



Therapeutic Effect of Red Ginger Leaf Ethyl Acetate Fraction Gel on *Staphylococcus aureus*-Infected Excision Wounds on Rat: A Preclinical Study

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Abstract | A wound is an injury caused by a disruption in the body's protective barriers resulting in damage to a specific area of the skin. Insufficient wound care may lead to further complications such as wound infections. *Staphylococcus aureus* is a pathogenic bacterium capable of causing wound infections. This study aimed to evaluate the effectiveness of gel formulations containing the ethyl acetate fraction obtained from red ginger leaves in facilitating the healing process of excision wounds. The study utilized 30 male Wistar rats, divided into six groups with five rats each, to ensure robust statistical analysis. The ethyl acetate fraction was extracted using a detailed maceration and fractionation process with 96% ethanol, followed by formulation into a gel. The gel formulations were evaluated for their antimicrobial properties and effectiveness in facilitating wound healing. The findings demonstrated that gel formulations containing 1%, 3%, and 5% concentrations of red ginger leaf extract exhibited significant antibacterial activity against *Staphylococcus aureus*, with the 5% concentration showing the largest zone of inhibition (11.20 ± 0.30 mm). Histological analysis revealed that the 5% gel concentration significantly enhanced epidermal thickness ($52,407.77 \pm 1.34$ nm) and promoted angiogenesis (26.33 ± 0.31) in the wound area. These results indicate that the 5% ethyl acetate fraction gel is the most effective in promoting wound healing by enhancing both epidermal regeneration and angiogenesis.

Keywords | Antibacterial, Ethyl acetate, Fraction, Gel, *Staphylococcus aureus*, Wound

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INTRODUCTION

A wound refers to an interruption in the typical arrangement and operation of the skin and underlying tissues.

Possible causes of this condition include trauma, surgical procedures, animal bites or scratches, and contact with corrosive substances (Tottoli *et al.*, 2020). When a wound arises, it can cause physiological disruptions in the afflict-

ed area, resulting in discomfort, edema, and the possibility of infection (Malone and Schultz, 2022). Effective wound management is crucial for avoiding problems and facilitating the healing process. This entails the act of cleansing the wound, using appropriate coverings, and occasionally utilizing medications or therapies to facilitate the healing procedure (Jose *et al.*, 2023). The selection of treatment is contingent upon the degree, placement, and root cause of the wound. The primary goal of wound management is to reduce complications, relieve pain, and expedite the restoration of normal function in the affected area (Eriksson *et al.*, 2022). Advanced treatments may include surgical procedures, administration of growth factors, or other specialist therapy to improve healing and minimize the likelihood of long-lasting wounds. It is essential to treat both the wound itself and any underlying disorders that could hinder the healing process in order to achieve a good recovery and restore the skin's integrity.

Insufficient wound care may lead to further complications, such as wound infections. *Staphylococcus aureus* is a pathogenic bacterium capable of causing wound infections (Cheung *et al.*, 2021). Untreated infections induced by these bacteria can progress into chronic wounds and result in the formation of biofilms composed of bacterial toxins, which are hazardous substances generated by the bacteria (Vestby *et al.*, 2020). The infection can be classified as either systemic, which indicates that it impacts the entire body, or localized, which indicates that it is confined to the specific place where the infectious agent is present. An α -Toxin possesses the capacity to induce the demise of host cells, particularly macrophages situated in the perivascular area of the skin (Olaniyi *et al.*, 2018). This hinders or slows down the process of recruiting polymorphonuclear cells. The impaired migration of polymorphonuclear cells to the perivascular space hampers the synthesis of hydrogen peroxide, which is essential for the immune response to infection in the wound (Raziyeva *et al.*, 2021). In the event of a failure occurring during the inflammatory phase, it will result in a prolonged inflammatory phase, subsequently leading to the development of a chronic lesion. Chronic wounds produce reactive oxygen species (ROS) that destroy extracellular matrix proteins and cause cellular injury (Dryden, 2020).

The purpose of drug utilization in wound treatment is to accelerate the wound healing process. There is the choice to provide either pharmaceutical or conventional remedies. The difference lies in the fact that extended utilization of chemical drugs may result in the emergence of resistance and provide a greater likelihood of negative consequences in contrast to conventional therapy. Empirically, Indonesians use red ginger leaf (*Zingiber officinale* Var. *Rubrum*) as a natural medicine for wound healing. Recent studies have shown its effectiveness in accelerating perineal wound

healing in postpartum mothers (Idayanti and Anggaraeni, 2022; Simarmata, 2022). The plant's potential for wound healing is attributed to its antioxidant, antimicrobial, and anti-inflammatory properties (Kanedi *et al.*, 2022). The substance is pulverized into a fine powder and thereafter administered directly onto the affected area. The foliage of red ginger comprises metabolite constituents including alkaloids, flavonoids, tannins, saponins, and steroids (Dania *et al.*, 2024; Yuandani *et al.*, 2023). These chemicals possess the ability to accelerate wound healing and prevent infection by impeding the proliferation of microorganisms.

Gel preparation is a highly recommended dosage form for wound healing since it has the ability to equally spread over the skin's surface and provide a soothing and painless feeling when applied. Previous studies have shown that red ginger leaf extract has significant antibacterial and wound healing properties (Aleem *et al.*, 2020; Wang *et al.*, 2020). However, there is limited research on the formulation of red ginger leaf extract into a gel for topical application. This study hypothesizes that a gel formulation containing the ethyl acetate fraction of red ginger leaves can enhance the wound healing process of excision wounds contaminated with *Staphylococcus aureus* by leveraging its antibacterial and anti-inflammatory properties.

MATERIALS AND METHODS

TOOLS AND MATERIALS

The following tools were used in the investigation: paper disc, scalpel, Fisherbrand Elite 10-100 μ l micropipette, Mettler Toledo electric balance, Astec HLF 1200L[®] laminar air flow cabinet, Pyrex glassware, and Z-2000 Hitachi[®] atomic absorption spectrophotometer. The components used in the study were red ginger leaves, a culture of the bacteria *Staphylococcus aureus*, dimethylsulfoxide (Merck[®]), ethanol (Merck[®]), ethylacetate (Merck[®]), glycerin, HPMC, methyl paraben, and TEA.

EXTRACTION AND FRACTIONATION PROCESS OF RED GINGER LEAF

First, Red Ginger Leaf extract was made by maceration using 96% ethanol. The dried sample was placed in a dark container, covered with 75 parts of the liquid filter, and left for 5 days while stirring frequently. After shredding and squeezing, the dregs were washed with the liquid filter. The solution was transferred to a closed vessel and left in a cool, dark place for 2 days. The clear liquid was poured off to avoid wasting the sediment. The macerate was evaporated using a rotary evaporator at $\pm 40^{\circ}\text{C}$ until a thick extract was obtained. The ethyl acetate fraction was made by dissolving 100 g of the thick extract in a mixture of 96% ethanol and distilled water (2.5:7.5). This solution underwent three rounds of extraction with 100 ml of ethyl acetate solvent each

time in a separating funnel. The mixture was filtered, and the filtrate was concentrated using a rotary evaporator at 40°C to obtain the ethyl acetate fraction. (Marianne *et al.*, 2021; Elebiyo *et al.*, 2023).

PHYTOCHEMICAL ASSESSMENT

Qualitative approaches were employed to identify the presence of various phytochemicals, including alkaloids, flavonoids, glycosides, tannins, saponins, triterpenoids, and steroids. The flavonoids were identified using a reagent consisting of concentrated hydrochloric acid, magnesium powder, and amyl alcohol. The alkaloids were identified using Mayer's, Bouchardat's, and Dragendorff's reagents, respectively. The foam test was employed to detect the presence of saponins. Tannins were detected by employing ferric chloride as a reagent, whereas the existence of steroids and terpenoids was assessed using the Liebermann-Burchard reagent (Sharma and Kaushik, 2021). Furthermore, a thin layer chromatography were use to profiling the phytochemical content, then the chromatogram were analyzed under UV 366.

GEL FORMULATION OF ETHYL ACETATE FRACTION OF RED GINGER LEAF

Three gel formulations were developed using the ethyl acetate fraction of red ginger leaves, all based on the same gel base formula. The modified gel preparation formula for the ethyl acetate fraction of red ginger leaves is detailed in Table 1 (Awaluddin *et al.*, 2020).

Table 1: Gel Formulation.

Material	Formula Concentration (%)		
	F1	F2	F3
Red Ginger Leaf Ethyl Acetate Fraction	1	3	5
HPMC	10	10	10
Propylenglycol	10	10	10
Triethanolamine	5	5	5
Glycerin	10	10	10
Distilled water	ad 100 ml	ad 100 ml	ad 100 ml

The gel formulation of the ethyl acetate fraction of red ginger leaves involved a multi-step process. Initially, hydroxypropyl methylcellulose (HPMC) was dispersed in 60 ml of hot distilled water and then homogenized to form Phase A. In the subsequent step, Phase B was prepared by combining the ethyl acetate fraction of red ginger leaves with propylene glycol, glycerin, and triethanolamine, followed by thorough homogenization. The two phases were then blended in a mortar and stirred continuously until a homogeneous mixture was achieved. The resulting gel formulation was subjected to a series of evaluations to ensure its quality and effectiveness. These evaluations included sensory properties assessment, which examined the gel's

appearance, texture, and odor; viscosity measurement to determine the gel's thickness and flow characteristics; uniformity analysis to ensure consistent distribution of the active ingredients throughout the gel; pH determination to measure the acidity or alkalinity of the gel; spreadability examination to assess the ease with which the gel can be spread on a surface; and stability evaluation to test the gel's stability under various temperature conditions, including ambient, low, and high temperatures. These tests were conducted to verify the formulation's consistency, stability, and suitability for use (Ansong *et al.*, 2023).

CONTROL VARIABLES

Control variables included temperature, sterilization, and animal housing. All extractions and gel formulations were performed at room temperature unless specified. Sterilization measures were strictly adhered to, with all equipment and media being sterilized before use to prevent contamination. Animal housing conditions were carefully controlled, maintaining a 12-hour light/dark cycle, a constant temperature of 22°C ± 2°C, and humidity levels at 55% ± 10%.

EVALUATION OF ANTIBACTERIAL ACTIVITY RED GINGER LEAF ETHYL ACETATE FRACTION GEL AGAINST *STAPHYLOCOCCUS AUREUS*.

The antibacterial activity of the gel formulations was assessed using the paper disk diffusion method, specifically tailored to evaluate efficacy against *Staphylococcus aureus*. A single colony of *Staphylococcus aureus* ATCC 25923 was inoculated into 10 mL of sterile nutrient broth and incubated overnight at 37°C. The bacterial culture was standardized to a 0.5 McFarland standard using sterile saline solution. Mueller-Hinton agar (MHA) plates were prepared by pouring 20 mL of sterile molten MHA into sterile Petri dishes and allowing them to solidify. Sterile paper disks (6 mm in diameter) were impregnated with 100 µL of gel formulations at concentrations of 1%, 3%, and 5% ethyl acetate fraction of red ginger leaves. Positive control disks were impregnated with Voltaren Gel, and negative control disks with dimethyl sulfoxide (DMSO). The standardized bacterial inoculum was spread evenly over the MHA plates using a sterile cotton swab. The impregnated paper disks were then placed on the inoculated MHA plates, ensuring even spacing. The plates were incubated at 37°C for 24 hours. After incubation, the diameter of the inhibition zones around each disk was measured using a digital caliper, recorded in millimeters (mm), and averaged over three independent experiments. The results were compared with those of the positive and negative controls to determine the gel formulations' efficacy (Patil *et al.*, 2020).

TREATMENT REGIME

The efficacy of incision wound treatments was evaluated using male white rats (*Rattus norvegicus* L.), aged 2–3 months, with a body weight of 200–250 grams. A total of 30 rats were divided into six groups as follows:

- Group 1: Infected wound without treatment
- Group 2: Infected wound with gelbase treatment
- Group 3: Positive control group (Infected wound with Voltaren Gel treatment)
- Group 4: Infected wound with F1 gel treatment
- Group 5: Infected wound with F2 gel treatment
- Group 6: Infected wound with F3 gel treatment

Each test animal had the hair removed from its back and was anesthetized with ketamine. Incisions were made using a 10-mm biopsy punch. The wounds were sterilized with a sodium chloride (NaCl) solution and inoculated with 1 milliliter of *Staphylococcus aureus* culture (Liang *et al.*, 2024). After 24 hours, bacterial contamination was observed, indicated by inflammation and erythema. Each group received specific treatments according to their test variables, and wound healing was assessed daily over 21 days. Histological examinations were conducted to evaluate angiogenesis and the development of the epidermal layer in the wound tissue. The wound perimeter was measured using high-resolution photographs, and the Macbiohotonics Image J software was used for quantitative analysis. Furthermore, The blood plasma samples obtained will then be examined TNF- α concentration using the Sandwich ELISA method. TNF- α specific antibody has been prepared on 96 well plate and calculate using microplate reader.

WOUND SIZE MEASUREMENT

Initially, the rats' backs were shaved, and a sterile biopsy punch created a 10 mm diameter full-thickness wound. Immediately after, high-resolution digital photographs were taken from a consistent distance and angle. Daily, wounds were cleaned with sterile saline, and photographs were taken with consistent lighting and positioning, using a ruler or scale for size calibration. Wound area measurement was conducted using ImageJ software (version 1.53c, National Institutes of Health, USA). The software was calibrated using the ruler or scale in each image to ensure consistent measurements. A blinded researcher manually traced the wound margins in the software to avoid measurement bias, and the traced area was calculated to determine the wound size in square millimeters (mm²) (Suarez-Arnedo *et al.*, 2020).

HISTOLOGICAL EXAMINATIONS

On day 21 post-injury, skin tissue samples were collected from each treatment group and immediately fixed in 10% neutral-buffered formalin to preserve tissue structure. The fixed tissues were processed through a series of alcohol and xylene baths and embedded in paraffin wax. Paraffin-embedded tissue blocks were sectioned at a thickness of 5 micrometers using a rotary microtome, and sections were stained with hematoxylin and eosin (HE) to assess overall tissue structure and angiogenesis. For quantitative analysis, stained sections were examined under a high-resolution

digital microscope equipped with a camera. Images were captured at 200x magnification to visualize blood vessels in detail. The number of blood vessels per high-power field (HPF) was counted manually. Additionally, epidermal regeneration also observed (Huang *et al.*, 2020).

ETHICAL CLEARANCE

The utilization and management of experimental animals in research laboratories adhere to the ethical guidelines for animal research outlined in the Declaration of Helsinki. Additionally, ethical clearance has been obtained from the animal research ethics committee of Universitas Sumatera Utara, with approval number: 0599/KEPH-MIPA/2023.

STATISTICAL ANALYSIS

The in vivo results were analyzed using ANOVA with Tukey's Multiple Comparison Test. P-values for significance were set at $P < 0.05$. Values for all measurements are expressed as mean $\pm$ SD. The statistical analysis was conducted using GraphPad Prism Software 9.0 to ensure the reliability and accuracy of the data interpretation. Detailed statistical methods were applied to assess the significance of differences between groups, ensuring robust analysis and interpretation of the data.

RESULTS AND DISCUSSION

PHYTOCHEMICAL SCREENING

Phytochemical screening is a qualitative test that is used as an initial step for ginger leaf extract. The purpose of phytochemical screening is to determine the secondary metabolites contained in extract and fraction of ginger leaf. The results of phytochemical screening can be seen in Table 2, 3 and Figure 1.

Table 2 shows the presence of various compounds in the ethanol extract, n-hexane fraction, ethyl acetate fraction, and residual fraction of red ginger leaves. Alkaloids were found in the ethanol extract, ethyl acetate fraction, and residual fraction, but not in the n-hexane fraction. This suggests the potential pharmacological value of red ginger leaves, as alkaloids are known for their analgesic and antibacterial properties (Aleem *et al.*, 2020). Terpenoids, detected in the ethanol extract and n-hexane fraction. The solubility of terpenoids in non-polar solvents like n-hexane explains their presence in this fraction. Tannins were found in the ethanol extract, ethyl acetate fraction, and residual fraction, known for their antioxidant and anti-inflammatory activities. This comprehensive screening highlights the diverse bioactive compounds present in red ginger leaves, indicating their potential therapeutic applications. In addition, the existence of these chemicals is further confirmed using Thin-layer chromatography (TLC) examination (Figure 1) and the determination of R_f values (Table 3). The unique R_f values observed under UV-366 nm and after spraying

Table 2: Phytochemical screening results of ethanol extract. *n*-Hexan fraction. ethyl acetate fraction and residual fraction of red ginger leaves .

No.	Secondary Metabolites	Ethanol Extract	N-Hexane fraction	Ethyl acetate fraction	Residual Fraction
1.	Alkaloids	+	-	+	+
2.	Terpenoids	+	+	-	-
3.	Tannins	+	-	+	+
4.	Flavonoids	+	-	+	-
5.	Saponins	+	-	-	+
6.	Glycosides	+	-	+	-

(+): contains a class of compounds; (-): does not contain the compound class.

Table 3: Results of Rf values of ethanol extract; *n*-hexane fraction; ethyl acetate fraction and residual fraction of red ginger leaves.

Rf Value of Ethanol Extract		Rf value of <i>n</i> -hexane fraction		Rf value of Ethyl Acetate fraction		Rf value of Residual fraction	
UV -366	After spraying	UV -366	After spraying	UV -366	After spraying	UV -366	After spraying
0.3	-	0.3	0.3	0.1	0.1	0.1	-
0.4	-	0.5	0.5	0.3	0.3		
0.8	-	0.6	0.6	0.5	0.5		
0.8	-	0.7	0.7	0.6	0.6		
	-	0.8	0.8	0.7	0.7		
				0.8	0.8		

provide evidence for the identification and differentiation of phytochemicals in the various fractions. It has been demonstrated that the Ethyl Acetate fraction exhibits a larger chromatogram spot compared to the other extract. An in-depth analysis of the phytochemical composition of red ginger leaves is essential for comprehending the therapeutic capabilities they possess.

GEL EVALUATION

The research involved a comprehensive evaluation of gel formulations to ensure their suitability for commercialization and widespread adoption. The evaluation tests conducted included viscosity measurement, homogeneity assessment, pH determination, spreadability examination, and stability testing at various temperatures (room temperature, low temperature, and high temperature) (Ansong *et al.*, 2023). A three-month trial period was conducted for the gel formulations, with weekly measurements averaged to determine the evaluation findings. The results of the gel preparation evaluation test for the ethyl acetate fraction of red ginger leaves are presented in Table 4.

The homogeneity test aims to ensure the uniform distribution of components within the gel mixture. A homogeneous gel is characterized by its smooth consistency, absence of lumps, and even color throughout its application. The results of the homogeneity test for the gel formulation of the ethyl acetate fraction of red ginger leaves demonstrated uniformity, with no coarse particles detected during the test.

The pH test evaluates the safety of the gel formulation by determining its potential to cause skin irritation

(Hayat *et al.*, 2023). Gels with a low or acidic pH can irritate the skin, while those with a high pH can cause

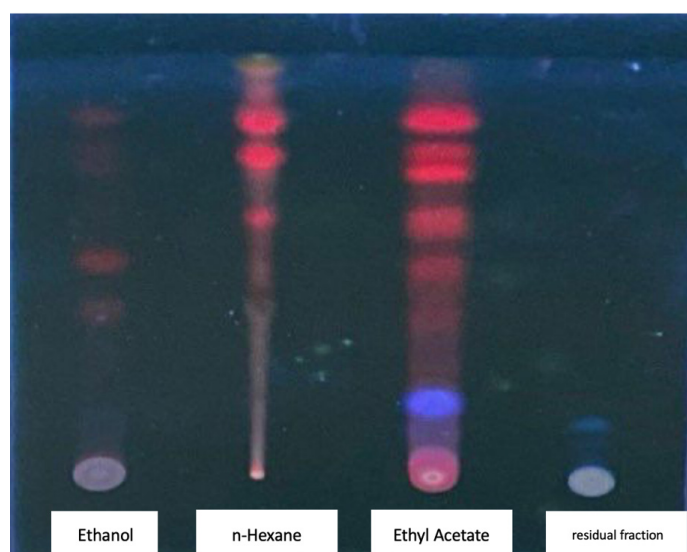


Figure 1: Thin Layer Chromatogram in UV 366.

dryness (Lukić *et al.*, 2021). Topical medications must maintain specific pH standards to ensure safety and efficacy. Viscosity testing measures the exact viscosity of the gel. Higher viscosity values correspond to thicker preparations. According to the Indonesian National Standard (SNI), the gel should have a viscosity between 2,000 cp and 50,000 cp (Sammulia *et al.*, 2023). The test results indicated that all gel formulations met the required viscosity criteria. Finally, the spreadability test, which is significantly influenced by viscosity, determines how easily the gel spreads. Higher viscosity results in a smaller spread

Table 4: Gel preparation evaluation results of Ethyl acetate fraction of red ginger leaves.

Evaluation Testing	Evaluation Results Average Measurement $\pm$ SD		
	F1	F2	F3
Homogeneity	Homogeneous	Homogeneous	Homogeneous
pH	6.41 $\pm$ 0.01	6.39 $\pm$ 0.01	6.35 $\pm$ 0.00
Viscosity	1714.4 $\pm$ 1.23	1730.9 $\pm$ 0.09	1905.7 $\pm$ 0.90
Spreadability	6.3 $\pm$ 0.02	6.2 $\pm$ 0.04	5.9 $\pm$ 0.06
Sensory Properties	Smooth, non-greasy, cooling sensation	Smooth, non-greasy, cooling sensation	Smooth, non-greasy, cooling sensation

Table 5: Results of the gel stability test for the ethyl acetate fraction of red ginger leaves.

Testing	Gel Formula	Evaluation				
		Homogeneity	pH	Viscosity (cps)	Spreadability(cm)	Sensory Properties
Room Temperature	F1	Homogen	6.35 $\pm$ 0.01	1008.6 $\pm$ 0.04	7.0 $\pm$ 0.12	Smooth, non-greasy, cooling sensation
	F2	Homogen	6.06 $\pm$ 0.01	1299.4 $\pm$ 0.01	6.3 $\pm$ 0.08	Smooth, non-greasy, cooling sensation
	F3	Homogen	5.80 $\pm$ 0.01	1343.8 $\pm$ 0.08	6.4 $\pm$ 0.45	Smooth, non-greasy, cooling sensation
Cold Temperature	F1	Homogen	6.20 $\pm$ 0.01	1018.1 $\pm$ 0.29	7.5 $\pm$ 0.08	Smooth, non-greasy, cooling sensation
	F2	Homogen	6.16 $\pm$ 0.01	1368.0 $\pm$ 0.46	7.2 $\pm$ 0.14	Smooth, non-greasy, cooling sensation
	F3	Homogen	5.91 $\pm$ 0.01	1388.6 $\pm$ 0.33	6.5 $\pm$ 0.57	Smooth, non-greasy, cooling sensation
Heat Temperature	F1	Homogen	6.11 $\pm$ 0.01	946.6 $\pm$ 0.98	7.0 $\pm$ 0.12	Smooth, non-greasy, cooling sensation
	F2	Homogen	6.08 $\pm$ 0.01	1254.1 $\pm$ 0.67	8.2 $\pm$ 0.24	Smooth, non-greasy, cooling sensation
	F3	Homogen	6.03 $\pm$ 0.02	1262.9 $\pm$ 0.47	8.8 $\pm$ 0.63	Smooth, non-greasy, cooling sensation

diameter, while lower viscosity leads to a larger spread diameter. Additionally, The sensory properties of the gel formulations were assessed for texture, feel, and user acceptance. The gels were smooth, non-greasy, and provided a cooling sensation upon application, enhancing overall user comfort and satisfaction. These pleasant sensory attributes are crucial for the potential commercial success of the product.

GEL STABILITY TEST

Stability testing of formulations was carried out to evaluate their ability to maintain quality over the storage period. This testing involves storing the formulations at various temperatures (room temperature, cold temperature, and hot temperature) and assessing their homogeneity, pH, viscosity, and spreadability over a period of three months. The results of the stability evaluation are presented in Table 5. The test findings indicate that the preparation maintained its quality standards throughout the three-month evaluation period. An ideal gel pH should closely match the skin's pH, typically between 4.5 and 6.5 (Lukić *et al.*, 2021). The one-way ANOVA test revealed a pH significance result of 0.405 (>0.05) during storage, suggesting no statistically significant difference in pH over time. The spreadability test indicating no significant variation in spreadability. Increasing the ethyl acetate content in red ginger leaves enhances the preparation's distribution. Homogeneity testing showed a p-value of 0.503 (>0.05), confirming the formulas' homogeneity in the viscosity test. The one-way ANOVA test for viscosity had a significance value of 0.065 (<0.05),

indicating no significant difference in the average viscosity test results, thus satisfying the homogeneity assumption. The stability of the gel formulations over extended periods and under different storage conditions was not thoroughly evaluated. Future studies should include long-term stability tests to ensure the consistency and effectiveness of the gel during storage and use.

ANTIBACTERIAL ACTIVITY OF GEL PREPARATION AGAINST *STAPHYLOCOCCUS AUREUS*

The research conducted an initial test to evaluate the antibacterial efficacy of gel formulations, focusing on the inhibition of bacterial growth in wounds, specifically targeting *Staphylococcus aureus*. This involved testing the antibacterial activity of gel formulations containing the ethyl acetate fraction of red ginger leaves (*Zingiber officinale* var. *rubrum*). The results of the antibacterial efficacy against *Staphylococcus aureus* are presented in Figure 2.

The 5% ethyl acetate fraction gel exhibited a significantly larger inhibition zone compared to the Voltaren Gel, indicating superior antibacterial activity. The increased zone of inhibition suggests that the red ginger leaf extract has potent antibacterial properties that are more effective than the conventional treatment. The lack of inhibition by DMSO confirms that the observed antibacterial activity is due to the active compounds in the red ginger leaf extract rather than the gel base or solvents used. The test results indicate that the gel formulation, which includes a 5% concentration of the ethyl acetate fraction derived from red ginger leaves, showed

Table 6: Wound healing results.

Groups	Wound Diameter (mm ² ± SD)							
	Day to							
	0	3	6	9	12	15	18	21
1	374.28±73.52	323.57±44.93	291.43±71.10	230.43±78.57 ^c	204.26±81.29 ^c	172.93±81.45	117.98±50.48 ^c	109.46±27.06 ^c
2	376.09±60.49	330.78±58.85	273.42±78.83	211.60±62.26 ^c	166.23±25.65 ^c	127.90±18.59 ^c	112.91±12.51 ^c	82.00±21.37 ^c
3	380.59±42.94	294.06±67.78	186.33±42.77 ^a	136.36±31.60 ^{ab}	100.28±29.98 ^{ab}	75.72±36.45 ^b	28.75±16.21 ^{ab}	8.65±1.57 ^{ab}
4	349.48±56.20	293.13±47.02	239.81±24.39	151.45±18.36	112.34±18.68 ^{ab}	85.98±14.10 ^b	63.99±12.87 ^{bc}	44.49±10.63 ^{abc}
5	403.34±74.71	331.39±65.34	279.85±71.04 ^c	194.87±51.79	118.47±13.2 ^b	89.02±20.74 ^b	65.62±20.10 ^{bc}	39.35±14.14 ^{abc}
6	377.75±88.01	309.78±86.13	217.36±93.50	146.74±29.86	107.79±20.18 ^b	81.82±11.23 ^b	39.79±26.50 ^b	21.60±19.9 ^{ab}

a: There is a significant difference with the untreated group ($p < 0.05$); **b:** There is a significant difference with the gel base group ($p < 0.05$); **c:** There is a significant difference with the voltaren gel group ($p < 0.05$).

highest inhibitory diameter. Dimethyl sulfoxide (DMSO) was employed as a negative control to ascertain its lack of inhibitory effects or interference with the results. *Staphylococcus aureus*, a gram-positive bacteria, possesses a sturdy cell wall structure characterized by a dense and inflexible peptidoglycan layer that incorporates teichoic acid (Rohde, 2019). The red ginger leaves have undergone phytochemical screening, revealing the presence of antimicrobial components such as tannins, flavonoids, and terpenoids, which are recognized for their antibacterial activities (Al-eem et al., 2020; Wang et al., 2020). The results indicate a direct relationship between the amount of ethyl acetate in red ginger leaves and their ability to effectively inhibit the growth of *Staphylococcus aureus*.

EXCISION WOUND HEALING EVALUATION

We conducted an experiment to assess the efficacy of wound excision in rats as a model organism. The rats were injured and monitored for a reduction in the size of their wounds over a period of 21 days. Result can be see in Table 6.

The measured lengths of excision wounds were analyzed statistically to evaluate the differences between treatment groups. Measurements taken on days 0, 3, and 6 showed a significant difference between the voltaren gel-treated group and the untreated group, but no significant difference was observed between the voltaren gel-treated group and the group treated with a 3% ethyl acetate fraction gel ($p > 0.05$). By day 9, a statistically significant difference ($p < 0.05$) was noted between the untreated group and both the voltaren gel-treated group and the gel base-treated group. On day 12, the assessment of excision incision lengths revealed significant differences between the untreated group and the groups treated with voltaren gel and 1% extract in gel formulation ($p < 0.05$). The gel base-treated group also showed a statistically significant difference compared to the groups treated with voltaren gel and ethyl acetate fraction gel at 1%, 3%, and 5% concentrations ($p < 0.05$).

By day 15, a notable disparity was observed between the gel base preparation group and the groups using voltaren gel

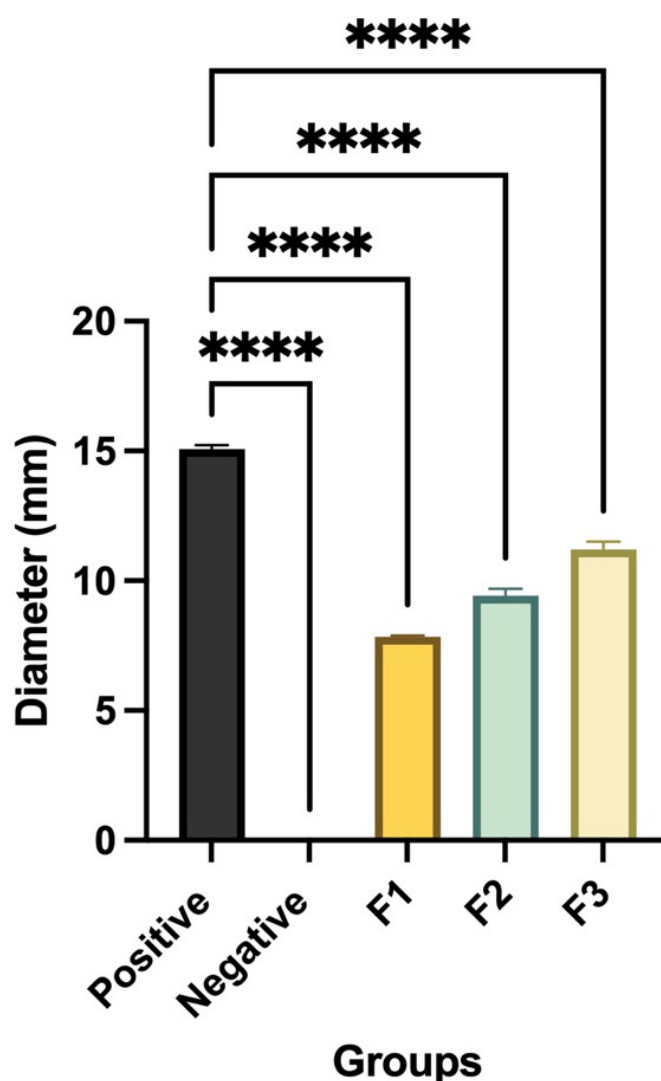


Figure 2: Graph of antibacterial activity test of gel preparation. Asterisk * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$ represents significant differences to positive control group and NS represents not significant ($P > 0.05$).

and ethyl acetate fraction gel at 1%, 3%, and 5% concentrations ($p < 0.05$). On day 18, measurements indicated significant differences between the untreated group and the voltaren gel-treated group ($p < 0.05$), as well as between the

gel base-treated group and the voltaren gel-treated group at 1%, 3%, and 5% concentrations ($p < 0.05$). Additionally, the voltaren gel-treated group showed significant differences compared to the untreated group at 1% and 3% concentrations ($p < 0.05$).

By day 21, there were significant differences between the untreated group and the groups treated with voltaren gel and ethyl acetate fraction gel at 1%, 3%, and 5% concentrations ($p < 0.05$). Differences were also noted between the gel base-treated group and the groups treated with voltaren gel and ethyl acetate fraction gel at 1%, 3%, and 5% concentrations ($p < 0.05$). Gel contained 5% ethyl acetate fraction showed highest activity, the results indicated dose dependent manner. Furthermore, The untreated group showed the slowest wound healing progress, with significant differences ($p < 0.05$) observed compared to all treatment groups by day 9. This indicates the necessity of treatment for effective wound healing. The gel base group showed better healing compared to the untreated group but was significantly less effective than the Voltaren Gel and the ethyl acetate fraction gels, particularly from day 12 onwards ($p < 0.05$). The group treated with Voltaren Gel showed significant improvement in wound healing compared to the untreated and gel base groups. By day 21, the wounds treated with Voltaren Gel were almost fully healed (8.65 ± 1.57 mm). The 5% ethyl acetate fraction gel group demonstrated the most effective wound healing, comparable to the Voltaren Gel group by day 21. The results indicated a dose-dependent manner in which higher concentrations of the ethyl acetate fraction led to faster and more effective wound healing. These comparisons clearly illustrate the superior efficacy of the ethyl acetate fraction gels, particularly at higher concentrations, in promoting wound healing and antibacterial activity compared to both untreated and control groups.

Red ginger leaves, a variety of *Zingiber officinale*, are rich in phytochemicals such as gingerols, shogaols, homovanillyl alcohol, alkaloids, flavonoids, saponins, tannins, phenolics, steroids, triterpenoids, and essential oils (Hendra *et al.*, 2022). These compounds have been found to possess antibacterial, anti-inflammatory, and antioxidant properties (Zhang *et al.*, 2022). The lipophilicity of these compounds, which is directly related to their wound healing activity, has been demonstrated in a study by (Mao *et al.*, 2019). The wound healing properties of red ginger leaves can be attributed to their phytochemical content, which includes flavonoids, alkaloids, saponins, and phenolic compounds (Vitale *et al.*, 2022). These compounds have been shown to have anti-inflammatory, antimicrobial, and antioxidant effects, all of which are important in the wound healing process (Yahyazadeh *et al.*, 2024; Mosae, 2018).

The comparison with the Voltaren-treated group, a conventional anti-inflammatory agent, was intended to

benchmark the efficacy of our red ginger leaf ethyl acetate fraction gel. Although Voltaren primarily addresses inflammation, it is a standard treatment in wound care. Our findings suggest that the gel not only exhibits significant antibacterial activity but also promotes wound healing by reducing inflammation and enhancing tissue regeneration. This comprehensive evaluation underscores the potential of the red ginger leaf ethyl acetate fraction gel as an effective therapeutic agent in managing infected wounds.

HISTOPATHOLOGY ANALYSIS OF EPIDERMAL THICKNESS

Due to its position as the outermost layer of the skin, the epidermis is highly susceptible to damage from impact or contact with external objects. Figure 3 illustrating the epidermal histological image of the test group and each individual group. Moreover, epidermal Thickness (nm) analysis presented in Table 7.

Table 7: Epidermal Thickness analysis.

Group	Epidermal Thickness (nm) (Mean $\pm$ SD)
Group 1	21937.79 $\pm$ 2733.41 ^c
Group 2	25813.55 $\pm$ 5495.68 ^c
Group 3	66493.89 $\pm$ 20345.10 ^{ab}
Group 4	37264.43 $\pm$ 2573.67 ^c
Group 5	45423.84 $\pm$ 7235.03
Group 6	52407.77 $\pm$ 3958.34 ^{ab}

a: There is a significant difference with the untreated group ($p < 0.05$); b: There is a significant difference with the gel base group ($p < 0.05$); c: There is a significant difference with the voltaren gel group ($p < 0.05$).

In the untreated group (Group 1), the epidermal layer was the thinnest, averaging $21,937.79 \pm 2733.41$ nm, indicating poor wound healing and inadequate epidermal regeneration, making the wound more susceptible to infection and delayed closure. The gel base group (Group 2) showed a slight increase in epidermal thickness to $25,813.55 \pm 5495.68$ nm compared to the untreated group, but it remained thinner than the positive control and ethyl acetate gel-treated groups, suggesting limited efficacy in promoting epidermal regeneration.

The positive control group treated with Voltaren gel (Group 3) exhibited a significant increase in epidermal thickness, reaching $66,493.89 \pm 20345.10$ nm, indicating effective wound healing and substantial epidermal regeneration due to the anti-inflammatory properties of Voltaren. The group treated with 1% ethyl acetate fraction gel (Group 4) displayed moderate epidermal thickness of around $37,264.43 \pm 2573.67$ nm, demonstrating a beneficial effect in promoting epidermal regeneration, though less pronounced than higher concentrations. The 3% ethyl acetate gel group (Group 5) showed significant epidermal thickness, averag

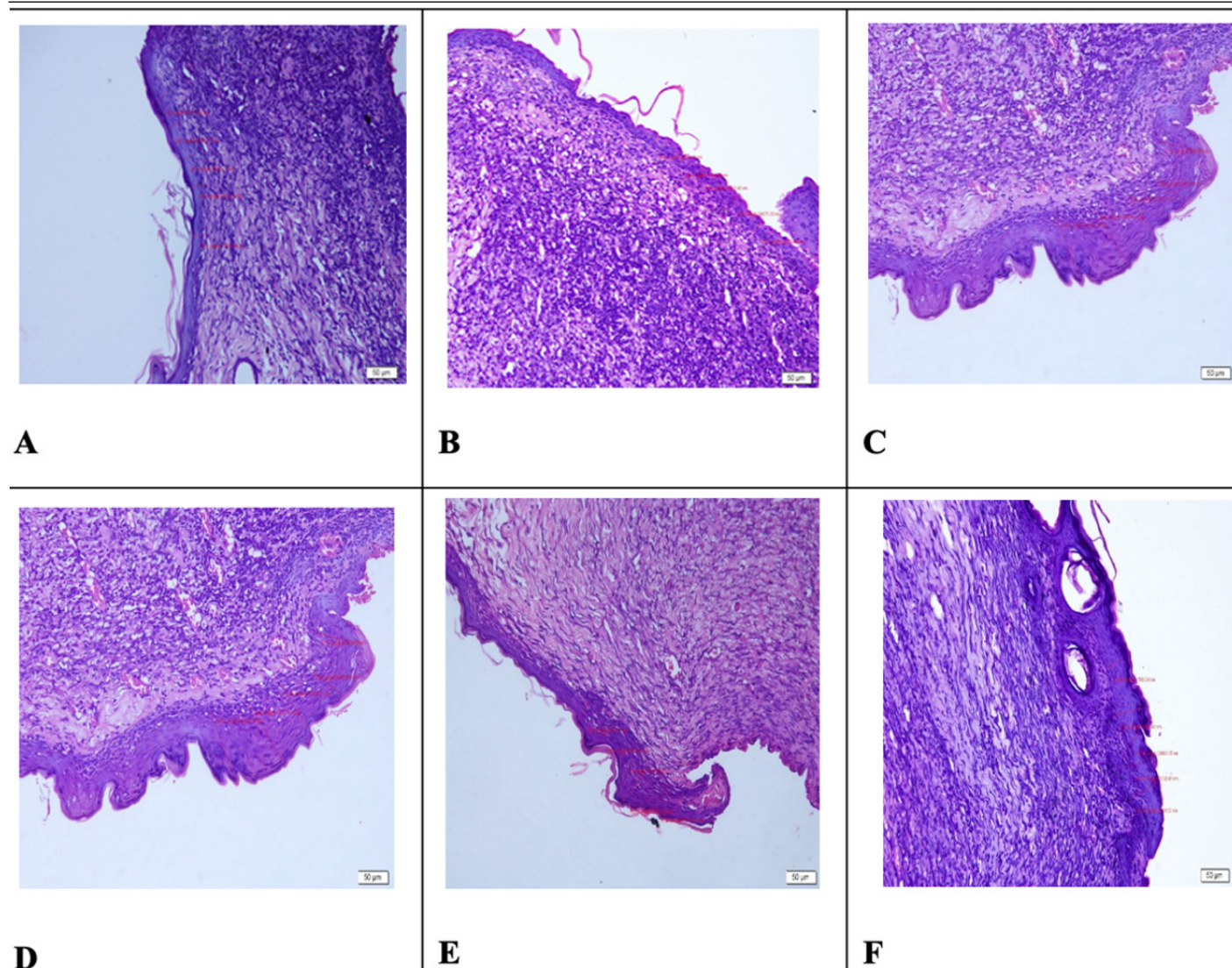


Figure 3: Histopathology of Epidermal Thickness, **A:** Group 1; **B:** Group 2; **C:** Group 3; **D:** Group 4; **E:** Group 5; **F:** Group 6.

ing $45,423.84 \pm 7235.03$ nm, suggesting enhanced wound healing efficacy with the higher concentration of ethyl acetate fraction. The 5% ethyl acetate gel group (Group 6) exhibited the highest epidermal thickness at approximately $52,407.77 \pm 3958.34$ nm, indicating the most effective epidermal regeneration among all groups, with a thick and continuous epidermal layer suggesting robust wound healing and tissue repair.

Stained sections were examined and imaged using a high-resolution digital microscope equipped with a high-definition camera. Images were captured at magnifications ranging from 40x to 100x to ensure sufficient detail for accurately measuring epidermal thickness. The image resolution was set to at least 300 dpi (dots per inch) to maintain clarity and sharpness, which is crucial for precise analysis. Epidermal thickness is crucial in evaluating wound healing treatments, as a thicker epidermis signifies better regeneration of the skin barrier, essential for protection against infections and further injury. The increased epidermal thickness observed in the groups treated with

ethyl acetate fraction gels, particularly at higher concentrations (3% and 5%), highlights the efficacy of these formulations in promoting wound healing. The bioactive compounds in the ethyl acetate fraction, such as flavonoids, tannins, and terpenoids, likely contribute to enhanced cellular proliferation and tissue regeneration, leading to a thicker and more robust epidermal layer. Comparison with the Voltaren-treated group indicates that while Voltaren gel promotes significant epidermal regeneration, the 5% ethyl acetate fraction gel formulation also shows considerable potential, underscoring the therapeutic efficacy of the red ginger leaf ethyl acetate fraction in accelerating wound healing and restoring skin integrity. The squamous epithelium, found in the outer skin layer, primarily protects against abrasion and bacterial invasion (Pereira and Sequeira, 2021). Damage to the epidermis can prompt significant new tissue formation, with the duration of healing affecting the epidermis thickness, potentially leading to abnormal epithelial tissue development (Pereira and Sequeira, 2021).

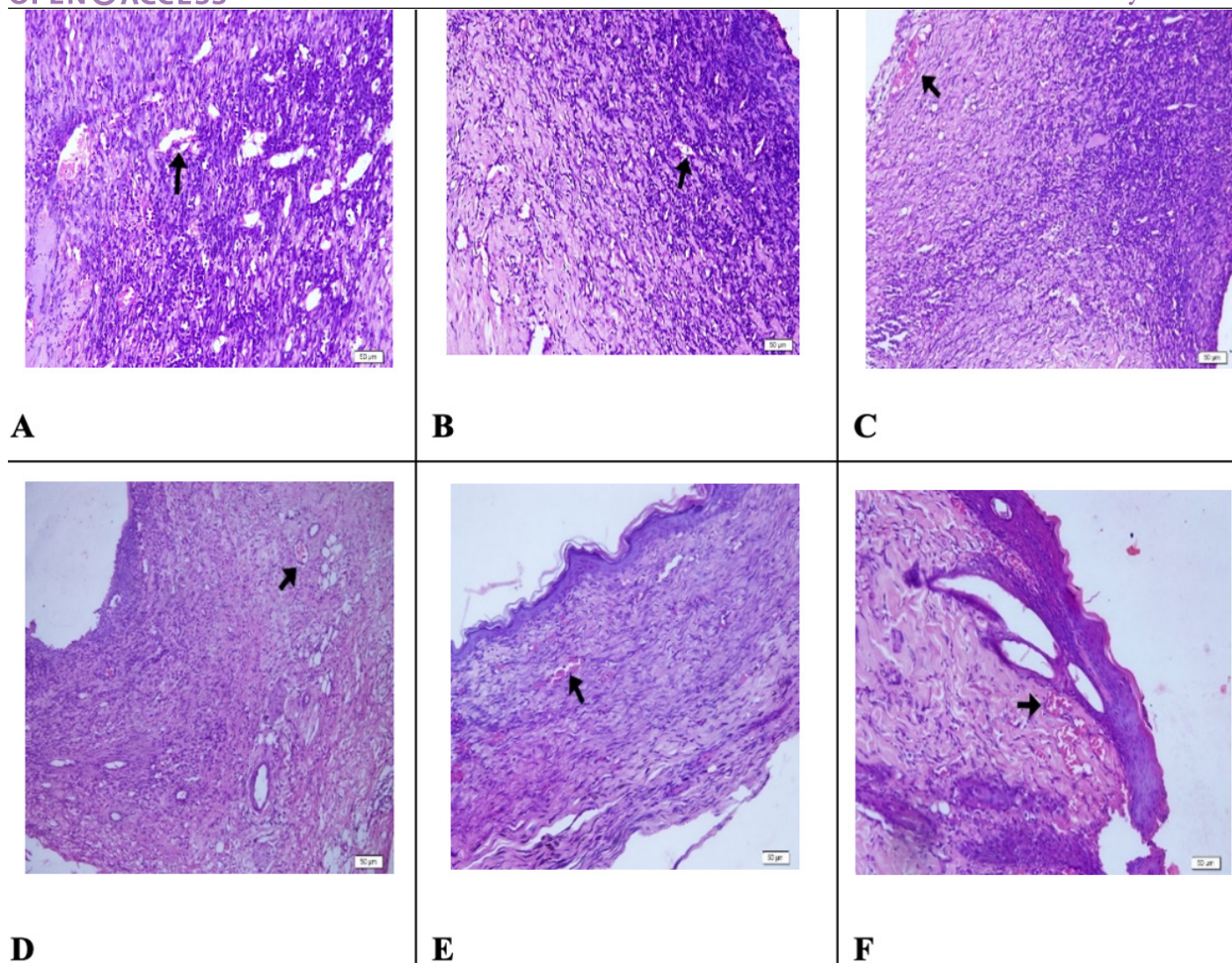


Figure 4: Angiogenesis histopathology test results of the treatment group.

ANGIOGENESIS ANALYSIS

Angiogenesis is the process through which new blood vessels are formed from pre-existing blood vessels in injured tissues (Dudley and Griffioen, 2023). Figure 4 and Table 8 present the angiogenesis dystopathology results of the experimental group undergoing therapy.

Table 8: Results of angiogenesis examination on excisional wound healing.

Group	Amount of Angiogenesis (Mean $\pm$ SD)
Group 1	14.20 $\pm$ 1.40 ^c
Group 2	14.67 $\pm$ 1.82 ^c
Group 3	30.60 $\pm$ 4.95 ^{ab}
Group 4	17.87 $\pm$ 2.23 ^c
Group 5	19.87 $\pm$ 1.70 ^c
Group 6	26.33 $\pm$ 0.31 ^{ab}

a: There is a significant difference with the untreated group ($p < 0.05$); **b:** There is a significant difference with the gel base group ($p < 0.05$); **c:** There is a significant difference with the voltaren gel group ($p < 0.05$).

Figure 4 exhibits the histological picture that indicates the angiogenesis segment of each treatment group. The figure clearly illustrates a noticeable contrast between the untreated group and the treatment group. Angiogenesis was absent in the control group, however in each experimental group, the onset of angiogenesis became evident.

The results of the angiogenesis examination on excisional wound healing showed that the untreated group (Group 1) exhibited the lowest number of blood vessels, averaging 14.20 $\pm$ 1.40 vessels per HPF, indicating poor angiogenesis and insufficient blood supply for effective wound healing. The gel base group (Group 2) showed a slight increase in angiogenesis compared to the untreated group, with an average of 14.67 $\pm$ 1.82 vessels per HPF, indicating minimal impact on promoting angiogenesis. The Voltaren gel group (Group 3) demonstrated a significant increase in angiogenesis, with an average of 30.60 $\pm$ 4.95 vessels per HPF, reflecting the anti-inflammatory properties of Voltaren, which promote improved blood supply and faster healing. The 1% ethyl acetate gel group (Group 4) displayed mod-

erate angiogenesis, with an average of 17.87 ± 2.23 vessels per HPF, indicating a beneficial effect of the ethyl acetate fraction in promoting new blood vessel formation. The 3% ethyl acetate gel group (Group 5) exhibited significant angiogenesis, with an average of 19.87 ± 1.70 vessels per HPF, suggesting enhanced wound healing efficacy with the higher concentration of ethyl acetate fraction. The 5% ethyl acetate gel group (Group 6) showed the highest level of angiogenesis, with an average of 26.33 ± 0.31 vessels per HPF, indicating the most effective promotion of new blood vessel formation and robust wound healing.

Table 9: TNF- α Measurement Results.

Test Group	Before Treatment Level (ng/mL) (Mean $\pm$ SD)	After Treatment Level (ng/mL) (Mean $\pm$ SD)
Group 1	75.00 ± 2.92^c	115.60 ± 15.07^{bc}
Group 2	73.80 ± 3.56^c	92.01 ± 10.42^{ac}
Group 3	82.60 ± 3.21^{ab}	63.62 ± 7.55^{ab}
Group 4	71.80 ± 3.83^c	82.77 ± 1.55^{abc}
Group 5	75.60 ± 7.99	76.37 ± 4.94^{abc}
Group 6	76.00 ± 1.87^c	68.67 ± 9.67^{ab}

a: There is a significant difference with the untreated group ($p < 0.05$); **b:** There is a significant difference with the gel base group ($p < 0.05$); **c:** There is a significant difference with the Voltaren gel group ($p < 0.05$).

Angiogenesis is critical in wound healing, providing necessary nutrients and oxygen to regenerating tissue. The increased number of blood vessels observed in groups treated with ethyl acetate fraction gels, particularly at higher concentrations (3% and 5%), highlights their efficacy in promoting angiogenesis. Bioactive compounds in the ethyl acetate fraction, such as flavonoids, tannins, and terpenoids, likely enhance blood vessel formation and tissue regeneration. The 5% ethyl acetate fraction gel showed comparable or superior efficacy to the Voltaren gel, underscoring its therapeutic potential in enhancing angiogenesis and supporting wound healing.

Injury-induced tissue cell damage can trigger the secretion of potent angiogenesis factors such as FGF-1 and FGF-2 (Farooq *et al.*, 2021). The ethyl acetate fraction of red ginger leaves contains secondary metabolites including alkaloids, flavonoids, tannins, saponins, steroids, and triterpenoids. Flavonoids have anti-inflammatory properties that help decrease cellular inflammation in excised wound tissue, thereby accelerating the healing process. Additionally, flavonoids contribute to the activation of HIF-1 (Hypoxia Inducible Factor), which increases the activation of VEGF, expediting angiogenesis (Subbaraj *et al.*, 2021). The dosage given during the wound healing phase can also influence angiogenesis. Gel dosage forms are known for their ability to maintain moisture in wounds, stimulating the release of growth factors such as VEGF, FGF-2, angiopoietin-1, thrombospondin, and angiogenesis by macrophages (Naito *et al.*, 2020).

THE EFFECT OF RED GINGER LEAF (ZINGIBER OFFICINALE VAR. RUBRUM) ETHYL ACETATE FRACTION ON TUMOR NECROSIS FACTOR ALPHA (TNF- α) EXPRESSION DURING GEL THERAPY

Tumor Necrosis Factor- α (TNF- α) is a pro-inflammatory cytokine that is synthesized by macrophages and neutrophils in response to tissue damage during the inflammatory phase of wound healing (Raziyeve *et al.*, 2021). In this study TNF- α were measured during pre- and post-treatment. Results of TNF- α concentration can be seen Table 9.

Statistical analysis was performed on TNF-alpha levels obtained after treatment. There was a significant difference between the no treatment group and all treatment groups ($p < 0.05$), the gel base group and all treatment groups ($p < 0.05$). The results showed that the test group treated with 5% ethyl acetate fraction gel showed the highest concentration of TNF- alpha. this shows that the effect of the gel is dependent on the dose.

Many things influence the expression of cytokines that act as inflammatory mediators. in this case, non-specific immune cells of the skin will interact with and identify *Staphylococcus aureus* germs, initiating an inflammatory cell infiltration response to eradicate bacteria through phagocytosis. Pathogenic microbial components stimulate pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), which are non-specific immune cells in the skin. These receptors identify pathogen-associated molecular patterns (PAMPs) of the microbes, leading to the immediate elimination of the microbes through processes such as initiation of phagocytosis, production of anti-bacterial compounds, and release of inflammatory mediators (Saha *et al.*, 2024). If the bacteria are not eradicated, proliferation of inflammatory cells will occur, leading to the progression of acute inflammation into chronic inflammation, one of which is characterized by increased expression of TNF-alpha.

Although no adverse effects were observed in the treated rats during the study period, the possibility of side effects or allergic reactions in humans cannot be ruled out. Further studies are required to assess the safety and potential side effects of the gel in human subjects.

CONCLUSIONS AND RECOMMENDATIONS

The findings demonstrate that the ethyl acetate fraction significantly enhances wound healing, evidenced by improved epidermal thickness and increased angiogenesis. The 5% ethyl acetate gel showed the most significant improvement in wound closure, reducing wound size more effectively than the untreated, gel base, and Voltaren gel

groups. While the study demonstrates potential, limitations include a 21-day study period and the use of a rat model. Future research should explore long-term effects, molecular mechanisms, and conduct clinical trials. The findings suggest significant potential for clinical use in managing infected wounds, though further research is necessary for clinical validation.

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NOVELTY STATEMENT

The novelty of this study is the evaluation of the effect of red ginger leaf ethyl acetate fraction on the treatment of infected excision wounds.

AUTHOR'S CONTRIBUTION

All authors contributed equally to the manuscript.

CONFLICT OF INTEREST

The authors assert that they do not have any conflicts of interest, whether financial, personal, authorship-related, or otherwise, that could potentially impact the research and the findings described in this paper.

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