized cages within the laboratory animal breeding and maintenance department of Semnan University. To facilitate adaptation and minimize stress, a one-week adjustment period was implemented. Throughout the study, the rats were subjected to uniform environmental conditions encompassing temperature, humidity, and lighting. Their nutrition, including meal frequency and food ration, remained standardized, and access to water was unrestricted. The care and management of the animals adhered to the guidelines set forth by the laboratory animal committee at Ethics Committee in Biological Research, Faculty of Veterinary Medicine, Semnan University, with ethical approval obtained under code IR.SU.REC.E-00-06 from the biological research ethics committee. The research protocol was executed in complete alignment with the principles articulated in the World Medical Association Declaration of Helsinki. The animals were categorized into five distinct groups as outlined in Table 1. Treatments were administered every 48 hours. Utilizing a sterile swab, the designated ointment was applied onto the wound surface. For the application of leech saliva, 100 µl was gently applied using a sterile swab. In the negative control group, which received no treatment, only dressing changes were performed.

Animal Skin Wound Model

A total of 75 adult male Wistar rats underwent visual and health assessments. The rats were subjected to intraperitoneal anesthesia using a combination of 10% ketamine hydrochloride (50 mg/kg) and 2% xylazine hydrochloride (5 mg/kg). Utilizing a conventional razor, the hair on the dorsal region between the shoulder blades was meticulously shaved. Following the shaving procedure, the shaved area underwent disinfection using alcohol. A square region measuring 1.5 × 1.5 cm in length and width was excised from the animal's skin using a scalpel blade. The excision included removal of the epidermis and dermis layers to expose the underlying muscle tissue, as delineated by Dwivedi *et al.*[20] Subsequently, the wound was thoroughly rinsed using physiological serum, and it was made ready for the inoculation of *Staphylococcus aureus* bacteria.

Bacterial Inoculation

Staphylococcus aureus bacteria (ATCC29213), prepared with a 0.5 McFarland dilution measured at 600nm wavelength in spectrophotometry, and exhibiting an optical absorption range of 0.08 to 0.1, contained a bacterial concentration of 1.5×10^8 colony forming units per milliliter (cfu/ml). For the purpose of infecting the wounds created in all samples, 100 µl of *Staphylococcus aureus* bacteria (ATCC29213) with a density of 0.5 McFarland, dissolved in BHI broth, were introduced to the wound site. Following the bacterial inoculation, sterile gauze was positioned over the wound and secured in place using a Fixed Dressing Roll. After a 24-hour interval from the bacterial inoculation, the dressing was unfastened, and treatment was administered at subsequent 48-hour intervals within a sterile environment. During treatment sessions, the ointments and leech saliva were applied onto the wounds of the samples using a sterile swab, after which the wounds were dressed with bandages.

A day following bacterial inoculation, a microbial swab was obtained from all samples and streaked onto nutrient agar culture medium to verify the accuracy of the inoculation. After a 24-hour incubation period, the test results were examined.

Wound Closure

At time intervals of 0, 7, 14, and 21 days, the wound surface dimensions were assessed using a digital caliper (150 mm), while the wound sites were documented through digital photography. The rate of wound healing among the various groups was subsequently compared and assessed utilizing Image J software. The calculation of the percentage of wound healing was achieved using the following formula:

$$\text{Recovery percentage} = \frac{(\text{Wound surface on the first day} - \text{Wound surface on the day of imaging})}{\text{Wound surface on the first day}} \times 100$$

Tissue Collection

At predetermined time intervals of 7, 14, and 21 days, the rats were subjected to intraperitoneal anesthesia (ketamine hydrochloride 10% (50 mg/kg) + xylazine hydrochloride 2% (5 mg/kg). Using a sterile scalpel, the wounds were excised at a distance of 0.3 to 0.5 mm from the wound edges. Tissue samples extracted from the animal wounds were meticulously preserved in separate plastic containers following immersion in a 10% formalin solution. Subsequent to 24 hours, the formalin solution was substituted with a fresh solution of the same composition. From the wound sites, tissue blocks were meticulously prepared, which were subsequently subjected to staining with hematoxylin-eosin and Masson trichrome stains. This staining process facilitated the

observation of tissue repair progress and assessment of the contributing factors in wound healing. The evaluation and verification of the slides' scoring criteria were conducted using a semi-quantitative approach in accordance with the methodology outlined by Gal *et al*.[21] (Table2).

Microbial Investigation

One gram portion of the wound sample was isolated for microbial analysis. The tissue was homogenized within a sterile mortar using a peptone water culture medium. Subsequently, from the tissue broth designated for cultivation in nutrient agar culture medium plates, a 0.1 volume was drawn with a sampler and introduced into tubes containing 0.9 peptone water culture medium. A series of six tubes were employed for conducting serial dilutions. For each wound sample, the process of lawn culture was executed twice on nutrient agar plates. Subsequent to this, the plates were placed within an incubator operating at a temperature of 37 degrees Celsius for duration of 24 hours. Following incubation, the quantity of colonies that had developed within the culture medium was quantified using a colony counter. The enumeration of the colonies was carried out in accordance with the method established by Stratford et al., thus facilitating the determination of the bacterial count within each sample.[22]

Statistical Analysis

The statistical evaluation of the collected data was conducted employing SPSS version 23 software. For parameters such as collagen, fibroplasia, new vessels, and epithelization within the studied groups, rank data were subjected to analysis using Kruskal-Wallis's test. Meanwhile, the outcomes of microbial tests were assessed using the One-Way ANOVA test, followed by Tukey's post hoc analysis, at a significance level of $p < 0.05$.

Results

Microbial Load of Wounds

The quantification of bacterial colonies isolated from the wound sites across various groups, post cultivation, was performed employing the methodology established by Stramford *et al*.[22] The findings are documented in Table 3. As per the data derived on the seventh day, it is evident that the leech saliva ointment (T2) group exhibited a lower rate of bacterial colony proliferation when compared to the negative control, positive control, and T3 groups. Similarly, the leech saliva-only group (T1) demonstrated diminished bacterial growth relative to the negative control, positive control, and T3 groups.

Upon assessing the count of bacterial colonies isolated from the wound sites across different groups during the second week (day 14), it was observed that the number of bacteria experienced a decline in all groups except the negative control group, which exhibited a relative increase in bacterial count compared to the seventh day. This decrease was particularly noticeable in the leech saliva ointment group (T2) as compared to the negative control group. Remarkably, the leech saliva ointment group (T2) exhibited superior performance compared to the leech saliva group (T1), as well as significant reduction in bacterial colony growth when compared to the positive control and T3 groups. Furthermore, the leech saliva group (T1) demonstrated a reduction in bacterial colony growth relative to the positive control, negative control, and T3 groups. This declining trend in

Table 2. Clarification of semi-quantitative evaluation scale for histological sections.

Scale	Epithelization	Polymorphonuclear Leucocytes	Fibroblasts	Angiogenesis	Collagen
0	Thickness of cut edges	Absent	Absent	Absent	Absent
1	Migration of cells (< 50%)	Mild ST	Mild ST	Mild SCT	Minima 1 GT
2	Migration of cells (≥ 50%)	Mild DL/GT	Mild GT	Mild GT	Mild GT
3	Bridging the excision	Moderate GL/GT	Moderate GT	Moderate GT	Moderate GT
4	Keratinization	Marked DL/GT	Marked GT	Marked GT	Marked GT

Surrounding tissue (ST), demarcation line (DL), subcutaneous tissue (SCT), granulation tissue (GT).

Table 3. Mean ± SD of bacterial counts extracted from the wound sites on the specified study days.

Group	Day 7	Day 14	Day 21
Control -	$134.2 \pm 5.4 \times 10^{5\,a\,\#}$	$141.2 \pm 6.5 \times 10^{5\,a\,\#}$	$150.4 \pm 3.67 \times 10^{5\,a\,\#}$
Control +	$24.2 \pm 5.07 \times 10^{6\,b\,*\,\#}$	$6.6 \pm 2.73 \times 10^{5\,c\,*\,\#}$	$1.2 \pm 0.8 \times 10^{5\,d\,\#}$
T1	$15.4 \pm 2.46 \times 10^{6\,b\,\#}$	$2.2 \pm 0.37 \times 10^{3\,d\,\#*}$	$0.8 \pm 0.6 \times 10^{4\,d\,\#}$
T2	$7.2 \pm 1.02 \times 10^{6\,c*\,\#}$	$1.2 \pm 0.5 \times 10^{5\,d*\,\#}$	$0.4 \pm 0.24 \times 10^{3\,d\,\#}$
T3	$27 \pm 3.73 \times 10^{5\,b*\,\#}$	$23 \pm 2.47 \times 10^{4\,b*\,\#}$	$19.8 \pm 1.78 \times 10^{5\,b\,\#}$

Within each row of the table, statistical dissimilarity between the groups is indicated by non-identical letters, while the same letters denote a lack of significant statistical distinction between the groups. Asterisk (*) signifies a statistical variance at a significance level below 0.05, while the hashtag (#) indicates a statistical variance at a significance level below 0.0001.

Table 4. Trajectory of pathological parameters across distinct groups: negative control group (untreated), positive control (treated with nitrofurazone ointment), group T1 (treated with leech saliva), group T2 (treated with leech saliva ointment), and group T3 (treated with Eucerin ointment).

Groups	Control -	Control+	T1	T2	T3
Epithelization	7 ± 3.5[b]	9.8 ± 4.56[b]	14.6 ± 3.13[b]	40.6 ± 3.13[a#]	13.30 ± 1.56[b]
Inflammation	36.4 ± 4.14	33.6 ± 3.67	30.8 ± 4.14	21 ± 3.13[a*]	41.3 ± 4.95[b]
Fibroblast	32.9 ± 3.67	37.8 ± 3.32	30.10 ± 5.07	28.7 ± 3.32	32.9 ± 5.31
Collagen	24.5 ± 5.19	23.8 ± 2.71	27.3 ± 4.83	32.9 ± 3.32	26.6 ± 1.92
Angiogenesis	28.7 ± 5.96	30.8 ± 3.83	22.4 ± 4.49	29.4 ± 6.83	34.3 ± 3.83

* denotes statistical significance at $p < 0.05$, while # indicates statistical significance at $p < 0.0001$.

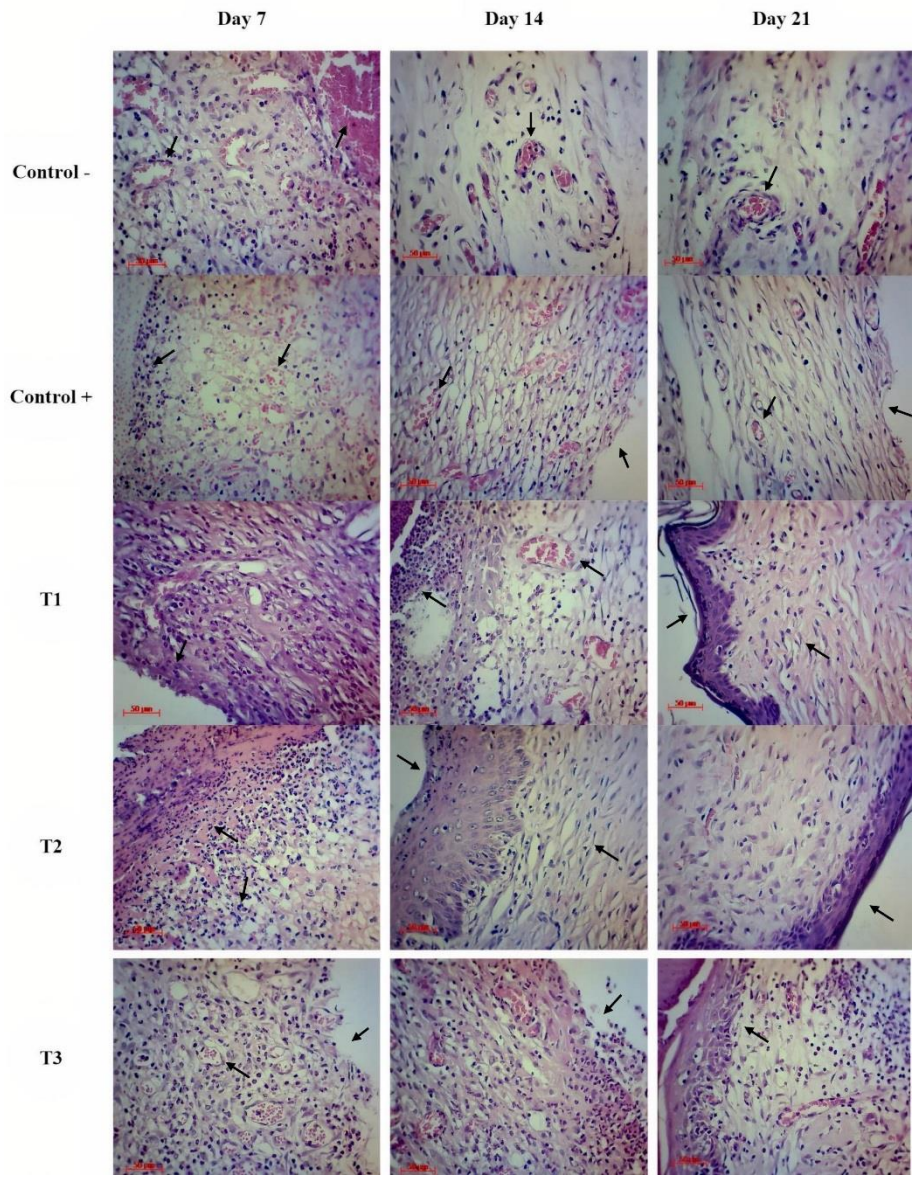

Figure 1. Microscopic visualization of the analyzed groups utilizing hematoxylin-eosin staining, observed at a magnification of 400×. Pathological images focus on the wound's central edge. In the negative control group on days 7, 14, and 21 (indicated by black arrows), notable inflammatory cell and angiogenesis presence is observed. The positive control group on these days shows concurrent inflammatory cell and angiogenesis activity, along with wound perimeter delineation. Leech saliva treatment (T1) reveals significant inflammatory cell presence on day 7; by day 14, angiogenesis increases with reduced inflammatory cells. By day 21, epithelial cell formation yields a scarred region. In the Eucerin group (T2) on day 7, inflammatory cells, angiogenesis, and immature fibroblasts are seen. Day 14 sees epithelial cell formation, reduced inflammation, and angiogenesis. By day 21, epithelial cells exhibit damage. Conversely, in Eucerin ointment group (T3) across days 7, 14, and 21, absent epithelial cells coincide with persistent inflammatory cells and angiogenesis at the wound edge.

bacterial counts, observed in various groups except the negative control, persisted until the 21st day.

In a broader context, leech saliva ointment (T2) exhibited notably superior antimicrobial efficacy compared to the different groups ($p < 0.05$). Additionally, the antimicrobial impact of leech saliva alone (T1) demonstrated superiority over other groups, barring leech saliva ointment (T3), Table 3.

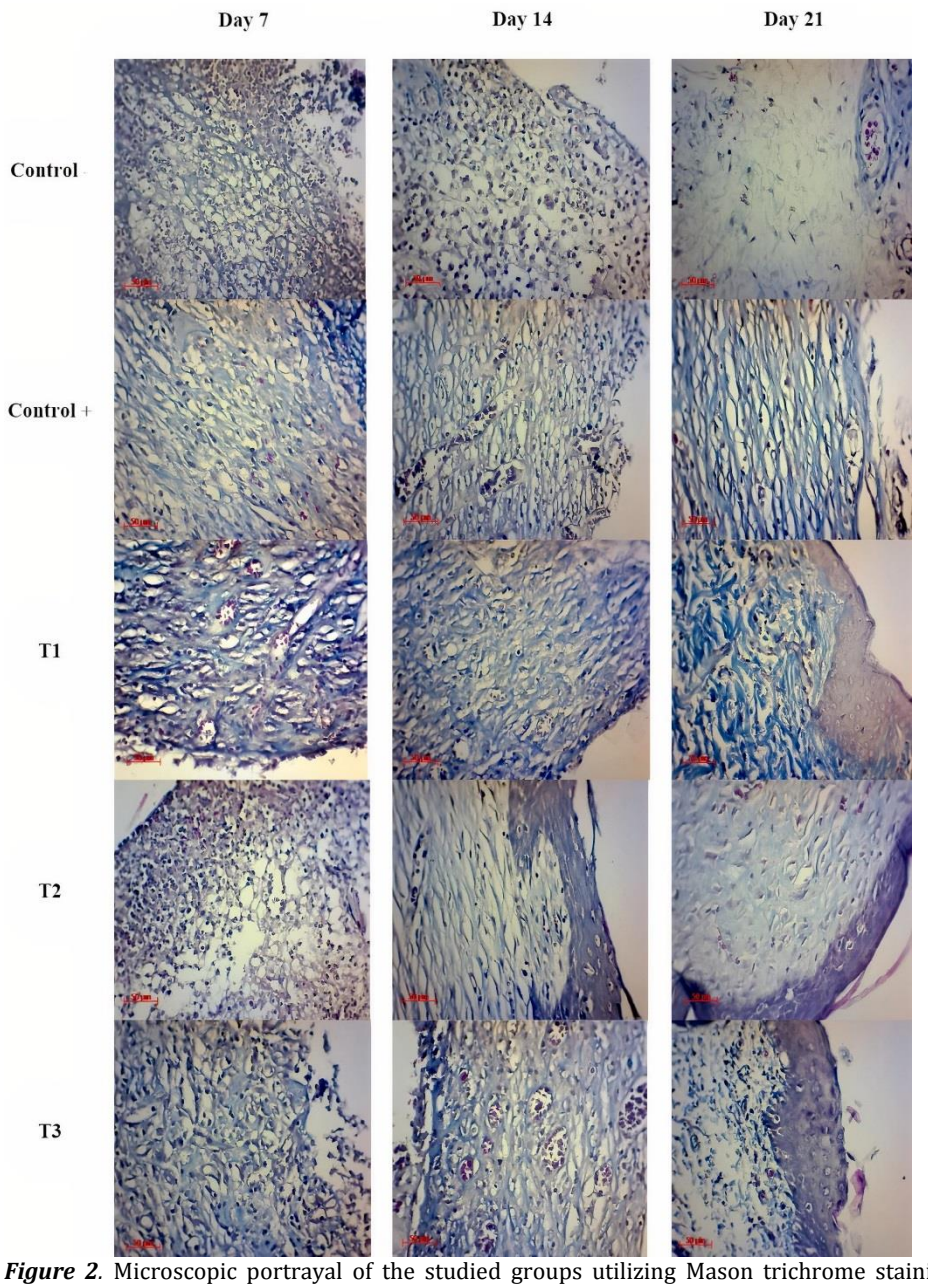

Figure 2. Microscopic portrayal of the studied groups utilizing Mason trichrome staining, observed at a magnification of 400×. Pathological images capture the wound's central edge. In the negative and positive control groups on days 7, 14, and 21, collagen cells display disoriented and thickened patterns. In the leech saliva group (T1), collagen cells exhibit consistent orientation and thickness on day 21, aligning with other groups. Within the 5% leech saliva ointment group (T2), collagen cells maintain uniform orientation and structure on day 21, mirroring days 14 and 7, as well as other groups. Conversely, the Eucerin ointment group (T3) manifests disordered collagen cell orientation on days 7, 14, and 21.

Pathological Observations

In this study, we assessed the wound healing progression within different treatment groups. To evaluate the wound healing dynamics across various samples, we investigated factors encompassing angiogenesis, fibroplasia, inflammatory cell counts, collagen density, and maturation. Given the ordinal nature of the data, non-parametric tests were employed, and the Kruskal-Wallis test was utilized due to the presence of more than three groups. Results are presented as mean ranks within each group. The trend of changes was analyzed using Area under curve (AUC) curves based on the mean area under the curve, along with standard deviation. The data analysis revealed significant variations among groups concerning epithelialization and inflammation, as indicated by the employed tests. Histopathological examinations of the groups treated with leech saliva ointment (T2) and leech saliva alone (T1) showcased a swiffer enhancement process compared to other groups. In the leech saliva ointment (T2) group, a more substantial reduction in inflammatory cells (Table 4) was noted, with a marked difference between treatment days of 7 and 21. Moreover, as depicted in Figure 1, the presence of inflammatory cells in the wound area of group T2 exhibited statistically significant variance compared to both positive and negative control groups, highlighting the effective anti-inflammatory influence of leech saliva in

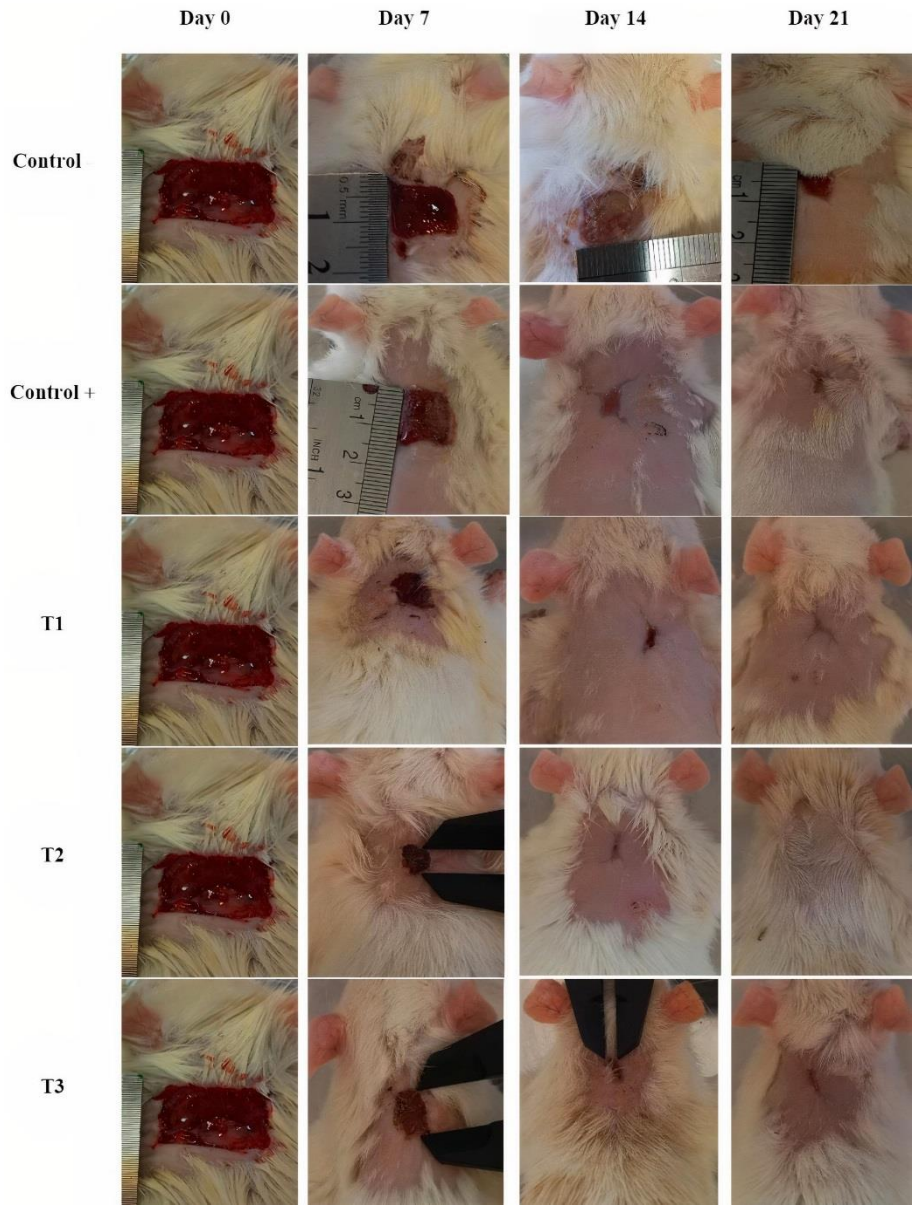

Figure 3. Observation of wound healing progression across varying time intervals and groups from a macroscopic perspective.

Table 5. Tabulated data depicts the average percentage of wound healing across different groups at various time intervals.

Groups	Day7	Day 14	Day 21
Control -	43.02	52	78.2
Control +	25.77	80.44	91.55
T1	42.67	87.11	99.55
T2	36	98.5	100
T3	30	83	95

curbing inflammation at the wound site. Collagen formation (Table 4) displayed an ascending trajectory across all groups. Particularly in the T2 and T1 treatment groups, as gleaned from Figures 1 and 2, a higher orientation and thickness of collagen fibers were apparent compared to other groups. This phenomenon, however, lacked significant differences among the various groups. Additionally, the escalating trend of collagen fiber presence within the wound area was evident from days 7 to 21. The degree of angiogenesis within the wound tissue (Table 4) showcased a descending trend from day 7 to 21 across the treatment groups. Notably, in group T1, as evident in Figures 2 and Table 4, this downward trend was more pronounced compared to other groups. While there was no statistically significant difference across the groups concerning overall angiogenesis, there were notable variations during the treatment days of 7 to 21. The presence of fibroblast cells during the treatment period from days 7 to 21 displayed discernible variability among the treatment groups. Referring to the data in Table 4 and the tissue slide images in Figure 1, it can be inferred that there is no significant overall difference in the fibroblast factor among all groups. In line with Table 4, the process of epithelialization exhibited a statistically significant difference in group T2 compared to other groups. Moreover, during the 7 to 21-day timeframe, all treatment groups displayed significant differences. As indicated by Figure 1, in the process of epithelialization, groups T2 and T1 exhibited the highest trend of wound closure relative to other groups.

Changes in Pathology Parameters in Different Groups

Histopathological analysis delved into the intricate dynamics of wound healing across discrete groups. Parameters spanning fibroplasia rate, angiogenesis, inflammatory cell count, collagen fiber density, and maturation underwent rigorous scrutiny. Detailed findings are elaborated in Table 4. Notable differentials surfaced during comparisons of epithelialization degrees: T2 versus negative control, T2 versus positive control, T2 versus T3, and T2 versus T1 demonstrated statistically significant disparities ($p < 0.001$). Notably, analysis of inflammatory factors unveiled statistically significant contrasts ($p < 0.05$) between T2 and T3. However, with respect to fibroblast, collagen, and angiogenesis, no statistically significant inter-group disparities were observed.

Macroscopic Results of the Wound

Wound dimensions were meticulously evaluated and quantified across the examined groups on days 0, 7, 14, and 21, employing a digital caliper. Upon scrutiny of the data presented in Table 5, it is evident that by the seventh day, both the negative control group and the cohort subjected to leech saliva treatment (T1) manifested a discernible reduction in wound area in contrast to the assorted groups. As the study progressed, specifically by the 14th day, the leech saliva ointment group (T2) exhibited a notably elevated percentage of wound healing, surpassing the corresponding rates observed in the negative control, nitrofurazone ointment (control+), and Eucerin ointment (T3) groups. In aggregate, the application of leech saliva ointment (T2) prominently showcased an enhanced rate of wound closure in comparison to the utilization of leech saliva alone (T1) (Figure 3).

According to the data in the Table. 4, the 21st-day assessment revealed a progressive rise in wound healing rates across all groups. Remarkably, the leech saliva ointment (T2) group exhibited full wound closure, while leech saliva alone (T1) was similarly effective in achieving complete restoration. Notably surpassing diverse groups, encompassing the negative control, both leech saliva ointment (T2) and leech saliva (T1) demonstrated superior wound closure rates. Consequently, the prescribed time frame sufficed for comprehensive wound healing and closure, Table 5.

Discussion

The intricate course of wound healing seeks to reinstate the structural integrity of compromised tissues within the wound site. This intricate process unfolds across four meticulously orchestrated phases: hemostasis, inflammation, proliferation, and regeneration.[23] In cases of chronic wound infections, the healing process may be hindered, resulting in clinical repercussions and direct ramifications for the healthcare system.[24]

Leech saliva encompasses an excess of 90 biologically active substances.[24] Owing to its anti-inflammatory and antimicrobial attributes, along with its capability to expedite wound healing, it stands as a prospective candidate for evaluation in the context of healing skin wounds beset by *Staphylococcus aureus* infections. Our study aimed to scrutinize the roles of inflammation and proliferation stages in wound healing while concurrently addressing the reduction of microbial burden. Leech therapy's historical use has solidified its standing as an effective medicinal intervention.[25] Leeches secrete a diverse array of factors, such as hirudin and factor Xa inhibitors, into wounds, thwarting scab formation and hastening the healing process.[26] Numerous investigations have delved into leech application's impact on blood supply within ischemia-induced models spanning mice, rats, rabbits, and pigs. These studies collectively attest to the ability of leeches to augment blood flow velocity, ameliorate microcirculation, and expedite anastomosis.[27–32]

Furthermore, a separate study identified a substantial and enduring rheological reduction in the viscoelasticity of blood samples subsequent to leech application.[33] This amalgamation of historical and contemporary insights underscores the multifaceted benefits and mechanisms underpinning leech therapy.

This study delved into the impact of leech saliva, both independently and in conjunction with Eucerin, on the healing process of *S. aureus*-infected wounds. Histopathological observations revealed pronounced accumulations of blood cells at the sites of infected rat wounds. Conversely, the treated groups exhibited a discernible reduction in blood cell presence, indicative of diminishing tissue inflammation throughout the treatment period. Broadly, a noteworthy discrepancy in inflammatory factors was apparent between the 7th and 14th days across all groups. Moreover, the interval of 14 to 21 days witnessed diminished levels of inflammatory factors, implying that our treatment regimen effectively curbs inflammation arising from wound infections (*S. aureus*) within this timeframe. In the broader context, alleviating pain and inflammation forms a pivotal initial stride in the wound healing process, as underscored by Duque *et al*.[34]

Leech saliva's analgesic effects can be attributed to antistatins with a 15 kDa molecular weight, effectively inhibiting kallikrein.[7] Contributing to this anti-inflammatory dynamic, Eglin C and cathepsin G

additionally partake in restraining tissue inflammation.[35,36] Moreover, the presence of complement C1 inhibitor within leech saliva reinforces its potential as a favorable anti-inflammatory agent.[37]

Angiogenesis and collagen synthesis are integral components of the proliferative phase in wound healing. The onset of angiogenesis typically occurs around the 4th day post-injury, expediting the supply of oxygen, nutrients, and growth factors to the injured tissue, as elucidated by de Moura *el al*.[38] In our current investigation, the levels of angiogenic factors and collagen exhibited significant fluctuations between days 7 and 21. Notably, no significant disparities were noted among the treatment groups themselves. Given the presence of these factors within leech saliva (T1), it emerges as a compelling candidate for the treatment of diverse ailments. A noteworthy application is leech therapy for diabetic foot ulcers, a particularly efficacious medicinal utilization of leeches. Diabetes-related foot ulcers afflict approximately 5% of diabetic patients.[3] The realm of clinical exploration concerning diabetic foot ulcers, venous congestion, tissue damage, Buerger's disease, and cutaneous leishmaniasis underscores the expansive spectrum of medicinal and therapeutic effects encapsulated within leech therapy, as substantiated by Koeppen *et al*.[17]

Pharmacological trials and clinical observations in medicinal leech therapy (MLT) demonstrate substantial potential for healing diverse chronic wounds, alongside indications of efficacy in managing conditions such as arthritis.[16] As per the data in Table 4, the leech saliva treatment group displayed a 42.67% wound healing rate on day 7, while the nitrofurazone 0.2% group (Control +) exhibited an average of 25.77%. Similarly, on the 14th and 21st days, the leech saliva group (T1) demonstrated wound contraction rates of 87.11% and 99.55% respectively, while the nitrofurazone 0.2% group (Control +) showcased contraction rates of 80.44% and 91.55% correspondingly. On the 14th and 21st days, the application of 5% leech saliva ointment (T2) led to complete wound closure. In congruence with our findings, Darestani *et al*. (2014) demonstrated that leech saliva spurred more pronounced wound contraction in contrast to phenytoin ($p < 0.05$).[39] Additionally, during the study conducted by Zakian *et al*.,[40] a comparison between medicinal leech therapy and phenytoin ointment on a skin wound animal model highlighted that the medicinal leech therapy group displayed swifter wound healing, reduced inflammatory response, and heightened collagen regeneration when compared to other groups.

Leech saliva harbors active antimicrobial peptides like chlormystine and destabilase.[11] Destabilase, even when denatured, shows dose-dependent antimicrobial activity against *S. aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*.[12] Various antimicrobial peptides (AMPs) have been identified in different leech species, including theromyzin, theromacin, neuromacin, and hydramacin-1, with efficacy against Gram-positive and Gram-negative bacteria.[41]

Recent research on *H. medicinalis* revealed peptides, notably pept_356, 3967, and 536-1, with potent antimicrobial properties against both bacterial types.[10,41] *Staphylococcus aureus* forms biofilms in wounds, causing chronic infections and impeding healing.[4] By capitalizing on the antimicrobial attributes inherent to leech saliva, our investigation yielded noteworthy and significant outcomes when subjected to in vivo experiments involving rat skin wounds afflicted with *S. aureus* infections. The findings, as elucidated in Table 3, unveiled a pronounced and statistically significant differentiation ($p < 0.05$ and $p < 0.001$) between leech saliva and the 0.2% nitrofurazone ointment group (designated as Control +).In accordance with the outcomes of this study, the application of leech saliva ointment (T2) demonstrated a substantial capacity to expedite the healing process of infected wounds when contrasted against both the negative control groups and nitrofurazone ointment (Control +) ($p < 0.05$). This effect was accompanied by an accelerated and more comprehensive regrowth of hair follicles around the wound site, as discerned from macroscopic imagery.

The observed swifter hair follicle regeneration within the leech saliva (T1) and leech saliva ointment (T2) groups can plausibly be attributed to augmented nourishment of hair follicles, attributed to heightened blood circulation in the wounded area. This phenomenon is linked to the bioactive compounds present in leech saliva (T1), which exert biologically active influences on tissues. These same biologically active substances are encapsulated within leech saliva (T2) as elucidated in our current study. Our findings resonate with previous research, particularly the works of Amani *et al*.[42] and Zakian *et al*.[40] The positive impact of leech saliva on the recuperation of *S. aureus*-infected wounds can potentially be attributed to multiple factors. These include the facilitation of angiogenesis and new blood vessel formation, the presence of wound healing response modulators, anti-inflammatory effects, and the inherent antimicrobial properties found within leech saliva.

Leeches and their extracts have been employed in direct application or as leech-derived extracts for the treatment of various ailments, including wound healing. This research focuses on isolating leech saliva, known to harbor the majority of therapeutic compounds found within leeches, and evaluating its efficacy in the wound healing process, particularly in cases of *S. aureus*-infected wounds.

Given the pressing necessity to address wound

infections and the growing concern of antibiotic resistance, along with the need for swift healing of both acute and chronic wounds, the investigation explores the potential of utilizing a natural medicinal blend in expediting the healing process and promoting skin health in individuals afflicted by such conditions. The research yielded promising outcomes through the application of leech saliva composition, juxtaposed with a reference point of 0.2% nitrofurazone ointment. Notably, during histopathological examinations, the 5% leech saliva ointment and pure leech saliva groups exhibited more favorable results compared to alternative treatment groups. Furthermore, in microbial testing, the 5% leech saliva ointment group and the pure leech saliva group demonstrated substantial distinctions in comparison to the 0.2% nitrofurazone group. In summary, the amalgamation of leech saliva and Eucerin as a 5% leech saliva ointment showcased positive outcomes with regard to the mending of wound tissue and the eradication of *S. aureus* infections.

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Conflict of Interest

There was no conflict of interests in relation to this article.

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