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Histopathological evaluation of green betel leaf extract ointment on incision wounds infected with Staphylococcus aureus in wistar rats

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Abstract: The skin, as the largest organ in both humans and animals, functions as a protective barrier against various external threats such as sharp objects, temperature fluctuations, chemicals, and physical trauma. Incision wounds are particularly vulnerable to infections, with *Staphylococcus aureus* being a common causative agent of secondary complications, including prolonged inflammation and delayed healing. This study investigates the efficacy of green betel leaf extract ointment in accelerating the healing process of incision wounds in Wistar rats infected with *Staphylococcus aureus*. An experimental, post-test-only controlled group design was employed, involving five groups of five rats each. The groups were treated with an ointment base (control), mupirocin ointment (positive control), and green betel leaf extract ointments at concentrations of 10%, 15%, and 20%. Data were analyzed using normality tests, homogeneity tests, and ANOVA. Phytochemical analysis revealed that green betel leaf extract contains alkaloids, terpenoids/steroids, saponins, tannins, and glycosides, all of which are secondary metabolites known for their antimicrobial and wound healing properties. Among the treatment groups, the 15% green betel leaf extract ointment exhibited the most rapid wound closure (average wound healing of 1.44 mm), approaching the effectiveness of mupirocin ointment. Histopathological observations further demonstrated a significant increase in epithelialization and fibroblast proliferation in treated groups compared to controls. In conclusion, green betel leaf extract ointment, particularly at a 15% concentration, shows promising potential as a topical agent for treating *Staphylococcus aureus*-infected incision wounds in Wistar rats.

Keywords: *Staphylococcus aureus*, Incision wound, Skin histopathology

INTRODUCTION

Wound severity can occur due to bacterial infection with the presence of a number of microorganisms that attack the tissue in the wound area, especially in open wounds, causing worse consequences[1,2]. One of the bacteria that can cause wounds in a serious condition is *Staphylococcus aureus*, which is a round gram-positive bacterium that is pathogenic to humans causing infections and disorders of the skin, usually only acting as a carrier [3,4]. *Staphylococcus aureus* infection is one of the most serious problems in wound healing [5,6]. This bacterium can disrupt the healing process and cause serious complications due to its ability to form biofilms, a protective layer that shields the bacteria from antibiotics and the body's immune system, making treatment difficult and infection more durable[7]. *Staphylococcus aureus* has a variant known as Methicillin resistant *Staphylococcus aureus* (MRSA) that is resistant to many antibiotics[8]. This makes

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treatment of the infection more difficult and leads to excessive inflammation, tissue damage, and slows down the formation of new tissue[5].

According to research by Nadya et al. 2021 *Staphylococcus aureus* is one of the most common pathogenic bacteria found in humans and animals that can infect mild skin to more serious infections such as pneumonia, endocarditis, and sepsis. In this study, it was explained that the preparation of coriander seed extract ointment (*Coriandrum sativum* L.) provided good effectiveness on wound healing in mice infected with *Staphylococcus aureus* bacteria but not by knowing the histopathological picture of the skin of mice infected with *Staphylococcus aureus* bacteria[9].

Currently, synthetic and herbal medicines are widely used in wound healing[10]. One of the herbal plants in Indonesia used in traditional medicine is green betel leaf (*Piper betle Linn*) to help accelerate the wound healing process which is known to have a number of benefits and contain bioactive molecules that have the potential to support health, including in the wound healing process[11]. The content contained in green betel leaves tannins, alkoloids, saponins, and flavanoids function as antimicrobials and stimulate the growth of new cells in the wound[12,13]. According to research by Atika et al. 2021 green betel leaf extract (*Piper betle* L.) has an effect on wound healing in mice (*Mus musculus* L.) which contains alkoloid compounds as antibacterials[14].

The purpose of this study was to determine the effect of green betel leaf extract on wounds infected with *Staphylococcus aureus* and determine the extent to which the extract has antimicrobial properties and reduces inflammation, accelerating tissue regeneration in infected wounds[15]. In addition, this study also wanted to evaluate the potential of green betel leaf extract as an alternative or traditional treatment for infected wounds and determine the histopathological picture in rat skin by examining the number of fibrolas and re-epithelialization.

This study aims to know the effect of healing incision wounds infected with *Staphylococcus aureus* bacteria with green betel leaf extract ointment based on macroscopic, microscopic observations, the number of fibrolas and epithelialization in wistar strain rats.

MATERIAL AND METHOD

This study is an experimental study with post test only controlled group design on male wistar strain rats. The research was conducted at the Pharmacology Laboratory of the Faculty of Pharmacy, University of North Sumatra and the Anatomical Pathology Laboratory of the Royal Prima Hospital Medan for more than 3 months from October 2023 to January 2024. The experimental animal research protocol was approved by the Prima Indonesia University Health Research Ethics Committee (041/KEPK/UNPRI/X/2023).

The animal test used male wistar rats consisting of five treatment groups with different concentrations[16]. In this study, five treatment groups were used to be able to evaluate the extent to which increasing the concentration of green betel leaf extract affects wound healing and infection control.

Tools and Materials

The tools used in this research are scalpel and surgical scissors, microscoprotary evaporator, gloves, and Fourier Transform Infrared (FTIR) spectroscopy. The materials used in this study were green betel leaves, Nutrient Agar (NA), Staphylococcus aureus ATCC 25923 bacteria, mupirocin ointment, HE staining, 10% formalin, ketamine HCL-xylazine, xylol, PBS pH 7.4.

Green Betel Leaf Extract

Green betel leaf extract was obtained through maceration method using 96% ethanol for 7x24 hours to separate solid-liquid mixture from green betel leaf

simplisia. Maceration is continued by evaporation using a vacuum rotary evaporator to produce a solvent-free thick extract[14,17].

To get green betel leaf extract ointment, the researcher made an absorption base ointment formulation in table 1 below[18]:

Table 1. Ointment formulation

Concentration	Green betel leaf extract (g)	*Adeps lanae* (g)	Stearyl alcohol (g)	White Beeswax (g)	Vaseline yellow (g)	Total ointment (g)
10%	2	0.6	0.6	1.6	15.2	20
15%	3	0.6	0.6	1.6	14.2	20
20%	4	0.6	0.6	1.6	13.2	20
Ointment base	-	0.6	0.6	1.6	17.2	20

The ointment preparations to be made in this study have different concentrations of green betel leaf extract, namely 10%, 15% and 20%. The process of making betel leaf extract ointment uses an absorption base. Weighed each of the above ingredients. Stage I stearyl alcohol, white beeswax were melted by heating. Stage II added vaseline yellow and adeps lanae, then stirred until homogeneous, then cooled. Added green betel leaf extract and then crushed until homogeneous. After homogeneous, put it in a tube and label it[18].

Phytochemical screening of green betel leaf extracts

Phytochemical analysis was carried out to determine the type of secondary metabolites using alkaloid test, terpenoid/steroid test, flavonoid test, tannin test and saponin test[19,20].

Fourier Transform Infrared (FTIR) Spectroscopy

FTIR (Fourier Transform Infrared) is a method that uses infrared spectroscopy. In infrared spectroscopy, infrared radiation is passed through a sample. Part of the radiation will be absorbed by the sample and the other part will be passed or forwarded. FTIR can be used quantitatively because the energy absorbed at a particular wavelength is directly proportional to the amount of kinetic energy associated, so the higher the concentration of the analyte, the more energy is absorbed.How to use FTIR the first step is to turn on the FTIR spectrophotometer. Then the sample powder is placed on the ATR plate then the ATR lever is rotated until it presses the sample on the ATR plate, background measurements are taken every scanning, and has been connected to a computer that has been equipped with OPUS software which is used to control the work of the spectrophotometer in the range of 4000-600 cm-1 with a resolution of 4 cm-1 and scans 32 times. Spectra were stored as absorbance data in OPUS format with three replications[21].

Bacterial Cultures

Staphylococcus aureus ATCC 25923 bacteria were grown on Nutrient Agar (NA) medium by scratching bacteria from pure culture on the surface of a slanted agar after which it was incubated at 37ºC for 24 hours. Bacterial colonies were suspended in test tubes containing 10ml of Natrium Borth (NB) solution, the turbidity was measured with a visible spectrophotometer at a wavelength of 580 nm, until a transmittance of 25% was obtained[22,23].

Animal and Experimental Design

Wistar rats were placed in plastic cages lined with wire mesh covers with a base in the form of husks and consisted of 5 treatment groups with each group consisting of 5 animals in one cage[24]. All test animals were acclimatized for one week and fed ad libitum[25]. Before treatment, rats were anesthetized by injecting 0.5 cc ketamine in the muscle (intramuscular) to ensure that the rats were unconscious during the incision and infection process. An incision was made on the rat's back with a sterile slingshot with the length of 2cm and 0.5 mm wide and 200 uL of *Staphylococcus aureus* ATCC 25923 bacteria were applied to the rat wounds that had formed using a micropipette evenly[9]. The skin of the rat's back after applying the bacteria was observed for 2-5 days to see the occurrence of wounds and pus formed.

The incision wounds in group I (positive control) mupirocin ointment was applied, group II (negative control) was applied vaseline blank, group III was applied 10% concentration green betel leaf extract ointment, group IV was applied 15% concentration green betel leaf extract ointment and group V was applied 20% concentration green betel leaf extract ointment. The application was done twice a day in the morning and evening for 14 days. Macroscopic observation parameters observed the length of wound healing, measuring the length of the wound on days 1, 3, 6, 9, 12 and 14 before the ointment was reapplied. Microscopic observations were made by observing rat skin on day 14[14,26].

Histopathological Examination

Histopathological preparations were made using the paraffin method, to observe the presence of inflammatory cells, fibrolas and epithelial cells by taking samples of rat skin and performing Harris-hematoxylin eosin staining viewed in a binocular light microscope with 100x and 450 x magnification[10,27].

Macroscopic and Microscopic Observations

Macroscopic observations were made by observing the length of wound healing of incisions infected with *Staphylococcus aureus* ATCC 25923 bacteria. Observations were made of wound healing, the larger the diameter of the healing that occurs, the better the healing. Diameter measurements were taken on days 1, 3, 6, 9, 12 and 14 before the betel leaf extract ointment was reapplied.

Microscopic observation with the development of histological wound healing, namely by making microscopic observations in the epidermis, dermis and hypodermis by identifying the presence of inflammatory cells, fibroblasts, epithelium[28,29].

Data Analysis

Data analysis was performed macroscopically observing the disappearance of pus, erythema and the length of healing of incisions infected with *Staphylococcus aureus* ATCC 25923 bacteria and microscopically observing the development of wound healing in the epidermis, dermis and hypodermis by identifying the presence of inflammatory cells, fibrolas and epithelium through the ANOVA test[30].

The ANOVA test is used to compare the averages of populations represented by several sample groups together or to test the average difference in data from more than five groups. The basis for decision making is if the sig value < 0.05 then the data is not normally distributed, but if sig > 0.05 then the data is normally distributed[31].

RESULTS AND DISCUSSION

Phytochemical screening test results of Green Betel Leaf Extract

The results of the phytochemical screening test observations on green betel leaf extract arepositive for alkaloid, terpenoid/steroid, saponin, tannin and

glycoside compounds contained secondary metabolite compounds can be seen from the reagents formed in the following table.

Table 2. Compounds contained in green betel leaf extracts

Secondary Metabolite Compounds	Reagents	Result
Alkaloid	Bouchardart	-
	Maeyer	-
	Dragendroff	+
Terpenoids/steroids	Salkowsky	-
	Lieberman-Burchad	+
Saponins	Aquadest + 96% Alcohol	+
Flavonoids	$Mg_{(s)}+HCl_{(p)}$	-
Tannin	$FeCL_3$ 1%	+
Glycosides	Mollish	+

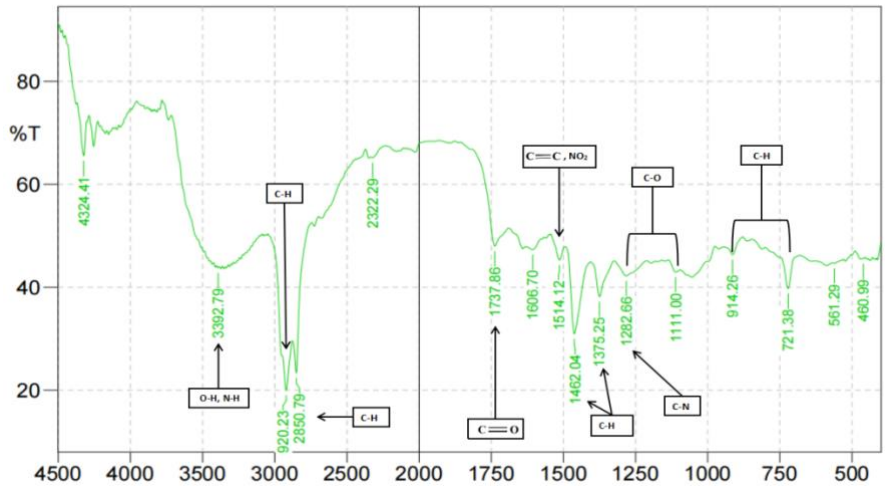

Figure 1. FTIR Test Analysis of Green Betel Leaf Extract Ointment

The percentage of transmission above 15 with a wave number of 3394.72 cm^1 indicates the presence of O-H functional groups in the form of phenols, nomoner alcohols, hydrogen bond alcohols that change, sometimes widening and N-H functional groups of amines, amides that are moderate. At wave number 2922.16 cm^1 shows a strong alkane C-H functional group. The wave number 1710.86 cm^1 indicates the presence of strong C=O aldehyde, ketone, carboxylic acid, ester functional groups. The wavelength of 1514.12 cm^1 shows the changing aromatic C=C functional group and strong NO2. The NO^2 functional group is also present at wave number 1367.53 cm^2 and in the range of wave numbers 1444.68 cm^1 and 1367.53 cm^2 shows a strong alkane C-H functional group. At wave number 1280.73 cm1 shows the presence of strong C-N amine, amide functional groups, and at wave numbers 1280.73 cm^1, 1112.93 cm^1, 1056.99 cm^1 shows the presence of strong C-O functional groups of alcohol compounds, ethers, carboxylic acids and esters. The alkane and aromatic C-H functional groups appear at wave numbers 912.33 cm1, 864.11 cm^1, 813.96 cm^1 and 758.02 cm^1.

Duration of Incision Wound Healing

Wound healing observations were made for 14 days, with measurements taken on days 1, 3, 6, 9, 12, and 14. The decrease in wound length is shown in Figure 2 below:

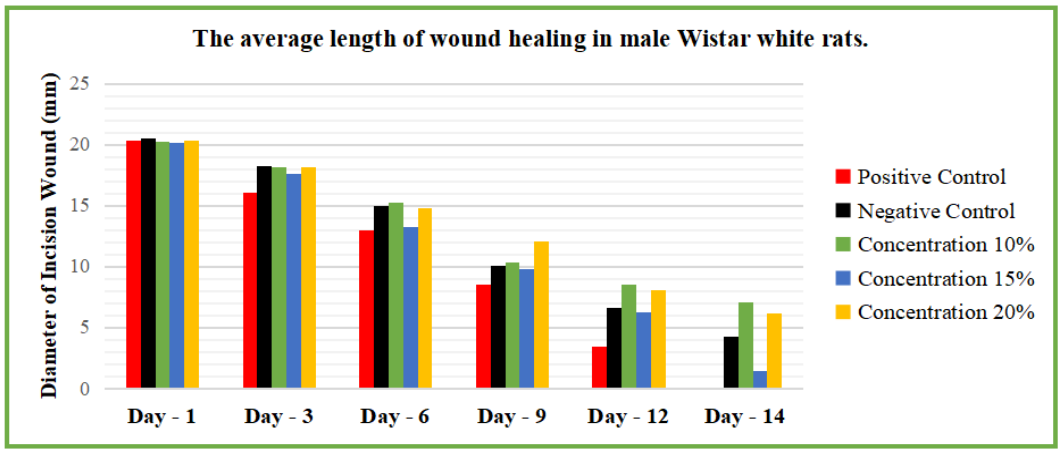

Figure 2. Visualization of the length of wound healing of incisions infected with *Staphylococcus Aureus* bacteria shows changes in the length of the wound in each different treatment group as thehealing time progresses.

The length of wound healing of incisions infected with *Staphylococcus aureus bacteria* on day 14, the negative control group was better than the average of the 10% concentration and 20% concentration groups, while the length of wound healing of the positive control incision was better than the length of wound healing in the negative control, 10% concentration, 15% concentration, and 20% concentration. The wound healing length of 15% concentration was significantly better than 10% concentration and 20% concentration. The graph depicts wound healing length on the vertical axis and group category on the horizontal axis.

Table 3. One-way Anova Test

Group	Mean	Std. Deviation	P-Value
Positive Control	10,23	7,713	
Negative Control	12,37	6,298	
Concentration 10%	13,27	5,415	0,917
Concentration 15%	11,49	7,275	
Concentration 20%	13,25	5,566	

Based on the One-Way ANOVA test results, a p value of 0,917 was obtained, which is greater than the predetermined significance level of 0.05. Thus, it can be concluded that there is not enough statistical evidence to reject the null hypothesis (H0). This analytical framework can accept the possibility that the null hypothesis is correct, indicating that there is no significant difference between the treatment groups.

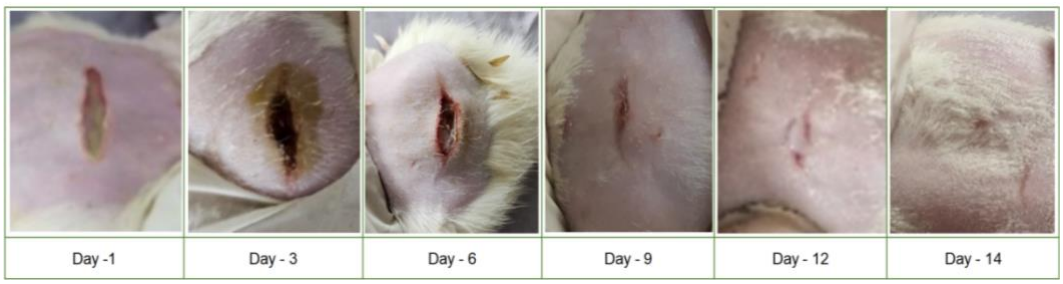

Figure 3. Wound healing with 15% concentration of Green Betel Leaf Extract Ointment Wound healing with 15% concentration of Green Betel Leaf Extract Ointment The use of green betel leaf extract at a concentration of 15% results in a healing time of 15% the fastest wound healing.

On day 14, the average length of wound healing in this concentration reached 1,44 mm.The average final wound size showed a significant difference compared to the other comparative concentrations.

Skin Histopathology in Wistar Rats

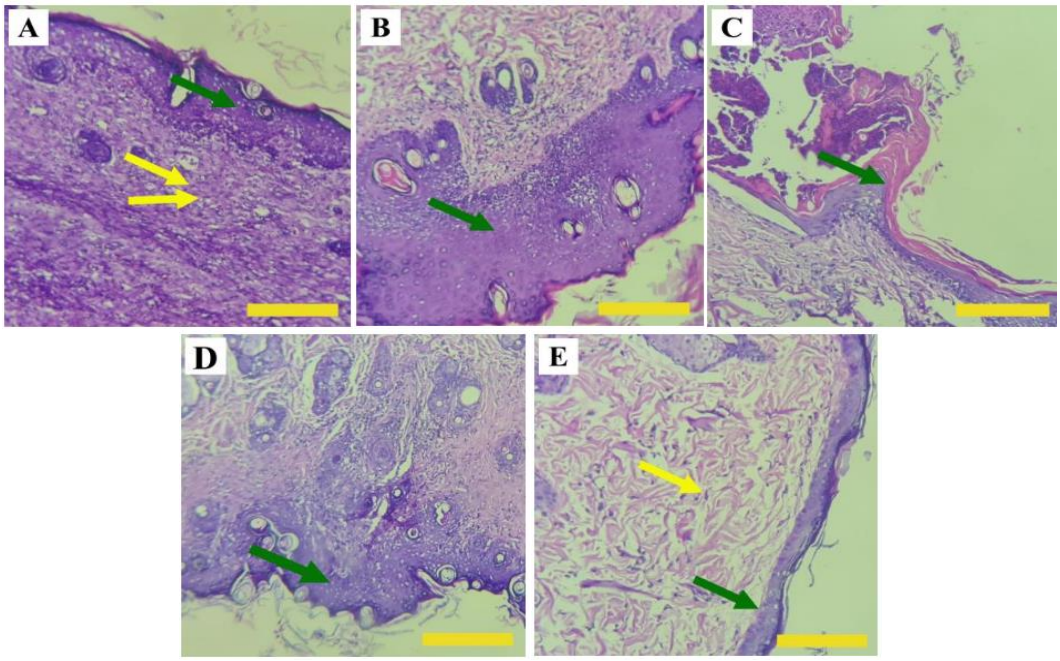

Figure 4. Positive control (A); negative control (B); 10% concentration of green betel leaf extract (C); 15% concentration of green betel leaf extract (D); 20% concentration of green betel leaf extract (E)

Microscopic picture of skin histopathology A: histopathology of skin with green betel leaf extract in the positive control group shows that the surface area of the tissue in the form of epithelium (green arrows) in the epidermis has been thickened and well formed and fibrolas cells (yellow arrows) in the dermis have been thickened, this indicates the process of epithelial cell migration in the normal skin layer has merged. B: Histopathology of the skin of the negative control group with green betel leaf extract shows that the surface area of the visible tissue has a barrier in the form of epithelium (green arrow) in the epidermis which has not yet fused with epithelial cells and is still in the inflammatory phase. Based on the description of the picture, it indicates that the wound is still in the process of tissue formation and is in the inflammatory phase. C: Histopathology of rat skin with 10% concentration of green betel leaf extract shows that the surface area of the tissue in the form of epithelium (green arrow) on the epidermis is still not well formed, this shows irregular epithelial cells and still does not show re-epithelialization. Based on the description of the picture, it indicates that the wound is still in the inflammatory phase. D: Histopathology of rat skin with 15% concentration of green betel leaf extract shows that the surface area of the tissue in the form of epithelium (green arrow) in the epidermis is thickened and epithelial cells begin to fuse in the normal skin layer, this indicates the process of re-epithelialization by epithelial cells that are almost complete and fibrolas cells in the dermis that begin to thicken and spread. Based on the description of the picture, it indicates that the wound is at the end of the inflammatory phase and then continues into the proliferation phase. E: Histopathology of rat skin with 20% concentration of green betel leaf extract shows that the surface area of the tissue in the form of epithelium (green arrow) on the epidermis is still not well formed and the epithelial cells have not fused with the normal skin layer, this indicates that the process of tissue formation has not been maximized. Fibrolas cells (yellow arrows) in the dermis are still scattered and look

tenuous. This description indicates that the wound is still in the early phase of tissue formation.

The results of phytochemical tests on green betel leaf extract contain alkaloid, steroid, and triterpenoid compounds. Alkaloid compounds are found in many plants and have various pharmacological properties, including antimicrobial, anti-inflammatory, and analgesic. Steroids can have anti-inflammatory and immunosuppressive effects, while triterpenoids are known as antioxidants and antimicrobials. Both groups of compounds may contribute to wound healing and antimicrobial effects. Saponins may play a role in inhibiting bacterial growth and supporting wound healing, and tannins have astringent and antimicrobial properties that may help reduce inflammation and wound healing by reducing infection and accelerating clotting[13,32].

The results showed that the p-value of each research group with concentrations of 10%, 15% and 20% were all <0.05, indicating that green betel leaf extract ointment has an effect on the healing of cuts infected with *Staphylococcus aureus* bacteria. In this study, it shows that betel leaf extract ointment with these concentrations has a significant effect on the healing of cuts infected with Staphylococcus aureus bacteria. Thus, the null hypothesis stating that there is no significant difference can be rejected, and we can conclude that the concentration of green betel leaf extract has a significant effect on wound healing. Based on the ANOVA output, it is known that green betel leaf extract ointment with a concentration of 15% has an average value of 11.49 with a standard deviation value of 7.275, providing evidence that this concentration is effective in accelerating wound healing infected with bacteria. The same results were also obtained from research conducted by Atika (2021) entitled The Effect of Green Betel Leaves (Pipet betle L.) Extract on Wounding Healing in mice (Mus musculus L.), where the results showed that green betel leaf extract can affect wound healing in mice (Mus musculus L.)[14].

That green betel leaf extract ointment has been proven to have a significant impact on the improvement of the macroscopic picture of the skin of rats that have incision wounds infected with *Staphylococcus aureus* bacteria. The macroscopic picture of the 10% green betel leaf extract ointment shows that the wound has not closed completely, the wound surface also still does not appear to have dried up. Green betel leaf extract with a concentration of 15% effectively heals wounds. The average increase in incision wound healing at a concentration of 15% is better when compared to the negative control group (K-) due to the content of active compounds in green betel leaf extract that act as anti-inflammatory and antibacterial. wound healing infected with Staphylococcus aureus bacteria in mice in the negative control group (K-) shows that the wound area is still in the inflammatory phase, which is characterized by the presence of reddish inflammatory characteristics (rubor), and has not dried completely. In contrast, in the positive control group (K+) the wound infected with *Staphylococcus aureus* bacteria had dried completely, and hair growth around the wound area began to increase. This is consistent with the statement of Athifah Royani Ma'sum (2018), which states that infection with *Staphylococcus aureus* can cause hair growth with a high number of bacteria in the wound can inhibit the wound closure and healing process. This study used three concentrations for three treatment groups with green betel leaf extract (Piper betle L.), namely with concentrations of 10%, 15%, and 20%, as well as one control group with measurements of wound diameter or 14 days [10], in line with Mawarti's research (2016) that there were differences in wound diameter observed even on the length of wound healing in mice (Mus musculus L.) day 14 of wound healing. This is done to determine the effective concentration for wound healing in mice (Mus musculus L.). The Anova test results showed that green betel leaf extract (Piper betle L.) was effective in healing wounds in mice. Betel leaf extract (Piper betle L.) at a concentration of 30% used by researchers is suitable as a wound healing drug. According to Akbar et al.

(2022), green betel leaves contain flavonoids, tannins, phenols, and saponins that play a role in the wound healing process because they have antimicrobial, anti-inflammatory, and antioxidant properties that affect the wound healing process and accelerate epithelialization[33].

CONCLUSION

Green betel leaf extract ointment is effective on the healing process of cut wounds infected with *Staphylococcus aureus* bacteria in wistar strain rats for 14 days of observation, the treatment groups show significant differences based on macroscopic observations, it can be concluded that the three concentrations of green betel leaf extract have an influence in healing cut woundsinfected with *Staphylococcus aureus* bacteria. The administration of green betel leaf extract ointment at a concentration of 15% showed a higher significance compared to the concentrationsof 10% and 20% in the healing process of cut wounds in wistar strain rats infected with *Staphylococcus aureus bacteria*. In addition, the administration of green betel leaf extract ointment at a concentration of 15% was able to improve the skin histopathology picture in wistar strain rats infected with *Staphylococcus aureus* bacteria, approaching the level of positive control (K+).

AUTHORS' CONTRIBUTIONS

Winda Irawati Zebua prepared the samples, designed the protocols, executed the protocols, and wrote the manuscript. Linda Chiuman and Edy Fachrial reviewed and supervised the manuscript. All authors have read and approved the final manuscript

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DATA AVAILABILITY STATEMENT

The utilized data to contribute to this investigastion are available from the corresponding author on reasonable request.

DISCLOSURE STATEMENT

There is no conflict of interest.

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