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Wound-Healing Effect of Ethyl Acetate Subfraction Ointment from Meniran Leaves (*Phyllathus niruri* L.) at 5% Concentration on White Male Rats

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Abstract

A study has been conducted on the healing effect of excision wounds using subfraction ointment of ethyl acetate of meniran leaves (*Phyllathus niruri* L.) with a concentration of 5% on the percentage of wound healing area, epithelialization time, and hydroxyproline levels in male white rats. This study consisted of 3 groups of rats based on ointment grouping, and each group consisted of 15 rats; from 15 rats, they were grouped again into 3 groups based on the day of taking the results of the wound healing parameters, namely days (5, 10 and 15); each group consisted of 5 rats, where group 1 was control with the administration of ointment base, group 2 was given (T® ointment) as a comparison, and group 3 was treated with the administration of the test preparation topically with subfraction ethyl acetate concentration of 5%. Each group was observed and measured for the percentage of wound healing area, epithelialization time, and hydroxyproline levels on days 5, 10, and 15. The results of data analysis using one-way ANOVA and two-way ANOVA followed by Duncan's test (SPSS 23.0) showed a significant difference between the control group and the treatment group in terms of the percentage of wound healing area, epithelialization time and hydroxyproline levels ($p<0.05$), so it can be concluded that the preparation of ethyl acetate subfraction ointment of meniran leaves with a concentration of 5% has an effect as an excision wound therapy.

Keywords: Phyllanthus niruri L, ethyl acetate subfraction, excision wounds, wound healing hydroxyproline

Introduction

A wound is a condition characterized by damage to body tissue. Damage to body tissue can involve connective tissue, muscle tissue, skin nerves and torn blood vessels that will disrupt the body's homeostasis (Abdulrahmat, 2014). The prevalence of one type of wound is an excision wound, which is caused by tissue being cut by a sharp object. Excision wounds can also be interpreted as wounds that occur due to contact with sharp objects such as knives. In excision wounds, the surface of the skin and the lower layer will be cut to varying depths (Priyandari, 2015).

The main goal in wound management is to achieve rapid healing with optimal function and good results. This can be achieved by preventing infection and trauma and then by providing an environment that can optimize wound healing (Priyandari, 2015). The main goal of wound treatment is to restore the function and shape of skin tissue to normal with minimal local complications. When a wound occurs, the tissue will undergo a healing process, which is a complex phenomenon and involves several processes. The wound healing process is a complex process consisting of 3 phases: the inflammatory phase, the proliferation phase, and the maturation phase (Primadina, et al., 2019).

Literature Review

The wound healing process can occur naturally through the wound healing mechanism. The wound healing process can be accelerated by treating the wound. In addition, a formula has been developed to help the wound healing process, from the development of the base and also the development of active substances from herbs (Handi, et al., 2017). According to research conducted by (Siahaan, et al., 2017), the administration of meniran leaf extract gel *(Phyllanthus niruri* L.) can increase epithelialization of wound tissue in male Wistar rats. Meanwhile, Kurhasi et al. (2015) also proved that meniran leaf extract can accelerate wound healing because it protects skin tissue from oxidative damage due to free radicals.

The development of the use of traditional medicines, especially from plants, to help improve public health has been quite widespread. One type of plant that can be used as medicine is meniran. Based on BPS data in 2015, meniran (Phyllanthus niruri L.) is included in 12 types of medicinal plants used in the traditional medicine industry. Meniran contains phenol, coumarin, terpenoid, and lignan (phyllanthin and hypophyllanthin) (Alegantina, 2015). Although it is a wild plant, this plant has many benefits. The most well-known benefit of meniran is that it can increase the immune system (Tjandrawinata, 2017). In addition, it also has an antihyperuricemic effect, can repair damage to pancreatic β cells and reduce blood glucose levels (Wahjuni, 2017), and helps heal cuts (Himawan, 2017).

Based on the activity of meniran (*Phyllanthus ninuri* L.), it is necessary to develop it into a pharmaceutical preparation to increase its use. This effort was made to facilitate the use of active compounds in meniran (*Phyllanthus ninuri* L.) as a wound healer, so a topical preparation formulation was made in the form of an ointment. The ointment preparation form

is suitable for skin treatment because the contact between the drug and the skin is longer and has a suitable consistency, making it easy to use (Sari, 2013).

In this study, observations will be made on the effects of administering ethyl acetate subfractions of meniran leaves on the wound healing process made in the form of an ointment preparation. The parameters observed were the percentage of wound healing area, epithelization time, and hydroxyproline levels in male white mice.

Hydroxyproline levels in tissue can be used as an index of parameters in the skin. The higher the hydroxyproline content, the greater the increase in collagen synthesis that is correlated in the wound-healing process (Rismana, 2013).

Research Method

This research was conducted at the LLDIKTI Region X Laboratory and the Pharmacology Research Laboratory of Universitas Perintis Indonesia. A sample of 1700 grams that has been dried is ground into powder. The powdered simplicia is macerated using 70% ethanol solvent. The macerate obtained is evaporated to obtain a thick extract, then diffracted using ethyl acetate (1:2), and the resulting fraction is evaporated to obtain a thick ethyl acetate fraction. After that, separation is carried out using column chromatography to obtain a thick ethyl acetate subfraction of meniran leaves. Silica gel as the stationary phase, while the mobile phase used is hexane and ethyl acetate with a ratio of (2:1). The results of the column chromatography obtained are grouped based on monitoring the spot pattern and Rf value, and monitoring is carried out using a TLC plate eluted using the mobile phase N-hexane: ethyl acetate (4:1).

The ointment preparation to be made in this study has a concentration of 5% ethyl acetate subfraction of meniran leaves, and the ointment preparation to be made is 30 g. Put the weighed meniran leaf subfraction (Phyllanthus niruri L.) as much as 1.5 g into a mortar, then weigh the ointment base as much as 28.5 g and put into the mortar, then grind until homogeneous. Remove from the mortar and put into the prepared container.

In this study, mice were divided into three groups, where each mouse was given treatment according to its group. The division of the groups is as follows:

1. Group I (control) is a group of mice that will be given wounds without being given treatment, and only ointment base is applied to the wound and the percentage of wound healing area, epithelialization time, and hydroxyproline levels in male white mice are examined on days 5, 10, and 15.
2. Group II (comparison) is a group of mice that will be applied with a circulating ointment preparation, namely T®, to the wound and the percentage of wound healing area, epithelialization time, and hydroxyproline levels in male white mice are examined on days 5, 10, and 15.
3. Group III (treatment) is a group of mice that are applied with subfraction ointment with a concentration of 5% to the wound, and the percentage of wound healing area,

epithelialization time, and hydroxyproline levels in male white mice are examined on days 5, 10, and 15.

The 500 ppm hydroxyproline stock solution was prepared by weighing 50 mg of standard hydroxyproline powder, putting it into a 100 mL measuring flask, and dissolving it with distilled water. The determination of the maximum absorption wavelength of hydroxyproline was made by taking the 500 ppm stock solution, which was pipetted as much as 0.12 mL, added with 1 mL of distilled water, and then added 1 mL of 0.01 M CuSO4, 1 mL of 2.5 N NaOH, and 1 mL of 6% H2O2. The solution was then stirred and incubated at 80ºC for 5 minutes. After the incubation process was complete, the solution was cooled, and 4 mL of 3 N H2SO4 and 2 mL of 5% 4-dimethylaminobenzaldehyde were added. The solution was re-incubated at 70oC for 16 minutes and cooled at 20ºC, and the absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 200-800, and the maximum wavelength was determined (Shila & Natasa, 2008).

The making of calibration curve was done by taking from the 500 ppm stock solution; five different concentration variations of the solution were made in a 10 ml measuring flask, as follows:

- 0.04 mL, containing 2 ppm hydroxyproline
- 0.08 mL, containing 4 ppm hydroxyproline
- 0.12 mL, containing 6 ppm hydroxyproline
- 0.16 mL, containing 8 ppm hydroxyproline
- 0.2 mL, containing 10 ppm hydroxyproline

The 500 ppm stock solution was pipetted as much as 0.04 mL; 0.08 mL; 0.12 mL; 0.16 mL; 0.2 mL was put into a 10 ml measuring flask, added with aquabidest up to 1 ml, then added 1 mL of 0.01 M CuSO4, 1 mL of 2.5 N NaOH, and 1 mL of 6% H_2O_2. The solution was then stirred and incubated at 80ºC for 5 minutes. After the incubation process was complete, the solution was cooled, and 4 mL of 3 N H_2SO_4 and 2 mL of 5% 4-dimethylaminobenzaldehyde were added. The solution was re-incubated at 70ºC for 16 minutes and cooled at 20ºC. Then, the absorbance was measured at the maximum wavelength, and a calibration curve was made to obtain the regression equation $y = a + bx$. This equation is used to determine the levels of hydroxyproline in skin tissue (Shila & Natasa, 2008). The data from wound healing parameters, namely the percentage of wound healing area and hydroxyproline levels, were analyzed statistically using one-way ANOVA testing, while epithelialization time was analyzed statistically using two-way ANOVA.

Result and Discussion

In researching the effects of wound healing, the sample used was meniran leaves as the test material. This plant was taken in the Jorong Tapian Kandis area, Kenagarian Salareh Aia, Palembayan District, Agam Regency. Before the research was conducted, the sample was first

identified in the ANDA herbarium, Department of Biology, Faculty of Mathematics and Natural Sciences, Andalas University. This is an initial step to determine the identity of the sample so that there are no mistakes in the plants that will be used for research.

Based on the identification that has been carried out, it is known that the sample used is Meniran (*Phyllanthus niruri* L.) family from Phyllanthaceae. After obtaining the ethyl acetate subfraction of meniran leaves, an organoleptic examination was carried out. The results of the examination showed that it was a thick liquid, blackish green in color, and had a distinctive odor. The ethyl acetate subfraction obtained contained flavonoid and phenolic compounds.

This compound was proven after a phytochemical test was carried out, where these compounds play a role in wound healing. After that, a drying shrinkage examination was carried out to determine the percentage of compounds lost during the heating process, not only water but also other volatile compounds. Drying shrinkage examination obtained a result of 6.86%, where the value of the drying shrinkage of the ethyl acetate subfraction of meniran leaves was no more than 14% (Ministry of Health of the Republic of Indonesia, 2008), thus meeting the requirements. After the subfraction was evaluated, the obtained subfraction was prepared for ointment. Evaluation of the ethyl acetate subfraction ointment of meniran leaves was an organoleptic test. The results of organoleptic observations of the ethyl acetate subfraction ointment of meniran leaves showed a semi-solid preparation, a distinctive odor, and a greenish color. After that, a homogeneity examination of the ointment preparation was carried out. The preparation showed homogeneity, which was indicated by the absence of lumps in the application results. The results of the ointment pH test showed that the 5% concentration ointment had a pH of 5. The ointment has a good pH value because it is acording the pH value of human skin, which is 4.5 - 6.5 (Ministry of Health, 1995).

The experimental animals used in this study were white male mice. The experimental animals were previously acclimatized for 7 days. The experimental animals used were male white mice which were divided into 3 main groups, namely the control group which was given an ointment base, the comparison group which was given a circulating ointment preparation T®, and the treatment group by giving ethyl acetate subfraction ointment of meniran leaves with each group consisting of 15 mice, from the 15 mice were grouped again into 3 groups based on the day of examination of the healing effect of the test animal's wounds, this examination was carried out on the 5th day, the 10th day and the 15th day to see the effect of administering the test preparation on the healing of excision wounds in mice.

The preparation was given to each group topically 2 times a day in the morning and evening, the duration of administration depended on the grouping of days, namely the group that received the test preparation for 5 days, 10 days and 15 days. Measurement of the wound diameter was carried out on the first and the last day of the wound. The percentage of wound healing area observed was the measurement of the initial wound area and the measurement of the final wound area on the 5th day, the 10th day, and the 15th day.

The measurement of the area of the healed wound is the first parameter used to assess the wound healing effect, where a high percentage indicates effective wound healing with the

wound size decreasing from day to day. The purpose of selecting the examination of the wound healing effect on the 5th day, the 10th day, and the 15th day was to see the effect of excision wound healing in the proliferation phase. The proliferation phase takes place on days 3-24 after the wound. In this phase, fibroblast formation occurs.

Fibroblasts are mesenchymal cells in the form of collagen fibers that play a role in wound healing, where collagen is a parameter for the formation of tissue or skin regeneration. Collagen is found in the dermis layer of the skin. The formed fibroblasts will move towards the wound area and produce a large amount of collagen matrix so that the wound is filled with fibroblasts and the wound closes. From the results of measuring the percentage of wound healing area on the 5th day, the 10th day and the 15th day, the comparison group that was applied with the T® ointment preparation gave the largest average percentage of wound healing area compared to all groups, followed by the 5% ethyl acetate subfraction ointment group. While the control group gave the smallest average percentage of wound healing area among all groups.

Duncan[a,b] test of wound healing parameter by day

Day	N	Subset 1	Subset 2	Subset 3
Day- 5	15	13.2867		
Day-10	15		43.0280	
Day-15	15			77.0847
Sig.		1.000	1.000	1.000

Duncan[a,b] test of wound healing parameter by ointment concentration

Ointment	N	Subset 1	Subset 2	Subset 3
control	15	37.6313		
5% subfraction	15		45.5393	
comparative	15			50.2287
Sig.		1.000	1.000	1.000

The second parameter is the epithelialization time, which is recorded from the first day of peeling the scab without leaving any residual wounds. From the results of observations made for 15 days on experimental animals, the epithelialization time of the 5% ethyl acetate subfraction ointment treatment group occurred on the 8th day, while the control group occurred on the 9th day, and the comparison group occurred on the 8th day.

Duncan test of epithelialization time by ointment concentration

Ointment group		N	Subset for alpha = 0.05 1	2
Duncan[a]	Comparative	10	7.60	
	5% subfraction	10	7.90	
	control	10		8.90
	Sig.		.327	1.000

The third parameter is the determination of hydroxyproline levels. The first thing to do is to determine the maximum absorption wavelength of hydroxyproline; in this study, the maximum wavelength was 559 nm. Furthermore, a calibration curve was made to obtain a regression equation. The regression equation obtained from the calibration curve using a series

of standard solutions is y = 0.1661 + 0.06025x, with a correlation coefficient (r) = 0.99878. Hydroxyproline is an amino acid with no chromophore group, so it does not have absorption in the UV-Vis region. The chromophore group is an unsaturated covalent group that provides absorption in the ultraviolet and visible regions. Therefore, derivatization is carried out to determine the hydroxyproline levels. Derivatization aims to change hydroxyproline into color, and its absorption can be read on a UV-Vis spectrophotometer. The derivatization process is carried out by making a hydroxyproline solution with the desired concentration, then adding reagents to change the hydroxyproline solution into a colored solution (Afifah, 2016). The derivatization process in this study was carried out by making a hydroxyproline solution with a concentration of 2 ppm, 4 ppm, 6 ppm, 8 ppm, 10 ppm, then adding reagents to change the hydroxyproline solution into color, the reagents added to the hydroxyproline solution are buffer, CuSO4, NaOH, H2O2, H2SO4 and 4-dimethylamino benzaldehyde.

The addition of a buffer solution or buffer solution to the hydroxyproline solution serves to maintain the pH value (acidity) so that it does not change much during the reaction by adding a strong base and dilution with water, then the solution is made alkaline by adding NaOH then adding CuSO4 solution and H2O2 solution which functions as an oxidizer. Furthermore, the solution is incubated at a temperature of 80 ° C for 5 minutes. Allowed to cool, and a PDAB (para-dimethylamino-benzaldehyde) solution was used as a complexing agent to change the solution into color and sulfuric acid, which functions as a catalyst. The chemical mechanism of this process can be described as follows.

This process involves the oxidation of amino acids to pyrrole-2-carboxylate or pyrrole, then the formation of chromophores by adding Ehrlich's reagent (p-dimethylamino-benzaldehyde) (Darwin and Sidney, 1960). The resulting compound is a very colored quinoid compound (color depends on the substituent and varies from orange-purple) (N. Yu. Ignat'eva et al., 2007). The hydroxyproline solution that has been added with PDAB solution will be yellow, then re-incubated the solution will turn red, the function of heating itself so that the reaction occurs faster. The higher the hydroxyproline content, the more concentrated the red color produced.

Derivatized hydroxyproline has absorption in the visible area. After that, the hydroxyproline levels were examined in the mouse skin tissue. The scar skin was taken; previously, the experimental animal was sacrificed first, then the skin tissue above the scar was taken using tweezers and surgical scissors. The skin tissue is then dried for 12 hours at a temperature of 60^{o}C, the aim being to dry the water content in the skin.

Next, it is hydrolyzed with 6N HCl for 24 hours at a temperature of 110 ° C to destroy or break down skin tissue into smaller pieces with the help of heating. After hydrolysis, the sample is neutralized with 2 ml of NaOH, and 1 ml of aquabides and 1 ml of buffer solution are used so that the pH remains the purpose of adding buffer to maintain the pH value because there is a mixture of strong base with strong acid and dilution with water. The sample is pipetted as much as 200 µl ad with aquadest up to 1 ml and mixed with 1 ml of 0.01 M $CuSO_4$, 1 ml of 2.5 N NaOH, and 1 ml of 6% H_2O_2, all three as oxidants.

The addition of oxidant solution functions to convert hydroxyproline into pyrrole-2-carboxylate or pyrrole. The solution is then stirred and incubated at a temperature of 80^{o}C for 5 minutes; the incubation functions so that the oxidant solution can react optimally. After the incubation process is complete, the solution is cooled and added with 4 ml of 3 N H2SO4 as a catalyst or to accelerate the reaction and 2 ml of 5% 4-dimethylamino benzaldehyde functions as a color complexing agent to change the sample to yellow, the sample is re-incubated at 70^{o}C for 16 minutes, cooled at 20^{o}C the solution will turn red, the function of self-heating so that the reaction occurs faster. The higher the hydroxyproline content, the more concentrated the red color produced. Hydroxyproline resulting from derivatization has absorption in the visible region. The visible region is in the 380-780 nm region. Determination of hydroxyproline levels is carried out on the 5th day, the 10th day, and the 15th day after the wound because these days have entered the proliferation phase where the proliferation phase of fibroblast formation occurs.

Fibroblasts will synthesize collagen, which is the main element of the extracellular matrix which is useful for forming scar tissue strength in wounds. The amount of collagen in the skin can be determined by measuring hydroxyproline levels. From the calculation results of the percentage of hydroxyproline levels, the comparison group gave the highest average percentage of hydroxyproline levels compared to all groups, followed by the treatment group that was applied with ethyl acetate subfraction ointment with a concentration of 5%. The group that was applied with vaseline flavum gave the smallest average percentage of hydroxyproline levels among all groups. Based on the calculation results of the percentage of hydroxyproline levels in this study, it was seen from the maximum wavelength, determination of the regression equation and absorbance value in the sample solution.

Duncan[a,b] test of hydroxyproline levels by day

		Subset		
Day	N	1	2	3
Day-5	9	.7600		
Day-10	9		1.6911	
Day-15	9			2.2811
Sig.		1.000	1.000	1.000

Duncan[a,b] test of hydroxyproline levels by ointment concentration

		Subset		
Ointment	N	1	2	3
control	9	1.2167		
5%-subfraction	9		1.6444	
Comparative	9			1.8711
Sig.		1.000	1.000	1.000

From the results of Duncan's further test, the control group was significantly different from the treatment group. The 5% ethyl acetate subfraction ointment group had good results compared to the control group, which was only given an ointment base. This indicates the effect

of giving ethyl acetate subfraction of meniran leaves on the ointment base. The results of previous studies support the results of this study.

According to research conducted by (Siahaan, et al., 2017), giving meniran leaf extract gel (Phyllanthus niruri L.) can increase epithelialization of wound tissue in male Wistar rats. Meanwhile, Kurhasi et al. (2015) also proved that meniran leaf extract can accelerate wound healing because it protects skin tissue from oxidative damage due to free radicals.

The ethyl acetate subfraction obtained contains flavonoid, phenolic, and steroid compounds. The journal (Harrizul et al., 2013) showed that meniran herbs contain lignin and terpenoid compounds, which have the potential as antibacterials. The journal (Siahaan et al., 2017) shows that flavonoids can accelerate the wound healing process by increasing the rate of wound contraction, approaching the epithelialization period, increasing collagen deposition, and forming granulation tissue. Flavonoid cells and meniran leaf extract also contain other active compounds such as phenols, tannins, saponins, and sterols. These active compounds act as antioxidants that affect wound contraction and increase the rate of epithelialization.

In the study (Ahmed et al., 2012) it was proven that in a group of mice injured with excision wounds and given 5% meniran leaf extract, it could reduce the width of the scar, increase fibroblast proliferation, and increase the amount of collagen and angiogenesis on the 13th day while in the study (Gea., 2020) using the ethyl acetate fraction showed that on the 10th day there was an increase in fibroblast proliferation, as well as an increase in the amount of collagen and angiogenesis.

Conclusion

Based on the results of the study on the healing effect of excision wounds with 5% concentration of ethyl acetate subfraction ointment of meniran leaves (*Phyllanthus niruri* L.) on male white mice, it was concluded that administration of 5% concentration of ethyl acetate subfraction ointment of meniran leaves can provide a better effect in the wound healing process compared to the control group. Based on the results of statistical analysis with the ANOVA test, a significant value ($p < 0.05$) was obtained so that it can be concluded that there is a significant difference between the treatment group and the control group and Duncan's further test showed that the treatment group was significantly different compared to the control group but not significantly different from the comparison group. This indicates that ethyl acetate subfraction of meniran leaves has an effect as an excision wound therapy. From this study, it is suggested to further researchers to test the healing activity of infected wounds in mice using bacterial media to see the antibacterial activity of compounds in meniran plants and measure hydroxyproline levels as a parameter for healing infected wounds.

Declaration of Conflicting Interest

The authors declare that there is no conflict of interest in this work.

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