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Advances in Animal and Veterinary Sciences

Research Article

Enhanced Wound Closure and Tissue Regeneration in Streptozotocin-Induced Diabetic Rats Treated with a Topical *Usnea longissima* Extract and Hyaluronic Acid Cream

Erman Munir[1]*, Sony Eka Nugraha[2], Dwi Suryanto[1], Oky Kusuma Atni[1]

[1]Department of Biology, Faculty of Mathematic and Natural Sciences, Universitas Sumatera Utara, Medan 20155, Indonesia; [2]Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Sumatera Utara, Medan 20155, Indonesia.

Abstract | Diabetes mellitus impairs wound healing due to persistent inflammation, oxidative stress, and infection. This study aimed to evaluate the wound healing potential of a cream formulated with *Usnea longissima* extract and hyaluronic acid (HA) in streptozotocin (STZ)-induced diabetic rats. *Usnea longissima* was extracted using maceration with 70% ethanol, and its phytochemical profile was screened qualitatively. Thirty rats were randomly divided into six groups (n = 5/group), including normal, diabetic control, Bioplacenton-treated, and three treatment groups receiving cream formulations with increasing concentrations of extract and HA. Wound healing was assessed through visual observation, histological scoring, and serum cytokine levels. Antioxidant activity was measured using the DPPH assay, and statistical analysis was conducted using ANOVA with Tukey's post-hoc test ($p < 0.05$). Phytochemical screening of *Usnea longissima* of this study shows the presence of flavonoids, alkaloids, saponins, and tannins, which are known to possess antioxidant, anti-inflammatory, and antimicrobial activities. By day 10, the highest-dose formulation (F3) achieved a wound closure rate significantly higher than the diabetic control group ($p < 0.05$).Histological analysis showed increased collagen density (3.20 ± 0.38), fibroblast count (76.0 ± 5.99 cells/field), and angiogenesis score (15.73 ± 1.61) in the F3 group, compared to the diabetic control ($p < 0.05$). Serum analysis showed elevated VEGF and FGF-7, with decreased TNF-α in the F3 group versus diabetic control ($p < 0.05$). The extract also showed moderate antioxidant activity with an IC_{50} of 54.9 µg/mL. Although these outcomes suggest strong wound healing potential, they are based on preclinical data and do not establish clinical effectiveness. Additional research, including human clinical trials, is needed to confirm the formulation's therapeutic value.

Keywords | Cream, Diabetic, Extract, Lichen, Hyaluronic acid, Wound

Received | March 25, 2025; **Accepted** | June 13, 2025; **Published** | July 05, 2025
***Correspondence** | Erman Munir, Department of Biology, Faculty of Mathematic and Natural Sciences, Universitas Sumatera Utara, Medan 20155, Indonesia; **Email:** erman@usu.ac.id
Citation | Munir E, Nugraha SE, Suryanto D, Atni OK (2025). Enhanced wound closure and tissue regeneration in streptozotocin-induced diabetic rats treated with a topical Usnea longissima extract and hyaluronic acid cream. Adv. Anim. Vet. Sci. 13(8): 1651-1660.
DOI | https://dx.doi.org/10.17582/journal.aavs/2025/13.8.1651.1660
ISSN (Online) | 2307-8316; **ISSN (Print)** | 2309-3331

INTRODUCTION

Diabetes mellitus is generally a chronic metabolic disorder characterized by persistent hyperglycemia due to defects in insulin secretion, action, or both. It continues to be one of the major health burdens with increasing prevalence all over the world, including more than 537 million adults in 2021, and is expected to rise significantly over the next few decades (Sun *et al*., 2022). There are several complications associated with DM, impaired wound healing remains one of the important challenges (Nirenjen *et al*., 2023). Diabetic wounds, especially those from foot ulcers, continue to be the cause of great morbidity due to enhancing the likelihood of infection, amputation, and mortality rates in people with diabetes (Wukich *et al*., 2022). Mechanistically, impaired wound healing in the view of attenuated angiogenesis, extremely high oxidative stress, continuous inflammation, and microbial contamination are associated with such pathological wounds (Wang *et al*., 2023). Thus, with this, developing effective therapeutics that accelerates wound healing rate and lowering complication rates represents a growing therapeutic challenge that demands more targeted and multi-mechanistic interventions, particularly for diabetic foot ulcers that fail to respond to standard care (Burgess *et al*., 2021).

Numerous herbal-based interventions have been explored to improve wound healing in diabetic models. Extracts from plants such as *Curcuma longa, Azadirachta indica* and *Centella asiatica* have shown efficacy in accelerating wound repair due to their antioxidant, antimicrobial, and anti-inflammatory properties (Mishra, 2024). Moreover Herbal Medicine was clinically studied for the management of wound such as *Ypericum perforatum*, *Centella asiatica*, and *Calendula officinalis* (Ahmed *et al*., 2024). However, only limited studies have investigated lichen-derived compounds, particularly *Usnea longissima*, which contains a complex mixture of secondary metabolites, including flavonoids, tannins, and phenolic acids. Recent phytochemical analyses suggest that *Usnea* species possess promising bioactivities, but their application in topical diabetic wound formulations remains underexplored.

Usnea longissima Ach. is distributed widely across the temperate regions of the world and has been traditionally considered for its medicinal properties. Phytochemical studies revealed that it has a very rich bioactive profile, comprising flavonoids and phenolic compounds, with antioxidant, anti-inflammatory, and antimicrobial properties (Bharti and Nayaka, 2022; Sepahvand *et al*., 2021). These attributes provide a strong rationale for why it might be a potent candidate for the multifarious challenges posed by diabetic wound healing.

In parallel, hyaluronic acid (HA), a glycosaminoglycan naturally found in the extracellular matrix, is widely employed in wound dressings due to its hydrating, angiogenic, and anti-inflammatory properties (Marinho *et al*., 2021; Abatangelo *et al*., 2020). HA enhances fibroblast proliferation, angiogenesis, and extracellular matrix remodelling that beneficial for the effective repair of wounds (Shang *et al*., 2024; Polizzi *et al*., 2024). Recent investigations have highlighted the benefits of combining HA with other natural agents. For instance, Guan *et al*. (2024) demonstrated that tea polyphenol-modified injectable hyaluronic acid-based hydrogel significantly accelerated wound closure by inhibiting infection, diminishing oxidative stress, and promoting collagen deposition. These findings support the growing interest in multi-agent HA-based wound therapies for synergistic effects.

Although hyaluronic acid and Usnea longissima extract have shown individual potential in promoting wound healing through hydration, antioxidant, and antimicrobial mechanisms, their combined use in a topical formulation has not been previously studied in a diabetic wound context. Therefore, this study was conducted to evaluate the wound healing efficacy of a topical cream containing *Usnea longissima* extract and hyaluronic acid in streptozotocin-induced diabetic rats. The assessment included wound closure rate, histopathology, and inflammatory biomarkers. The research hypothesizes that the combination of *Usnea longissima* extract and hyaluronic acid in a topical cream formulation will significantly enhance diabetic wound healing, primarily through synergistic antioxidant, anti-inflammatory, and pro-angiogenic mechanisms.

METERIALS AND METHODS

Materials

The materials and tools used in this study include Lichen *Usnea longissima* (300 grams), Hyaluronic acid (Aogubio LLC-China) and 70% ethanol (3000 mL) (Merck). The formulation of creams involved the use of distilled water, Disodium EDTA (Merck), glycerin(Merck), propylene glycol (Merck), Carbopol Gel 1%(Merck), methyl paraben(Merck), glyceryl monostearate (Smart Lab), cetyl alcohol (Smart Lab), stearic acid (Smart Lab), Cremophor (Smart Lab), and stearyl alcohol (Smart Lab). Sterilized instruments, including tweezers, knives, and scissors, were used in the wound healing experiments. Histopathological observations were conducted using hematoxylin and eosin (H&E) staining solutions. For cytokine serum analysis, an ELISA microplate reader was employed alongside specific antibodies for VEGF, TNF-α, and FGF-7 (Solarbio). Additional materials included 0.1 M citrate buffer (pH 4.5), ketamine (50 mg/kg BW) and 0.9% w/v NaCl solution.

Sample Collection and Phytochemical Screening of *Usnea longissima* Extract

Usnea longissima, a type of lichen often found in high-altitude and humid environments, typically thrives in montane and subalpine forests. Sample of *Usnea longissima* were collected from Sibuatan Mountain area with an elevation of approximately 2,457 meters (8,061 feet) above sea level. Figure 1 shows a close-up of a thin tree branch with a cluster of Usnea longissima, a type of lichen, hanging from it. The lichen appears as a delicate, hair-like structure with a pale greenish-white color.

Figure 1: *Usnea longissima* Lichen sample.

The extraction of *Usnea longissima* was conducted utilizing the maceration method, as outlined in previous publications (Nugraha *et al*., 2024). The extraction was performed by macerating 300 g of dried Usnea longissima in 3000 mL of 70% ethanol at room temperature (~27°C) for 24 hours using a 1:10 solvent-to-sample ratio. The mixture was filtered, and the filtrate was concentrated using a rotary evaporator at reduced pressure. Phytochemical screening was performed to identify secondary metabolites, such as alkaloids, flavonoids, glycosides, tannins, saponins, and terpenoids/steroids (Bhardwaj *et al*., 2024). Furthermore, Gas Chromatography-Mass Spectrometry (GC-MS) investigations were conducted to identify certain bioactive chemicals employing standardized procedures (Pradhan *et al*., 2023).

Cream Formulation Preparation

The cream formulations combining *Usnea* extract and hyaluronic acid were prepared in three formulas (F1, F2, and F3) with varying concentrations of active ingredients, as shown in Table 1.

Phase A included distilled water as a solvent, Disodium EDTA as a chelator, Glycerin and Propylene Glycol as humectants, Carbopol Gel 1% as a thickener, and Methyl Paraben as a preservative. Phase B consisted of Glyceryl Monostearate and Cremophor as emulsifiers, Cetyl and Stearyl alcohols as stabilizers, and Stearic acid as an emollient. Phase C varied the amounts of *Usnea* extract (2.5–7.5 g) and hyaluronic acid (1.5 g), with higher concentrations in F2 and F3 expected to enhance antimicrobial, antioxidant, and moisturizing properties, improving skin hydration and elasticity. Furthermore, formula characteristics and stability also perform using standard procedure (Modi *et al*., 2024). The doses of *Usnea longissima* (2.5 g, 5 g, and 7.5 g) were selected based on preliminary range-finding experiments (Posobiec and Laffan, 2021).

Table 1: Cream Formula.

Phase	Composition	Master Formula (gram)		
		F1	F2	F3
Phase A	Distilled water	Q.s. 100	Q.s. 100	Q.s. 100
	Disodium EDTA	0.1	0.1	0.1
	Glycerin	3.0	3.0	3.0
	Propylene Glycol	5.0	5.0	5.0
	Carbopol Gel 1%	2.5	2.5	2.5
	Methyl Paraben	0.5	0.5	0.5
Phase B	Glyceryl Monostearate	2.0	2.0	2.0
	cetyl alcohol	4.0	4.0	4.0
	Steatic acid	1.0	1.0	1.0
	Cremophor	1.0	1.0	1.0
	Stearyl alcohol	1.0	1.0	1.0
Phase C	Usnea Extract	2.5	5.0	7.5
	Acid Hyaluronate	1.5	1.5	1.5

Wound Healing Experiment Procedure

The research was validated by animal research ethics committee of Universitas Sumatera Utara, with ethical approval no. 0828/KEPH-FMIPA/2024. The minimum number of animals per group was determined using the Federer formula and 30 rats were randomly allocated to six experimental groups using a computer-generated random number table to minimize selection bias. The following groups were (1) normal rats, (2) STZ-induced negative control rats without treatment, (3) Group 3 (positive control) received Bioplacenton® cream (0.1 g/day), a clinically used topical product containing neomycin sulfate and placenta extract, known for its tissue regenerative and antimicrobial properties, (4) STZ-induced rats treated with Formula 1 cream, (5) Formula 2 cream, and (6) Formula 3 cream. Streptozotocin (STZ) was administered intraperitoneally at a dose of 50 mg/kg BW in 0.1 M citrate buffer (pH 4.5). Blood glucose levels were monitored from day 1 to day 21 post-STZ induction, with diabetes defined as blood glucose levels exceeding 180 mg/dL (Igbashio *et al*., 2024). Following diabetes induction, the dorsal fur of each animal was shaved, and a standardized full-thickness excisional wound measuring 1 cm in diameter was created under anesthesia (ketamine, 50 mg/kg BW) using a sterile biopsy punch. The wound was made on the dorsal thoracic region to ensure uniform exposure and reduce variability due to

animal movement. The depth of the wound was controlled to include the complete removal of the epidermis and partial removal of the dermis, while avoiding penetration of the underlying muscle tissue (Greenwood *et al*., 2022). Creams were applied topically (0.1 g daily), and wounds were cleaned with 0.9% NaCl solution. Wound healing was monitored by measuring the wound diameter using a digital caliper (±0.01 mm accuracy) every day for 21 days. Furthermore, wound histological examination was conducted following standard hematoxylin and eosin (H&E) staining protocols and the levels of VEGF, TNF-α, and Fibroblast Growth Factor-7 (FGF-7) in blood rat serum were analyzed using an ELISA (Enzyme-Linked Immunosorbent Assay) Microplate Reader (Gondaliya *et al*., 2022).

Statistical Analysis

All data were first tested for normality using the Shapiro-Wilk test. One-way ANOVA was used for group comparisons, followed by Tukey's HSD post-hoc test ($p < 0.05$). All statistical analyses were performed using GraphPad Prism 9. The histogram data were constructed using GraphPad Prism Software 9.0 (Kim, 2017).

Table 2: Qualitative phytochemical screening result.

No.	Compounds	Reagent	Dried Sample	Extract
1	flavonoids	HCL(c), Mg powder. amil alcohol	+	+
2	alkaloids	Mayer	+	+
		Bouchardat	+	+
		Dragendorf	-	+
3	saponins	Foam test	+	+
4	tannins	FeCl3	+	+
6	steroids/terpenoid	Liberman Burchard	+	+

Description: (+): Present; (-): absence.

RESULT AND DISCUSSION

Results of Phytochemical Screening

The detected metabolites of the phytochemical constituents of the ethanol extract of *Usnea longissima* is presented in Table 2. Moreover, additional chemical detection is conducted using GCMS. Based on the GCMS analysis, a total of 10 compounds were detected. The result showed in Figure 2 and Table 3.

The phytochemical screening revealed bioactive compounds such as flavonoids, tannins, alkaloids, and saponins in *Usnea longissima*. Flavonoids are potent antioxidants that reduce oxidative stress, a major factor in diabetic wound healing impairment (Zulkefli *et al*., 2023). Tannins, known for their antimicrobial and astringent properties, promote tissue contraction and prevent infections (Huang *et al*., 2022). While this study focused on qualitative identification using GC-MS, quantitative analysis such as compound concentrations or peak area percentages was not conducted. Nevertheless, several of the identified constituents, including eugenol and n-hexadecanoic acid, are known in the literature for their roles in wound healing. Eugenol is reported to reduce inflammation by inhibiting pro-inflammatory cytokines such as TNF-α and IL-6, thereby promoting the proliferative phase (Mouro *et al*., 2019). Similarly, n-hexadecanoic acid has demonstrated antibacterial activity, supporting microbial control at the wound site and aiding re-epithelialization (Shaaban *et al*., 2021).

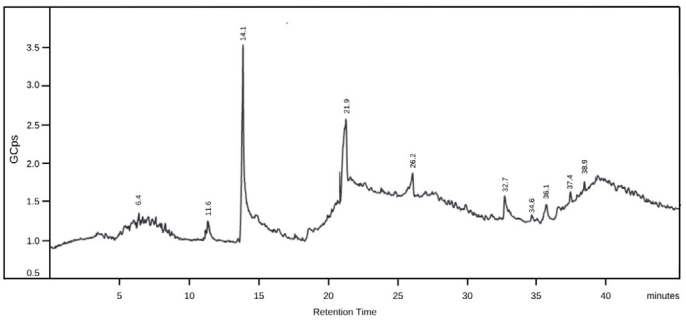

Figure 2: GCMS chromatogram of *Usnea longissima* extract.

Table 3: GCMS-phytochemical screening result.

No.	Retention time	Name	Molecular Formula	Molecular Weight (g/mol)
1	6,4863	Eugenol	$C_{10}H_{12}O_2$	164.20
2	11,6972	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42
3	14,1962	3-Buten-2-one, 3-methyl-4- (1,3,3-trimethyl-7-oxabicyclo [4.1.0]heptan-1-yl)-	$C_{14}H_{22}O_2$	222.32
4	21,9034	Tetrahydroxydioxotricosanoic acid	$C_{23}H_{46}O_2$	454.63
5	26,2865	β-Alectoronic acid	$C_{28}H_{32}O_9$	512.5
6	32,7465	Caperatic acid	$C_{21}H_{38}O_7$	402.5
7	34,6123	Hexadecadienoic acid	$C_{16}H_{28}O_2$	252.39
8	36,1151	Octasiloxane, hexadecamethyl-	$C_{16}H_{48}O_7Si_8$	577.2
9	31,4962	1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane	$C_{13}H_{39}O_5Si_6$	443.96
10	38,9327	Tetrasiloxane, decamethyl-	$C_{10}H_{30}O_3Si_4$	310.68

Antioxidant Analysis

The antioxidant activity of the sample was evaluated by calculating the percentage of inhibition at different concentrations. The results are presented in Table 4.

The relationship between the concentration and percentage inhibition was assessed using linear regression, and the

IC50 value was calculated. Table 3 shows that the percentage of inhibition increased with higher concentrations of the sample. At a concentration of 1000 µg/ml, the sample exhibited the highest inhibition percentage (80.452%), while the lowest percentage inhibition (45.526%) was observed at a concentration of 31.25 µg/ml. The IC_{50} value of the *Usnea longissima* extract was calculated to be 54.9 µg/mL, which indicates moderate antioxidant capacity. As a comparison, quercetin, used as the standard antioxidant in this study, exhibited a significantly lower IC_{50} value of 4.77 µg/mL, consistent with its well-established potent antioxidant properties.

Table 4: Antioxidant analysis result.

No	Sample	Concentration (µg/ml)	Corrected Sample Absorbance	% Inhibition	Linear Regression	IC50 (µg/ml)
1	*Usnea longissima* Ach. Extract	1000	0.075	80.452	y = 10,343x + 8,5693 R^2 = 0,9945	54.9
		500	0.1013	73.588		
		250	0.1343	64.987		
		125	0.164	57.255		
		62.5	0.189	50.738		
		31.25	0.209	45.526		
		0	0.403	0		
2	Quercetin Standard	100	0.0587	84.709	y = 12,044x + 31,161 R^2 = 0,9856	4.77
		50	0.0787	79.496		
		25	0.1077	71.937		
		12.5	0.1447	62.294		
		6.25	0.1900	50.478		
		3.125	0.2087	45.613		

Table 5: Physical and chemical stability evaluation results.

No	Sample	Organoleptic	Homogenity	pH	Viscosity (clycling test)	Separation test	Low temp. Storage	High temp. Storage
1	F1	Thick, brown colour	Homogen	6.43	547.0 cP	No	Stable	Stable
2	F2	Thick, brown colour	Homogen	6.38	563.5 cP	No	Stable	Stable
3	F3	Thick, brown colour	Homogen	6.31	582.6 cP	No	Stable	Stable

Cream Evaluation

The physical and chemical stability of the formulated cream samples (F1, F2, and F3) was assessed through organoleptic tests, homogeneity checks, pH measurements, viscosity analysis, and stability tests under varying conditions, including low- and high-temperature storage. The results are summarized in Table 5.

The stability of the cream formulations was assessed under accelerated and stress conditions by storing samples at low temperature (4 ± 2°C) and high temperature (40 ± 2°C) for a duration of four weeks. No phase separation, color change, or pH deviation was observed, indicating physical and chemical stability during storage. All formulations were thick, brown, and homogeneous, with no visible phase separation, indicating a uniform dispersion of ingredients. The pH values (6.31–6.43) were within the ideal range for skin compatibility, ensuring safety and minimal irritation. Viscosity varied slightly among F1, F2, and F3 (547.0–582.6 cP), all showing adequate thickness for good spreadability and stability. Stability testing confirmed robustness under low- and high-temperature storage, with no signs of separation or degradation. These results demonstrate the formulations' reliability, with F3's higher viscosity, which may enhance topical efficacy and retention on the wound site. Although in vivo wound application did not produce visible irritation in any group, formal dermal irritation or sensitization tests were not performed in this study. We acknowledge this as a limitation and plan to include standardized irritation and sensitization assays (e.g., Draize test) in future safety assessments.

Blood Sugar Examination

The effects of various treatments on blood sugar levels were evaluated over a 20-day period. The blood sugar levels of six groups were measured on day 1 and day 20, with the results presented in Table 6. Statistical significance was assessed in comparison to Group 2, which served as the untreated control group.

Table 6: Blood sugar evaluation results.

No	Group	Blood Sugar Day-1	Blood Sugar day-20
1	Group 1	109.8±9.0	126.8±25.2
2	Group 2	261.4±15.0*	272±15.4*
3	Group 3	358±112.7*	300.8±47.6*
4	Group 4	336±52.9*	338.8±47.6*
5	Group 5	276±38.7*	306.8±17.7*
6	Group 6	300.4±39.0*	312.2±56.6*

*Statistically significant at $p<0.05$ to Group1 (Normal Group).

The primary goal of maintaining diabetic conditions across all groups was achieved, except for Group 1, which exhibited blood sugar levels below the diabetic threshold. The treatments in Groups 3, 4, 5, and 6 effectively stabilized or reduced blood sugar levels while keeping the groups in a diabetic state, allowing for consistent comparisons.

Wound Dressing Evaluation

The wound healing process was visually evaluated over 10 days across six treatment groups, as shown in Figure 3. Im-

ages captured on days 1, 5, and 10 demonstrate the progression of wound closure. The results were further quantified by measuring wound diameters daily (Figure 4).

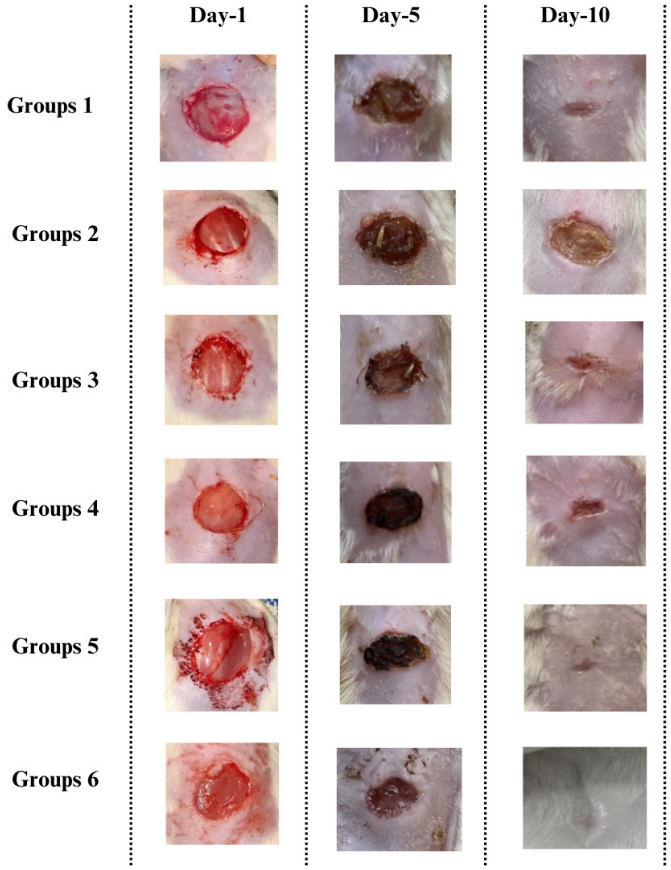

Figure 3: Wound visualization to day-10.

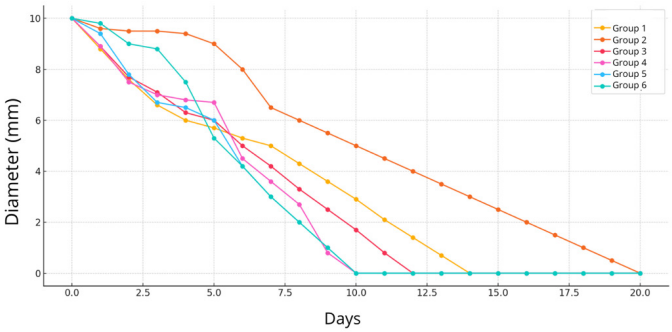

Figure 4: Wound diameter evaluation per day.

The observations indicate notable differences in wound healing across groups. On day 1, all groups exhibited open wounds of similar sizes. By day 5, Groups 4, 5, and 6 showed significant progress in wound contraction, with reduced wound diameters and less inflammation compared to Groups 1, 2, and 3. By day 10, Groups 5 and 6 demonstrated nearly complete wound closure, suggesting that these treatments were the most effective in promoting healing.

Histology Evaluation

The histological evaluation of wound tissues was conducted to assess the effects of treatments on collagen density, fibroblast numbers, and angiogenesis. Microscopic examination revealed significant differences between the treatment groups, as summarized in Table 7 and visualized in Figure 5.

Table 7: Histology examination score under 400× magnification levels.

NO	Group	Collagen Density Score (0-4)	Fibroblast number Cells per high-power field (HPF)	Angiogenesis (Number of blood vessels per high-power field (HPF)
1	Group 1	1.87 ± 0.32*	67.00 ± 8.49*	8.87 ± 2.47*
2	Group 2	1.47 ± 0.31	41.60 ± 7.09	3.60 ± 1.23
3	Group 3	2.20 ± 0.16*	67.00 ± 9.25*	8.87 ± 1.5*
4	Group 4	2.20 ± 0.15*	71.40 ±13.15*	13.33 ±1.15*
5	Group 5	2.80 ± 0.56*	65.60 ± 9.21*	15.33 ±1.61*
6	Group 6	3.20 ± 0.38*	76.00 ± 5.99*	15.73 ±1.61*

*Statistically significant at $p<0.05$ to Group2 (Untreated Group).

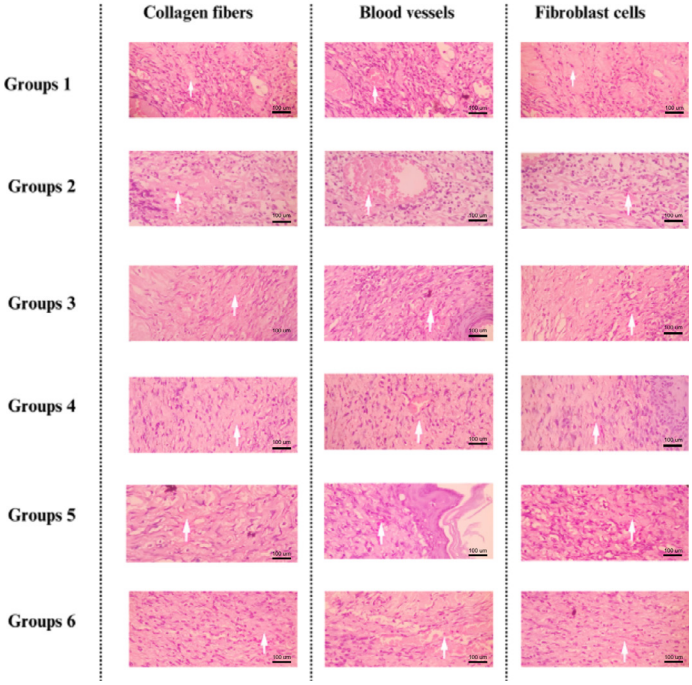

Figure 5: Microscopic examination on wound tissue (100× magnification).

Group 2, the untreated control, had the lowest scores across all parameters, reflecting minimal tissue regeneration and poor wound healing. In contrast, Groups 5 and 6 showed the most significant improvements, with Group 6 achieving the highest collagen density (3.20 ± 0.38), fibroblast number (76.00 ± 5.99), and angiogenesis (15.73 ± 1.61), indicating robust healing. Group 5 had similarly high scores, highlighting its effectiveness. Groups 3 and 4 also showed notable improvements over Group 2, with increased collagen density and angiogenesis, while Group 1 demonstrated moderate effects.

Serum Biochemical Evaluation

The serum levels of VEGF, TNF-α, and FGF-7 were evaluated to assess the biochemical response to different treatments. Figure 6 presents the data, showing significant differences between groups.

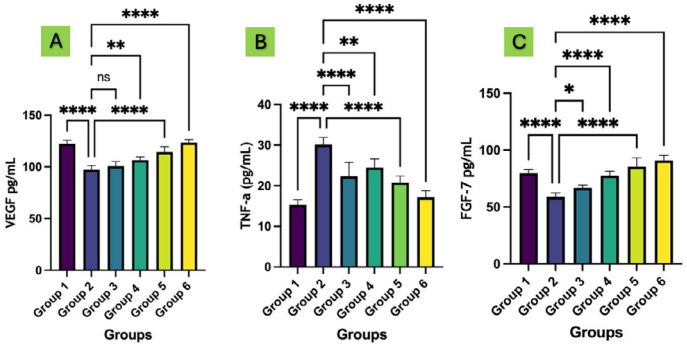

Figure 6: Serum biochemical evaluation, A. Serum VEGF; B. Serum Tnf-a; C. Serum FGF-7. Error bars represent mean ± SD (n = 5). Statistical significance determined by one-way ANOVA with Tukey's post-hoc test. *P ≤ 0.05, **P ≤ 0.01, *** P ≤ 0.001, ****P ≤ 0.0001 compared to the untreated diabetic control group; NS, not significant (P > 0.05).

The serum levels of VEGF, TNF-α, and FGF-7 were analyzed to assess the biochemical responses to different treatments, revealing significant differences between the groups (Figure 5). Group 6 exhibited the highest VEGF levels, significantly surpassing the untreated control groups (****P ≤ 0.0001), indicating enhanced angiogenesis. Groups 4 and 5 also showed significantly elevated VEGF levels (***P ≤ 0.001), while Groups 1, 2, and 3 demonstrated comparatively lower levels, with Group 2 (untreated control) being the lowest. TNF-α levels were significantly reduced in Groups 5 and 6 compared to the untreated control group (***P ≤ 0.001 and ****P ≤ 0.0001, respectively), suggesting strong anti-inflammatory effects. Groups 3 and 4 showed moderate reductions (**P ≤ 0.01), whereas Groups 2 exhibited the highest TNF-α levels, reflecting persistent inflammation as untreated control group. Similarly, FGF-7 levels were highest in Group 6 (****P ≤ 0.0001), indicating enhanced fibroblast activity and tissue regeneration. Groups 4 and 5 also demonstrated significantly elevated FGF-7 levels (***P ≤ 0.001), while Groups 1, 2, and 3 had lower levels, with the control group (Group 2) being the lowest. These results highlight the superior performance of treatments in Groups 5 and 6 in promoting angiogenesis, reducing inflammation, and enhancing tissue regeneration.

The treatment effects demonstrated a clear dose-dependent trend across multiple biological markers. In the VEGF profile (Figure 6A), levels increased progressively from Group 4 (low dose) to Group 6 (high dose), with Group 6 exhibiting the highest VEGF expression among all groups—indicating a dose-related pro-angiogenic response. Similarly, TNF-α levels (Figure 6B) showed a gradual decrease with increasing extract concentrations, suggesting a dose-dependent anti-inflammatory effect. Group 6 had significantly lower TNF-α levels compared to Group 2, the untreated diabetic control. FGF-7 levels (Figure 6C) also rose consistently from Group 4 to Group 6, supporting the hypothesis that higher concentrations of the cream formulation enhance fibroblast activity and tissue regeneration in a dose-responsive manner.

Our findings align with earlier research on natural product-based therapies for diabetic wound healing. For example, Zhang *et al.* (2021) reported that plant-based antioxidants can suppress pro-inflammatory cytokines like TNF-α and accelerate tissue regeneration, which mirrors our results showing TNF-α reduction and FGF-7 enhancement in treated HA-Lichen groups. Similarly, Subramanian *et al.* (2023) highlighted flavonoid-rich extracts improving angiogenesis and collagen deposition outcomes we also observed with *Usnea longissima*. However, there were limitied studies have examined lichen-derived compounds, and this research contributes novel data regarding the therapeutic potential of *Usnea longissima* in wound healing.

The cream formulations, particularly F3, significantly improved wound healing outcomes. By day 10, Groups 5 and 6 exhibited near-complete wound closure. This aligns with findings by Accipe *et al.* (2023), who reported accelerated wound closure through antioxidant and anti-inflammatory effects of plant-based extracts. Hyaluronic acid plays a complementary role by improving hydration, extracellular matrix remodeling, and fibroblast migration (Valachová *et al.*, 2022). Together, these mechanisms underpin the superior performance of the *Usnea longissima*-HA cream.

Histological analyses revealed increased collagen deposition, angiogenesis, and fibroblast proliferation in the treated groups. Collagen deposition reflects effective extracellular matrix remodeling, which strengthens wound integrity (Mathew-Steiner *et al.*, 2021) Additionally, enhanced angiogenesis, marked by elevated VEGF levels, improves vascularization critical for wound repair (Moreira and Marques, 2022). Similar findings were observed by Radomska-Leśniewska *et al.* (2017), highlighting natural antioxidants' ability to upregulate VEGF expression. The observed improvements in wound healing particularly the enhanced collagen density, fibroblast activity, and VEGF levels suggest accelerated tissue regeneration and angiogenesis in the preclinical setting, which may have translational relevance for managing chronic ulcers in diabetic patients, pending further validation in human studies.

The cream formulations significantly reduced TNF-α levels, reflecting strong anti-inflammatory effects. Chronic inflammation delays the proliferation phase of healing by

sustaining elevated pro-inflammatory cytokines such as TNF-α (Chae *et al*., 2011). These results align with studies demonstrating that plant-based antioxidants suppress pro-inflammatory cytokines to facilitate faster healing (Chae *et al*., 2011).

The synergy between *Usnea longissima* and HA is evident in the enhanced outcomes. HA contributes to cell proliferation and angiogenesis, while *Usnea longissima* reduces oxidative stress and microbial contamination. Similar synergistic effects have been reported in formulations combining natural extracts with HA (Juncan *et al*., 2021). This dual mechanism makes the cream a promising alternative to conventional treatments such as Bioplacenton, which primarily targets infection control but lacks significant antioxidant activity. Furthermore, the GC-MS screening revealed the presence of eugenol, a compound known for its wound healing and anti-inflammatory properties (Mouro *et al*., 2019). In addition, hexadecanoic acid, also identified in the extract, possesses antibacterial activity that may further support the wound healing process (Shaaban *et al*., 2021). Additionally, the elevated FGF-7 levels in Groups 5 and 6 confirm the role of the cream in stimulating tissue regeneration. The stability and skin compatibility of the formulations further underscore their potential for real-world application.

In addition to aligning with earlier findings on natural agents for wound healing, the study contributes distinct insights by focusing on the topical use of *Usnea longissima*. While many studies report favorable effects of plant-based treatments, conflicting evidence exists regarding the cytotoxicity of lichen-derived compounds such as usnic acid at higher doses or through systemic administration (Mariraj *et al*., 2025). Our results did not show adverse effects, likely due to the topical route and optimized dose. Moreover, although the antioxidant activity was moderate (IC_{50} = 54.9 µg/mL), the significant improvement in histological parameters suggests that even modest oxidative stress reduction, when combined with hyaluronic acid's regenerative properties, can produce therapeutic effects. These findings underscore the importance of formulation synergy and indicate the need for further mechanistic studies and comparative trials with other plant-based therapies.

Despite promising findings, this study has several limitations. First, although no visible skin irritation was observed during the treatment period, formal dermal safety assessments, such as skin sensitization or Draize irritation tests, were not performed. These tests are essential for regulatory approval and to fully evaluate the topical tolerability of the formulation. Second, this study did not include any pharmacokinetic (PK) evaluation of the cream formulation. The extent of cutaneous absorption, bioavailability of active compounds, and potential systemic exposure remain unknown. Such data are critical for defining safe and effective dosing, particularly for compounds like usnic acid, which may pose toxicity risks at higher systemic concentrations. Third, this work was conducted exclusively in rats, which exhibit faster wound contraction due to panniculus carnosus muscle a process that differs from human re-epithelialization. Therefore, future studies using large animal models or ex vivo human skin systems may provide better translational insight. Finally, this study did not examine long-term wound remodeling, scar formation, or recurrence, which are relevant to the chronic nature of diabetic wounds. Addressing these gaps in follow-up studies will strengthen the clinical potential of the formulation.

CONCLUSIONS AND RECOMMENDATIONS

This preclinical study demonstrates that a topical cream combining *Usnea longissima* extract and hyaluronic acid improves diabetic wound healing in rats through antioxidant, anti-inflammatory, and pro-angiogenic effects. Among the tested formulations, the highest dose (F3) showed the most significant improvement in wound closure, tissue regeneration, and cytokine modulation. While these findings are promising, they are limited to an animal model and should not yet be interpreted as clinical evidence of therapeutic efficacy. Further research is warranted to explore pharmacokinetic properties, perform longer-term safety studies, and validate these results in advanced preclinical models and human clinical trials to assess translatability and optimize dosing.

ACKNOWLEDGMENTS

We would like to express our profound gratitude to the Universitas Sumatera Utara, and Cendikia Lab for all facilities and means during the research. This research received funding from the Directorate of Research, Technology, and Community Service, Ministry of Education, Culture, Research, and Technology, Indonesia, under contract numbers 093/E5/PG.02.00.PL/2024 and 12/UN5.4.10.S/PPM/KP-DRTPM/2024.

NOVELTY STATEMENTS

This research represents the first investigation of a topical formulation integrating *Usnea longissima* extract with hyaluronic acid for the treatment of diabetic wounds. The combined formulation demonstrated a significant improvement in wound closure, attenuation of inflammatory responses, and enhancement of tissue regeneration in streptozotocin-induced diabetic rats, highlighting its potential as a synergistic therapeutic approach.

AUTHOR'S CONTRIBUTIONS

Erman Munir conceptualized the study, supervised the research, and reviewed the manuscript. Sony Eka Nugraha contributed to the experimental design, data collection, and manuscript writing. Dwi Suryanto assisted with histological analysis and data interpretation. Oky Kusuma Atni conducted phytochemical and antioxidant evaluations and contributed to data analysis. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that they have no conflicts of interest related to this research.

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