World Journal of Pharmaceutical Science and Research
www.wjpsronline.com
Research Article
ISSN: 2583-6579
SJIF Impact Factor: 5.111
Year - 2025
Volume: 4; Issue: 2
Page: 12-28

THE EFFECT OF 10% ETHYL ACETATE SUBFRACTION OINTMENT OF MENIRAN LEAVES (*PHYLLANTUS NIRURI* L.) ON THE EXPRESSION OF TNF-A IN WHITE MALE RATS

Sanubari Rela Tobat[*1,2], Satya WydyaYenny[3], Fatma Sri Wahyuni[4], Etriyel MYH[5], Suvina Rahmadani[2], Mimi Aria[2], Puspa Pameswari[2], Epi Supriwardi[2], Sandra Tri Juli Fendri[2]

[1]Doctoral Program, Department of Biomedical Sciences, Faculty of Medicine, Andalas University, Padang, West Sumatra, 25163, Indonesia.
[2]Faculty of Pharmacy, Perintis University Indonesia, Padang, West Sumatra, 25586, Indonesia.
[3]Department of Dermatology and Venereology, Faculty of Medicine, Andalas University, Padang, West Sumatra, 25163, Indonesia.
[4]Faculty of Pharmacy, Andalas University, Padang, West Sumatra, 25163, Indonesia.
[5]Surgical Department, Faculty of Medicine, Andalas University, Padang, West Sumatra, 25163, Indonesia.

Article Received: 13 February 2025 | | Article Revised: 03 March 2025 | | Article Accepted: 25 March 2025

***Corresponding Author: Sanubari Rela Tobat**
Doctoral Program, Department of Biomedical Sciences, Faculty of Medicine, Andalas University, Padang, West Sumatra, 25163, Indonesia.
DOI: https://doi.org/10.5281/zenodo.15108730

How to cite this Article: Sanubari Rela Tobat, Satya Wydya Yenny, Fatma Sri Wahyuni, Etriyel MYH, Suvina Rahmadani, Mimi Aria, Puspa Pameswari, Epi Supriwardi, Sandra Tri Juli Fendri (2025). THE EFFECT OF 10% ETHYL ACETATE SUBFRACTION OINTMENT OF MENIRAN LEAVES (*PHYLLANTUS NIRURI* L.) ON THE EXPRESSION OF TNF-A IN WHITE MALE RATS. World Journal of Pharmaceutical Science and Research, 4(2), 12-28. https://doi.org/10.5281/zenodo.15108730

ABSTRACT

Research has been carried out on the effect of giving meniran leaves (*Phyllanthus niruri* L.) in the form of an ointment with a concentration of 10% on the expression of TNF-α in male white rats. This study aims to determine the effect of giving meniran leaves (*Phyllanthus niruri* L.) in healing excision wounds assessed by the percentage of wound healing area, epithelialization time and TNF-α expression, on days 5, 10 and 15 of skin tissue. Experimental animals were grouped into 2 groups, namely, group 1 as a control by administering an ointment base (vaseline flavum) and group 2 as a treatment group, namely, by administering 10% meniran leaf (*Phyllanthus niruri* L.*)* ethyl acetate subfraction ointment. Both groups were observed and measured the percentage of wound healing, epithelialization time and the expression of TNF-α in inflammatory cell tissues (neutrophils, macrophages, and lymphocytes) on days 5, 10 and 15. The results of data analysis using one-way ANOVAfollowed by the Duncan test showed that there was significant difference between the control group and the treatment group in the percentage of wound healing area, epithelialization time and TNF-α expression ($p<0.05$), so it can be concluded that the preparation of meniran leaf ethyl acetate subfraction ointment with a concentration of 10% has an effect as excisional wound therapy.

KEYWORDS: *Phyllanthus niruri* L, *ethyl acetate subfraction, excision wounds, and wound healing, TNF-α expression.*

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 tearing of blood vessels which will result in bleeding.[1,16] One type of wound is an excision wound, where an excision wound is a wound caused by tissue being cut due to a scratch from a sharp object, usually such as a knife and so on.[2] Based on data from the United States Emergency Department in 2005, 11.8 million wounds were treated and more than 7.3 million lacerations were treated per year. Incision or puncture wounds account for approximately 2 million patients treated each year. The prevalence in Indonesia for open wound injuries is 25.4%, with the highest prevalence in Central Sulawesi province at 33.3%. Based on age groups, the highest prevalence of open wounds is in the 25 to 34-year age group (32%).[3]

When the body experiences an injury, an inflammation process occurs, which is the initial stage in repairing damaged tissue. During the inflammatory process, the role of inflammatory cells in the tissue or in the blood circulation, chemical mediators produced from inflammatory cells, endothelium, or chemical mediators dissolved in plasma (for example complement, blood clotting factors, etc.) is required.[4,17,27,38] Inflammatory cells consist of mononuclear inflammatory cells (monocytes and lymphocytes) and polymorphonuclear inflammatory cells (basophils, eosinophils, and neutrophils). Under normal conditions, these cells are in the blood vessels, but when injury occurs, these cells will emigrate into the dermis tissue, especially neutrophils.[5,33] Tumor Necrosis Factor Alpha (TNF-α) is a type of proinflammatory cytokine produced by neutrophils and macrophages. When an injury occurs, TNF-α level will increase. Increased levels of TNF-α can induce the release of the endothelial adhesion molecule, namely, intercellular 3 adhesion molecule 1 (ICAM-1), which will increase the attachment of neutrophils to endothelial cells before entering the extravascular space or intracellular space. Other inflammatory products will cause neutrophil chemotaxis towards the injured tissue.[6]

The main goal of wound treatment is to restore the function and shape of skin tissue to normal with as few local complications as possible. When an injury occurs, the tissue will undergo a healing process which is a complex phenomenon involving several processes.[7,18,26,35,39] There are many substances that can speed up wound healing, including several plant extracts, even though this use is actually a habit carried out by traditional communities.[8,19,28] Therefore, plants are increasingly being chosen as alternative and natural medicines for the treatment of various diseases. However, there is still not much research that examines their efficacy scientifically.[9]

To overcome the problem of healing excision wounds, a preparation is needed that has good penetration power and a long contact time. One of the preparations chosen is the ointment. The formulation of the ointment will affect the amount and speed of the active substance that will be absorbed. The active substance in the ointment is included in a base or carrier that will bring the medication into contact with the skin surface. The vehicle used for topical preparations will have beneficial effects if chosen appropriately.[10]

One of the herbal plants that play a role in the wound healing process is meniran (*Phyllanthus niruri* L). This plant can be found in almost all regions of Indonesia. Meniran phytochemical studies characterized the presence of various compounds such as lignans (Phyllanthin, hypophyllanthin), flavonoids, and tannins.[11,20,25,31] Meniran herb also has pharmacological activity as anti-inflammatory, antihistamine, antifungal, anti-oxidant and antimicrobial.[12,21-24,29-30,40,42,43] Based on the activity of meniran (*Phyllanthus ninuri*L), it needs to be developed into a pharmaceutical preparation to increase its use. This effort was made to facilitate the use of the active compounds in meniran

(*Phyllanthusniruri* L) for wound healing, so a topical preparation in the form of an ointment was formulated. The ointment dosage form is appropriate for skin treatment because the contact between the drug and the skin is longer and has the appropriate consistency, so it is easy to use.[13]

In previous research, testing ointment from the ethyl acetate fraction of meniran leaves (*Phyllanthus niruri* L) with a concentration of 10% affected wound healing in male white rats as seen in the percentage of hydroxyproline content, where treatment with a concentration of 10% had a better wound healing effect.[14] Meanwhile, according to the research conducted by Siahaan *et al*, 2017, administration of meniran (*Phyllanthus niruri* L) leaf extract gel increased wound tissue epithelialization in male Wistar white rats. Based on the description above, researchers are interested in continuing research on the effect of administering meniran leaf ethyl acetate subfraction ointment (*Phyllanthus niruri* L) with a concentration of 10% to male white rats with the observed parameters being the percentage of wound healing area, epithelialization time, and TNF-α expression in white male rats.[15]

Research Objectives: 1). To determine the effect of administering ethyl acetate subfraction ointment of meniran leaves (*Phyllanthus niruri* L) with a concentration of 10% on the percentage of wound healing, epithelialization time, and expression of TNF-α in male white mice. 2). To determine the effect of the duration of administration of meniran leaf ethyl acetate subfraction ointment (*Phyllanthus niruri* L) with a concentration of 10% on days 5, 10, 15 on the percentage of wound healing, epithelialization time, and TNF-α expression in male white mice.

RESEARCH METHODOLOGY

The research was carried out from September to December 2023 at the Pharmacology Research Laboratory, Faculty of Pharmacy, Indonesian Pioneer University and the Anatomical Pathology Laboratory, Faculty of Medicine, Andalas University.

Tools

Rat cage, cotton wool, hair clipper, scissors, droppers, rulers, razor blade (tiger®), digital weighing scale, animal scale, gloves, mask, oven, stir bar, knives, tweezers, measuring cup, *beaker glass*, tongs, spatula and universal pH stick, rotary microtome, Heat induced epitope, microwave, deck glass.

Material

The materials used in this research were rat food and drink, 100%, 96%, 70% ethanol, distilled water, vaseline, flavum, 10% meniran leaf ethyl acetate subfraction ointment, isoflurane, 5 % lidocaine prilocaine cream, formalin, poly- L-lysine, deparaffinization, xyline, distilled water, cutrate buffer pH 6, PBS pH 7.4, H2O2 , sodium goat serum (NGS), goat anti-rabbit IgG antibody (H+L), biotinylated BA- 1000 -1.5, avidin biotin, HCl buffer, hematoxylin, entellan.

Sampling

The samples used were fresh meniran leaves (*Phyllanthus niruri* L.) were taken from Tampuni, Kenagarian KambangTimur, Lengayang District, Pesisir Selatan Regency, and West Sumatra.

Sample Identification

Sample identification was carried out at the Andalas University Herbarium, Department of Biology, Faculty of Mathematics and Natural Sciences, Andalas University, Padang.

Preparation of Ethyl Acetate Subfraction of Meniran Leaves

A fresh sample of 9000 g was air-dried to obtain a dry sample of 2.700g, which was then ground to become powder. The simplicia powder was macerated using 70% ethanol solvent for 3 days. Then filter the results of the maceration, get macerate and dregs, and do the remaceration until you get a clear brown coloredmaserate. The resulting maserate was evaporated using *a Rotary Evaporator* until a thick extract was obtained, and then fractionated using ethyl acetate (1:2), and the resulting fraction was evaporated to obtain a thick ethyl acetate fraction.

Next, separation was carried out using column chromatography to obtain a thick subfraction of meniran leaf ethyl acetate. Silica gel was used as the stationary phase, while the mobile phase used was hexane and ethyl acetate in a ratio of (2:1). The column chromatography results obtained were grouped based on spot pattern and rf value monitoring. Monitoring was carried out using TLC plates eluted with the mobile phase n-hexane: ethyl acetate (4:1).

Evaluation of Ethyl Acetate Subfractions

1. Organoleptic Examination

The inspection was carried out visually, namely, by observing the shape, color, and smell.[44]

2. Determination of Sub-Faction Yield

The subfraction yield is calculated using the equation:

$$\% \text{ Yield} = \frac{Subfraction\ Powder\ Weight}{Condensed\ \ Fraction\ Weight}\ x\ 100\%$$

3. Drying Shrinkage Check

Dry porcelain crucible and lid in the oven at 105^0 C for 30 minutes, then let it cool and then weigh it. Put the subfraction into the crucible until the weight is 1-2 grams beyond the weight of the previously known crucible with lid. Shake the crucible gently so that the extract is evenly distributed and put it back in the oven, open the lid, and leave the lid in the oven. The crucible containing the subfraction was heated in an oven at 105^0 C until constant weight. After that, the crucible is removed and cooled in a desiccator, then weighed. Repeat as above until a constant weight is obtained.[44]

Calculate the drying loss using the formula:

$$\% \text{ drying shrinkage} = \frac{(B-A)(C-A)}{(B-A)}\ x\ 100\%$$

Explanation:

A = Weight of empty crucible

B = Crucible weight + ethyl acetate subfractionation before drying

C = Crucible weight + ethyl acetate subfractionation drying

Preliminary Examination of Chemical Content

The ethyl acetate subfraction from meniran leaves (*Phyllanthus niruri* L.) was put into a test tube, added with 5 ml of distilled water and 5 ml of chloroform acetate, left until two layers were formed.[45]

a. Flavonoid Test (Cyanidine Test Method)

Take a layer of 1-2 drops of water, drop it on the drip plate, and then add Mg powder and HCl (p), the formation of a red color indicates the presence of flavanoids.

b. Saponin Test

Take a layer of water, then shake vigorously in a test tube, then permanent foam forms (± 15 minutes) indicating the presence of saponins.

c. Terpenoid and Steroid Test ("Simes" Method)

Take a small layer of chloroform using a dropper containing cotton wool and norit. Drop the filtrate on the drip plate. Let it dry. The residue is added to 1 drop of anhydrous acid and 2 drops of H_2SO4 (p), the formation of a purple blue color indicates the presence of steroids, if a red color is formed, it indicates the presence of terpenoids.

d. Alkaloid Test ("Culvenore - Fristgerald" Method)

Take a small layer of chloroform and add 10 ml of 0.05 N chloroform ammonia, stir slowly, then add 2-3 drops of H_2SO4 2N then shake gently, and let stand until separated. Acid layers adding 2 drops of Mayer's reagent, a positive alkaloid reaction is indicated with white mist to white lumps.

e. Phenolic Test

Take a layer of 1-2 drops, drop it on the drop plate, and then add the FeCl3 reagentto form a blue color indicating the presence of phenolic content.

Preparation of subfraction ointment

The ointment preparation that will be made in this research has a concentration of 10% meniran ethyl acetate subfraction and the preparation that will be made is 30 g. Add 3 grams of meniran leaf subfraction (*Phyllathus niruri L*) into a mortar, then weigh 27 g of the ointment base (vaselin flavum), put it in a mortar and then grind until homogeneous. Remove from the mortar; put it into the prepared container.

Evaluation of Meniran Leaf Subfraction Ointment

1. Organoleptic Examination

The inspection is carried out visually, namely, by observing the shape, color, and smell.[44,45]

2. Homogeneity Check

The examination were carried out by smearing 0.1 gram of the preparation mass on a glass object, then leveling it with another glass object at a slope of 45^0 , pulling quickly with the same pressure. The arrangement was observed under a microscope and no coarse grains were visible.[44]

3. Ointment pH Check

The pH value was measured using a universal pH stick dipped in 0.5 grams of ointment. The pH value of a good ointment is 4.5 - 6.5 in accordance with the pH value of human skin.[44]

Experimental Animal Preparation

Testing the effect of administering the ethyl acetate subfraction of meniran leaves on wound healing will be carried out using experimental animals of male white rats weighing ±200 grams. A total of 18 mice were divided into 2 main groups, and then each group was examined on days 5, 10, and 15 after the mice were given excision wounds. On each day of examination, the animals were sacrificed using isoflurane and then the skin tissue of the experimental animals was taken for examination of TNF-α. Before use, mice were first acclimatized for 7 days. Animals are declared healthy if during acclimatization they do not show deviations in body weight of more than 10% and visually there are no symptoms of disease.

The day before the wound was made, the fur of the experimental animal was shaved on the back where the incision would be made, and then cleaned using cotton wool treated with 70% alcohol, and then anesthetized the mouse using isoflurane. Next, a circular wound was made with a diameter of ±2 cm and a depth of ±1 mm by lifting the rat's skin on the back with tweezers and then cutting it with surgical scissors.[46] In the research, each group of mice was given the following treatment:

- Group I (control) is a group of mice that will be given wounds without being given treatment and only smear vaselineflavum ointment based on the wound and check the percentage of wound healing area, epithelialization time, and expression of TNF-α in male white mouse on the 5^{th}, 10^{th}, and 15^{th} days.
- Group II (treatment) was a group of mice that applied subfraction ointment with a concentration of 10% to the wound and examined the percentage of wound healing area, epithelialization time, and expression of TNF-α in male white mice on days 5, 10, and 15.

Wound Healing Activity Assay

- The ointment wasapplied to the back of the rat 2 times/day.
- Rats were also given an anesthetic/analgesic cream, namely, 5% lidocaine prilocaine cream to treat pain 2 times /day.
- The ointment preparation was given to groups of mice that had been grouped.
- Next, the wound healing parameters were observed.[46]

Wound Healing Parameters

The percentage of wound healing area by calculating the wound area on the first day after being injured, and then the wound healing area on days 5, 10, and 15 in each group. Then look for the percentage of wound healing which is calculated using the formula:

$$\% \text{ wound healing} = \frac{initial\,wound\,area - final\,wound\,area}{initial\,wound\,areal} \; x\; 100\%$$

The time required for the formation of new epithelium to completely cover the wound area. In this case, the day the scar tissue peels from the wound is recorded without leaving a scar residue in the excision area.[47]

Immunohistochemical Observations

a. Immunohistochemical staining procedure

Paraffin blocks were cut with a rotary microtome to a thickness of 4 µm and placed on a glass coated with Poly-L-lysine. After that, deparaffinize with Xylene, then rehydrate with graded alcohol starting with ethanol 100%, 96%,

70%, distilled water for 5 minutes each. Epitope retrieval with heat induced epitope retrievalusing a microwave for 10 minutes in citrate buffer pH 6. Washing in PBS pH 7.4 3x5 minutes. Endogenous peroxidase blocking with 3% H_2O_2 in PBS pH 7.4 for 3 minutes, followed by 0.3% H_2O_2 in PBS pH 7.4 for 30 minutes. Washing in PBS pH 7.4 3x5 minutes. Non-specific protein blockwith 2% Normal Goat serum *(NGS)* in PBS pH 7.4 for 20 minutes at room temperature. Application of primary antibody and incubation in a humid chamber overnight 4^oC TNF α; dilution 1:100 (TNF alpha Polyclonal Antibody), washed in PBS pH 7.4 3x5 minutes. Incubated with secondary antibody at room temperature for 30 minutes using *Goat Anti-Rabbit IgG Antibody* (H+L), Biotinylated BA-1000-1.5, and dilution 1:100. Washing in PBS pH 7.4 3x5 minutes. Incubate with avidin biotin complex at room temperature for 30 minutes, at room temperature for 30 minutes. Application of DAB chromogen in Tris HCl buffer pH 7.6. Wash with distilled water for 3x5 minutes. Counterstaining with hematoxylin. Wash with distilled water for 10 minutes. Bluing in saturated solutions of lithium carbonate. Wash with distilled water for 10 minutes. Dehydration in graded alcohol; starting with 70%, 96%, 100% ethanol, 5 minutes each. Clearingin Xylene 2 x 5 minutes. Mounting deck glass with entellan.

b. Procedures for assessing TNF alpha expression by immunohistochemical staining

TNF-alpha expression appears as a brown color on immunohistochemical staining. The TNF-alpha staining pattern is matrix and cytoplasmic, with brown color detected in the extracellular matrix and cell cytoplasm. Area measurements were carried out by taking photomicrographs at 400x magnification (objective 40x), in 5 different fields, in the area of postwound granulation tissue. Expression measurements were carried out by measuring the proportion of the area (stained area fraction)colored brown, by isolating the area stained brown, binary conversion and measuring the area proportion to the ImajeG program (Image J 1.49v software, National Institute of Health, Bethesda, MD, USA). The size of the positively stained area is reported in percent area.

3.5.4 Data Analysis

The data analysis used in this research is analysis of variance (ANOVA). This ANOVA is used because the data obtained is objective, categorical, and numerical. In this study, data from wound healing parameters, namely, the results of the percentage of wound healing area, epithelialization time, and immunohistochemistry were used for statistical analysis using one-way ANOVA. If the results obtained are significant ($p<0.05$). Data analysis was continued with the Duncan test which aims to determine the significance of the differences in results between each treatment group.

RESULTS AND DISCUSSION

After conducting research on the effect of administering ethyl acetate subfraction ointment of meniran leaves (*Phyllanthus niruri* L) on the expression of TNF-α in male white mice, the following results were obtained:

1. Based on the results of sample identification, it shows that the sample used in this research is a meniran leaf plant (*Phyllanthus niruri* L.) from the family Phyllanthaceaewith sample identification code number: 615/K-ID/ANDA/IX/2023
2. Based on the information that passed the ethical review with number 543/UN.16.2/KEP-FK/2023 we have approved the protocol for this study.
3. The results of organoleptic observations of the meniran leaf ethyl acetate subfraction ointment showed that it was a semisolid preparation, dark green in color and contained white crystals, and had a distinctive smell.
4. The results of examining the pH of the meniran leaf ethyl acetate subfraction ointment showed a pH of 5.43 in the ointment preparation with a concentration of 10%.

5. The results of measuring the percentage of average wound healing area in the control group and 10% concentration, respectively, are:
   - Day 5 is 52.64% and 53.58%.
   - Day 10 is 73.15% and 85.64%.
   - Day 15 is 96.30% and 99.49%.
6. The average epithelialization time in the control group and 10% concentration, day 10 and day 8.
7. The results of the examination of TNF-α expression on day 5 of the control group and 10% concentration, respectively, were:
   - Day 5 is 50.15% and 43.68%.
   - Day 10 is 60.61% and 43.05%.
   - Day 15 is 63.70% and 55.22%.

DISCUSSION

In research regarding the effect of healing excision wounds, the sample used was an ethylacetate subfraction ointment of meniran leaves (*Phyllanthus niruri* L.) as a test material. Meniran plants were taken from Tampuni, Kenagarian KambangTimur, Lengayang District, Pesisir Selatan Regency, West Sumatra. Before the research was carried out, the samples were first identified in the ANDA herbarium, Department of Biology, Faculty of Mathematics and Natural Sciences, Andalas University. Identification is the first step so that the identity of the sample can be known so that there are no errors regarding the plants used in the research.

Evaluation of meniran leaf ethyl acetate subfraction ointment was an organoleptic test with a semisolid dosage form, dark green in color and containing white crystals and a distinctive smell, a homogeneous preparation characterized by the absence of lumps on the smear. Then a pH test of the ointment was carried out using a universal pH stick tool where the ointment preparation had a concentration of 10%, pH 5.43. This ointment has a good pH because it corresponds to the pH of human skin, namely, 4.5-6.0.[44]

The experimental animals used in this research were male white rats which had previously been acclimatized for 7 days. The experimental animals used were divided into 2 groups, namely, group 1 (control), rats which were injured and smeared with an ointment base (Vaseline flavum), and group 2 (treatment) rats which were injured and smeared with 10% concentration of meniran leaf ethyl acetate subfraction ointment. The total number of experimental animals was 18 mice, divided into 2 groups, with each group consisting of 9mice. This examination was carried out on the 5th day, 10th day, and 15th day.[32]

Topical ointment was given to each group twice a day in the morning and evening, given for 15 days at a rate of ±80 mg with the aim of observing wound healing in the proliferation phase. In this proliferation phase, the role of fibroblasts is very dominant in the repair process, which plays a role in preparing to produce protein products that will be used during the tissue reconstruction process. This proliferation phase starts from the third day and ends in two weeks marked by the formation of granulation tissue in the wound.[34,37,48]

Measuring the percentage of wound area that heals is the first parameter used to assess the effect of effective wound healing as the size of the wound decreases from day to day. The purpose of choosing to examine the effect of wound healing on day 5, day 10, and day 15 was to see the effect of healing excision wounds in the proliferation phase. In this

phase, fibroblast formation occurs. Fibroblasts are masenchymal cells in the form of collagen fibers which play a role in wound healing, where collagen is a parameter for tissue formation or skin regeneration. Collagen is found in the dermis layer of the skin. The formed fibroblasts will move towards the wound area and will produce large amounts of collagen matrix so that the wound fills and the wound closes.

From the results of measuring the percentage of wound healing on the 5th day, the 10th day, and the 15th day, the group of animals given 10% meniran leaf ethyl acetate subfraction ointment produced a higher average percentage of wound healing than the control group, especially on day 10 of wound healing. Wound diameter measurements were carried out on day 5, day 10, and day 15 to calculate the percentage of wound healing. The percentage of wound healing observed was the initial area measurement with final area measurements on the 5th day, 10th day, and 15th day. A high percentage was indicated by the smaller the size of the wound, the better the wound healing.

Table 1: Results of measuring the percentage of wound healing area on the 5th day.

Group	Animal	Initial Diameter	Final Diameter	Initial wound area	Final wound area	% wound healing
Control	1	2.83	1.83	6,286	2,628	58.19
	2	2.70	2.00	5,721	3,140	45.11
	3	2.76	1.36	5,978	2,712	54.63
Mean ± SD		2.76 ± 0.07	1.73 ± 0.33	6.00 ±0.28	2.83 ±0.27	52.64 ± 6.76
Subfraction 10%	1	2.90	1.93	6,600	2,923	55.71
	2	2.93	2.03	6,738	3,234	52.00
	3	2.86	1.96	6,418	3,014	53.00
Mean ± SD		2.90 ± 0.04	1.97 ± 0.05	6.59 ± 0.16	3.06 ± 0.16	53.58 ± 1.92

Table 2: Results of measuring the percentage of wound healing area by day10th.

Group	Animal	Initial Diameter	Final Diameter	Initial wound area	Final wound area	% wound healing
Control	1	2.83	1.50	6,286	1,764	71.93
	2	2.70	1.03	5,721	0.832	85.45
	3	2.76	1.70	5,978	2,267	62.10
Mean ± SD		2.76 ± 0.07	1.41 ± 0.34	6.00 ± 0.28	1.62 ± 0.73	73.15 ± 11.74
Subfraction 10%	1	2.90	1.03	6,600	0.832	87.39
	2	2.93	1.20	6,738	1,130	83.22
	3	2.86	1.03	6,418	0.879	86.30
Mean ± SD		2.90 ± 0.04	1.09 ± 0.10	6.59 ± 0.16	0.95 ± 0.16	85.64 ± 2.16

Table 3: Results of measuring the percentage of wound healing area per day15th.

Group	Animal	Initial Diameter	Final Diameter	Initial wound area	Final wound area	% wound healing
Control	1	2.83	0.60	6,286	0.282	95.50
	2	2.70	0.43	5,721	0.144	97.48
	3	2.76	0.56	5,978	0.244	95.91
Mean ± SD		2.76 ± 0.07	0.53 ± 0.09	6.00 ± 0.28	0.22 ± 0.07	96.30 ± 1.04
Subfraction 10%	1	2.90	0.16	6,600	0.018	99.72
	2	2.93	0.20	6,738	0.031	99.53
	3	2.86	0.26	6,418	0.050	99.22
Mean ± SD		2.90 ± 0.04	0.21 ± 0.05	6.59 ± 0.16	0.03 ± 0.02	99.49 ± 0.25

Based on the results of normality and homogeneity tests on the measurement data, the results were normally and homogeneously distributed as indicated by a significant value in each experimental group > 0.05. Because the parametric statistical assumption test has been met, further analysis can use the one-way ANOVA test. The results of statistical calculations of one-way analysis of variance (ANOVA) on wound healing in test animals showed significant differences expressed as P<0.05 between the control group and the 10% subfraction on days 5, 10 and 15.

The second parameter is epithelialization time. Epithelialization time is the time recorded from the first day of spontaneous scab peeling without leaving residual wounds. From the results of observations carried out for 15 days in experimental animals in the treatment group with 10% ethyl acetate subfraction, the average epithelialization time was on the 8th day, and in the control group the epithelialization time was on the 10th day.

Table 4: Epithelialization Time.

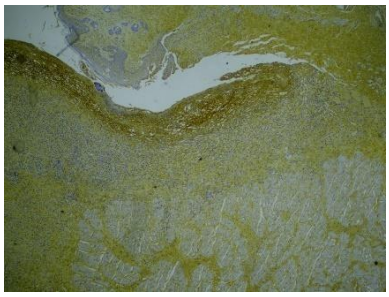
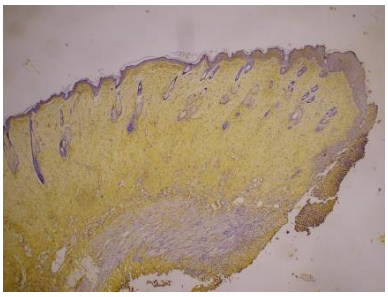
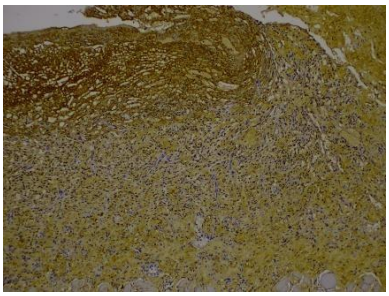
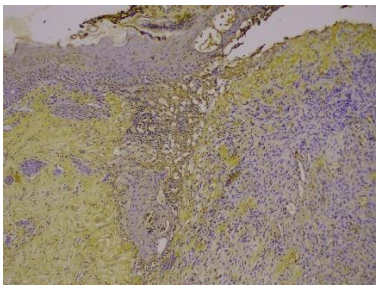
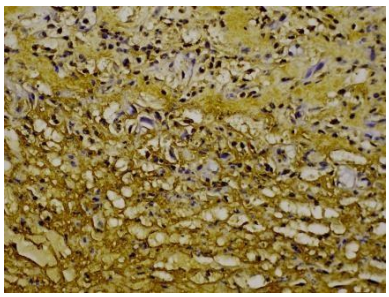
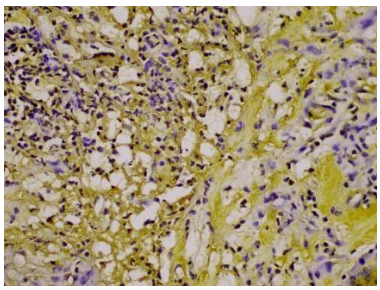
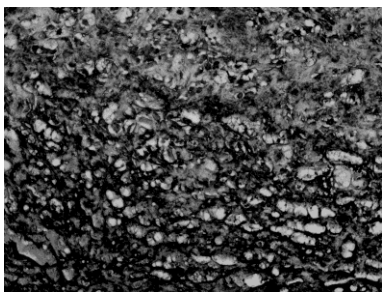
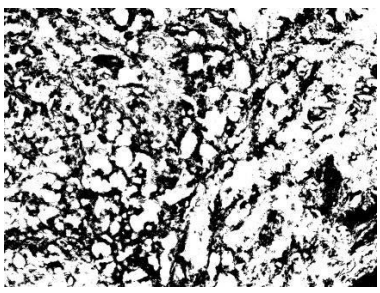
Group	Animal	Epithelialization time	Average ± SD
Control	1	9	9.67 ± 0.58
	2	10	
	3	10	
Subfraction 10%	1	8	7.67 ± 0.58
	2	7	
	3	8	

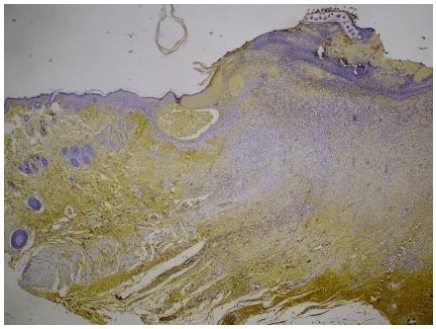
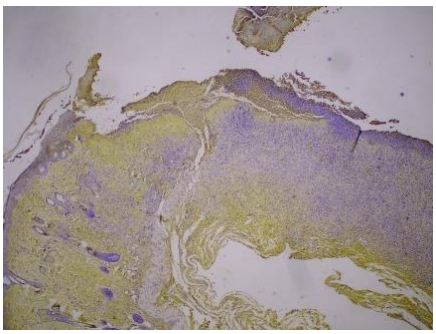
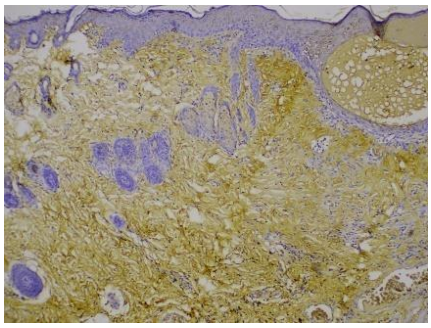
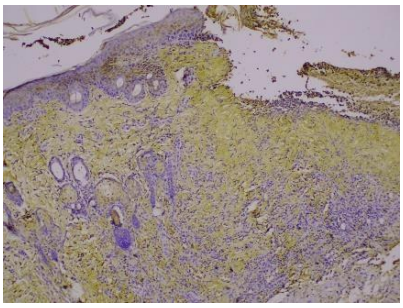
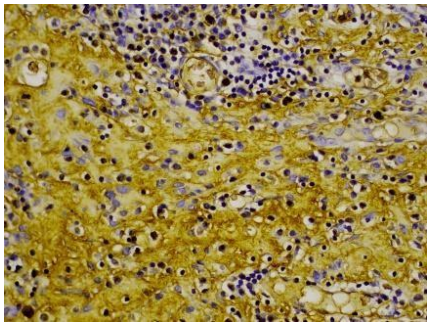
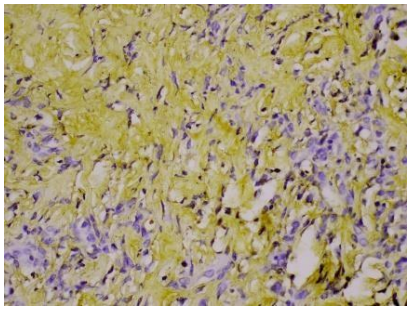
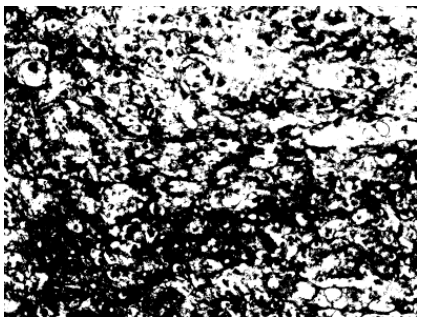
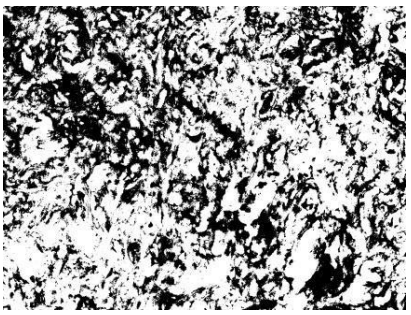
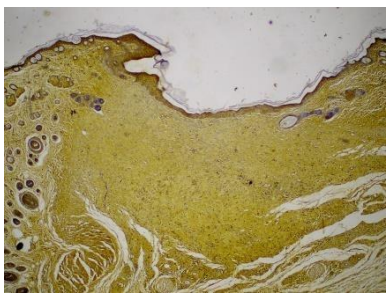
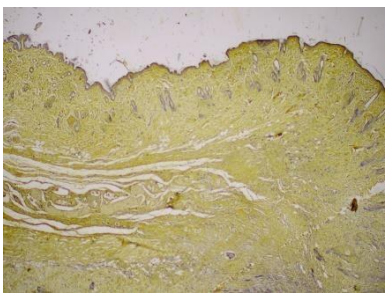
The third parameter is examining the expression of TNF-α in wound tissue. The description of TNF-alpha expression in skin tissue after excision of experimental animals shows differences between the control group and the treatment group. Meanwhile, TNF-alpha expression appears as a brown color in the cytoplasm of inflammatory cells and extracellular matrix stroma in the granulation area of the dermis after excision wound. In the control group on days 5, 10, and 15, TNF-alpha expression appeared high with strong intensity in the granulation area. Treatment with a 10% subfraction showed TNF alpha expression with color intensity and an area that was lower than the control on the same day. Lower TNF alpha expression indicates a lower inflammatory process, this means that the 10% subfraction influences the wound healing process by suppressing proinflammatory cytokines, thus providing better wound healing results compared to the control. It is known that high expression of TNF-alpha indicates excessive inflammation and fibrosis reactions, but too low expression also suppresses the healing process. TNF expression plays a role in wound healing which functions to epithelialize the surface, however, excessive expression will interfere with healing including the formation of wound epithelialization. This is the same as the microscopic picture of wounds in treatment with meniran subfraction, where the epithelium is thicker and more complete than the control.

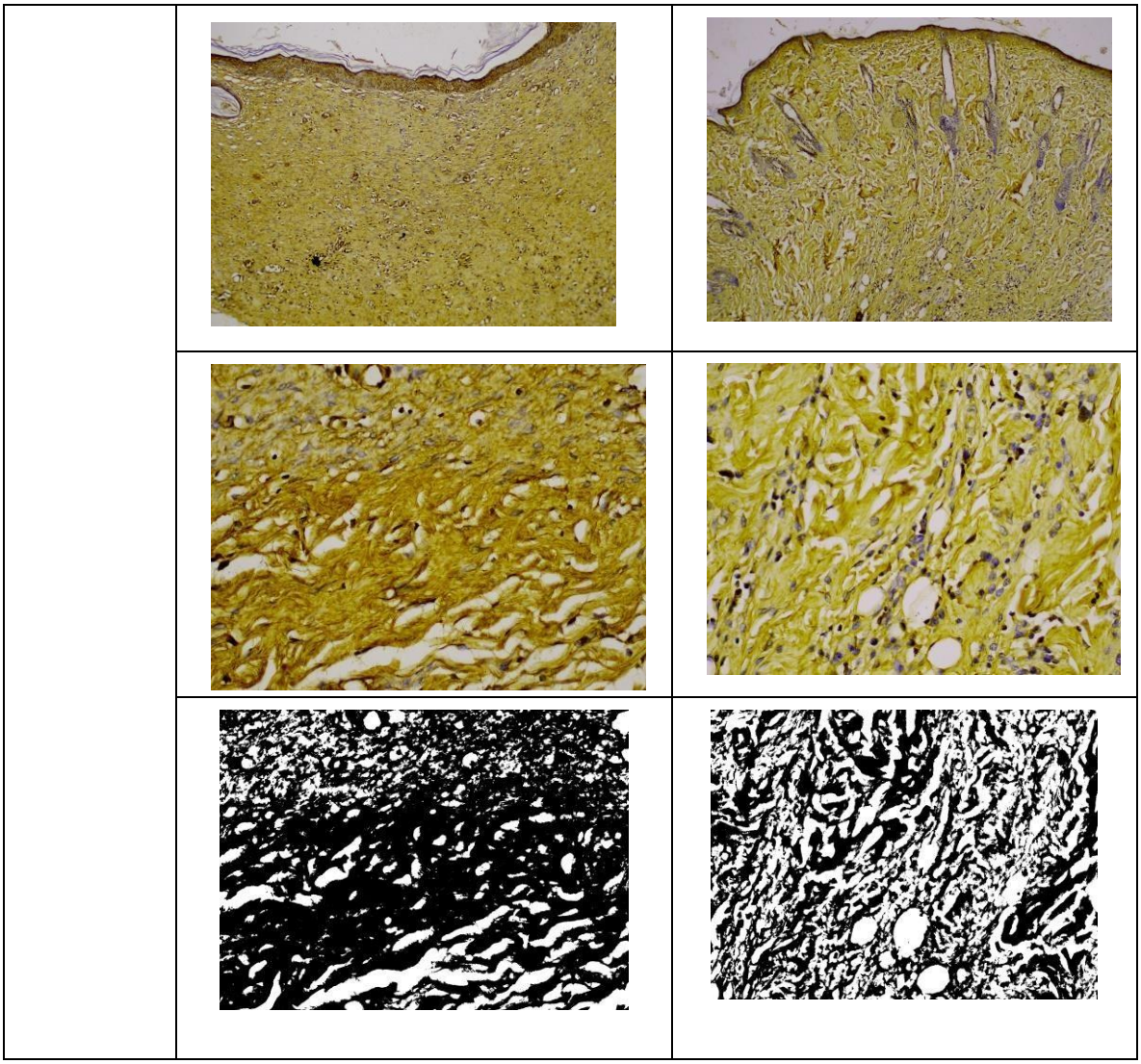
Table 5: Evaluation of TNF-α Expression Immunohistochemically.

Day	Group	Sample	TNF-α expression (% area)						
			Field of view						Group mean
			LP 1	LP 2	LP 3	LP 4	LP 5	Average	
5th	**Control**	1	56.08	50.08	51.22	46.31	59.12	52.56	
		2	40.32	47.33	46.33	65.76	45.09	48.97	
		3	46.43	45.53	61.01	48.33	43.28	48.92	**50.15**
	Subfraction 10%	1	42.52	41.59	43.03	42.52	44.17	42.77	
		2	45.51	45.52	41.52	42.53	45.54	44.12	
		3	44.52	42.12	42.53	46.51	45.12	44.16	**43.68**
10th	**Control**	1	56.54	53.54	63.83	66.58	63.81	60.86	
		2	66.54	65.51	65.54	55.54	52.51	61.13	
		3	57.52	57.58	66.54	66.11	51.52	59.85	**60.61**

	Subfraction 10%	1	39.72	36.82	37.74	38.71	37.77	38.15	
		2	43.52	49.62	39.75	44.74	46.72	44.87	
		3	39.72	44.75	49.76	49.72	46.75	46.14	**43.05**
15th	**Control**	1	74.21	64.26	74.21	64.25	74.24	70.23	
		2	54.26	54.25	54.26	46.23	54.21	52.64	
		3	64.21	64.26	74.25	64.21	74.23	68.23	**63.70**
	Subfraction	1	50.24	54.26	50.27	53.44	55.28	52.70	
		2	56.27	57.29	60.24	60.29	57.26	58.27	
		3	50.74	56.25	55.28	55.84	55.28	54.68	**55.22**

Testing Time	**Control**	**Subfraction 10%**
Day-5		
		
		
		

Day-10		
		
		
		
Day-15		



The results of statistical calculations of one-way analysis of variance (ANOVA) on the expression of tnf-α in test animals showed significant differences expressed as $P<0.05$ between the control group and the 10% subfraction on days 5, 10 and 15. Results of Duncan's follow-up test of tnf-α expression on the 5th and 10th days of the control group and the 10% subfraction group was not significantly different, however, the results of the Duncan's follow-up test of tnf-α expression on the 15th day of the control group and the 10% subfraction group were significantly different.

Based on the observations obtained, it can be concluded that the ethyl acetate subfraction ointment of meniran leaves affects wound healing; this is because the ethyl acetate subfraction, the main compound of which is phylantin, has anti-inflammatory, analgesic and antioxidant effects. Meniran leaves are also known to have anti-inflammatory effects from the flavonoid compounds contained in them, which can influence wound healing, where anti-inflammatory wounds can heal more quickly. The mechanism of phylantin as an anti-inflammatory is through inhibiting the COX-2 enzyme, so that it can inhibit the formation of prostaglandin E2 so that prolonged inflammatory processes can be prevented and inflammatory responses such as pain and swelling can be stopped.[49]

Wounds that dry more quickly are also caused by the tannin content in meniran leaves, which functions as an astringent. Astringent is a tightening agent that has the power to wrinkle and shrink skin tissue, so that bleeding from

wounds can stop quickly, and the wound dries up more quickly.[41,50] Inflammation is a stage of the wound healing process, where when inflammation is reduced or inhibited, pain mediators, namely, prostaglandins, cannot cause vasodilation of blood vessels or no stimulation of pain, so that the healing stage will be accelerated towards proliferation and maturation (*remodeling*). The steroids contained in piladang leaf extract may also inhibit the phospholipase enzyme, so that arachidonic acid and prostaglandins are not formed by blocking the release of enzymes, stabilizing the lysosomal membrane, inhibiting the release of inflammatory mediators and inhibiting the migration and infiltration of leukocytes.[51]

The results of this research are supported by the results of previous research. According to the research conducted by Siahaan*et al*, 2017, administration of meniran leaf extract gel (*Phyllanthus niruri* L.) can increase the epithelialization of wound tissue in male Wistar rats.[15] Meanwhile Kurhasi and Fuji, 2015 because it protects skin tissue from oxidative damage caused by free radicals.[36,52] Tumor Necrosis Factor Alpha is a proinflammatory cytokine whose amounts increase when inflammation occurs, such as when an incision occurs.[53] Tumor Necrosis Factor Alpha is expressed during injury because it has many functions during inflammation, such as increasing the pro-thrombotic role, stimulating leukocyte cell adhesion, inducing endothelial cells, regulating macrophage activity and others.[54] According to Navarro (2008), high levels of TNF-α expression indicate that the wound tissue is still in the inflammatory stage, while low levels of TNF-α expression indicate that the tissue has passed the inflammatory phase and entered the proliferation phase.[55]

CONCLUSION

Based on the results of research on the healing effect of excision wounds from meniran leaves (*Phyllanthus niruri* L) with a concentration of 10% on male white rats, it was concluded that:

1. Applying meniran leaf ethyl acetate subfraction ointment can provide a better effect in the wound healing process.
2. The duration of administration of meniran leaf ethyl acetate subfraction ointment can provide a good healing effect. Based on the results obtained in statistical analysis using the anova test, a significant value was obtained ($p<0.05$).

Grant information

This study was fully funded by the General Directorate of Higher Education through PDD Research Grant Fiscal year 2023 contract number: 0217/E5/PG.02.00/2023 and012/E5/PG.02.00.PL/2023.

The funders had no role in study design, data collection, and analysis, decision to publish, or preparation of the manuscript.

BIBLIOGRAPHY

1. Abdurrahmat AS, Wounds, Inflammation and Recovery. Entropy Journal, 2014; 9(1): 729-738.
2. Priyandari Y &Umatjina, SATMU, *Jatropha Curcas*Tree Sap Topical Accelerates the Healing Time of Excision Wounds in Mice. Journals of Nurses Community, 2015; 6(2), 198–206. http://journal.unigres.ac.id/index.php/JNC/arti cle/view/52.
3. Husaini ED, Wounds and Treatment, KTL. Journal of Community Nurses, 2010; 6(2): 198-206.
4. Arimbi A, Azmijah R, Darsono H, Plumeriastuti, TV Widiyanto and D. Legowo. Textbook of General Veterinary Pathology, 2nd ed. Airlangga University Press; 2015.

5. Wijaya YA, Kalangi SJR and Kaseke MM. Description of Postmortem Wound Inflammatory Reactions in Experimental Animals. e-Biomedic Journal, 2015; 3(1): 539-543.
6. Haq FF. The Effect of Incision Wounds on Comparison of TNF-α Levels in White Rats (*Rattus Norvegicus)* of the Wistar Line [Ph. D thesis], Unissula Press; 2016.
7. Primadina N, Basori A, &Perdanakusuma DS. Wound Healing Process Viewed from the Aspect of Cellular and Molecular Mechanisms. QanunMedika - Medical Journal, Faculty of Medicine, Muhammadiyah Surabaya, 2019; 3(1): 31. https://doi.org/10.30651/jqm.v3i1.2198.
8. Mathivanan N, Surendiran G, Srinivasan K, Malarvizhi K. Morindapubescens JE Smith (*Morindatinctoria* Roxb.). Fruit extract accelerates wound healing in rats. J Med Food, 2006; 4(1): 591-3.
9. Madhavi P. Evaluation of Anti-Inflammatory Activity of Citrulluslanatus Seed Oil by In-vivo and In-vitro Models. IRIJPAS Com, 2012; 2(4): 104-108.
10. Lachman L & Lieberman AA. Theory and Practice of Industrial Pharmacy. Universitas Indonesia. Jakarta; 1994.
11. Hakim MD, Kamruzzaman and ObydulHoq MD. A review on ethnomedicinal, phytochemical and pharmacological properties of Phyllanthus niruri. J Med Plants Stu, 2016; 4(6): 173-180.
12. Kaur N, Kaur B, Sirhindi G. Phytochemistry and Pharmacology of *Phyllanthus niruri* L. Review of Phytotherapy Research, 2017; DOI: 10.1002.
13. Sari WN. Effect of Giving Ethanol Extract of Meniran Leaves (*Phyllanthus niruri* L.) On the Activity and Phagocytic Capacity of Peritoneal Macrophages in Male White Mice. [Thesis], Padang: Indonesian College of Pharmacy, 2023.
14. Weli H& Tobat SR 2021. Effect of Giving 10% Ethyl Acetate Subfraction Ointment of Meniran (*Phyllanthus niruri* L*) Leaves* on Healing Excision Wounds in WhiteMaleRats. [Thesis], Padang: Indonesian Pioneer University, 2021.
15. Siahaan MSY, Pangkahila W & Aman IGM. Meniran leaf extract gel (*Phyllanthus niruri*) increases the epithelialization of wound healing in the skin of white male Wistar rats (*Rattus norvegicus*). Journal of Biomedicine (JBM), 2017; 9(1): 14–18.
16. Brunauer A, Verboket RD and Kainz DM. Rapid Detection of Pathogens in Wound Exudate via Nucleic Acid Lateral Flow Immunoassay. Biosensors (Basel), 2021; 11(3): 74.
17. Larouche J, Sheoran S, Maruyama K and Martino MM, "Immune Regulation of Skin Wound Healing: Mechanisms and Novel Therapeutic Targets," Adv Wound Care (New Rochelle), 2018; 1(7): 209-231, DOI: 10.1089/wound.2017.0761. PMID: 29984112; PMCID: PMC6032665.
18. Wulandari P, Hutagalung MR and Perdana Kusuma DS, "Deteksi Kadar Transforming Growth Factor (TGF-ß) Pada Luka Akut. Jurnal Rekonstruksi danEstetikJanuari – Juni 2021; 06(1). DOI: 10.20473/jre.v6i1.28225.
19. Tsala DE, Amadou D, Habtemariam S. Natural wound healing and bioactive natural products. Phytopharmacology, 2013; 4 (3): 532-560. ISSN 2046-1194. [Online] Available: https://api.semanticscholar.org/CorpusID:10977575.
20. Dalimartha S. Atlas tumbuhanobat Indonesia. Trubus Agriwidya. Jakarta, 2000.
21. Sutrisna EM, Maryati, Wahyuni S andTanti AS. Anti-inflammatory Effect of *Phyllanthus niruri* L. from Indonesia. Pharmacog J, 2019; 11(6): 1347-50.
22. Thyagarajan SP, Jayaram S, Gopalakrishnan V, Hari R, Jeyakumar P and Sripathi MS. Herbal medicines for liver diseases in India. J Gasrtoenterol Hepatol, 2002; 3: 370.
23. Farouk A. Antimicrobial activity of certain Sudanese plants used in folkloric medicine.Screening for antibacterial

activity. Fitotherapia, 1983; 54(1): 3–7.

24. Santos ARS, Filho VC, Yunes RA, Calixto JB. Further studies on the antinociceptive action of the hydro alcoholic extract from plants of the genus Phyllanthus. J Pharm Pharmacol, 1995; 47(1): 66–71.
25. Himawan H, Pramono, D. Resti. Uji Farmakologi Ekstrak Kental Daun Meniran (*Phyllanthus niruri* L.) Untuk Membantu Penyembuhan Luka Sayat Pada Tikus Putih Jantan. Jurnal Farmamedica, 2017; 2(1): 25-31, DOI: doi.org/10.47219/ath.v2i1.30
26. Singh S, *et al*. The physiology of wound healing, Surgery. 2017. [Online]. Available: http://dx.doi.org/10.1016/j.mpsur.2017.06.00
27. Gurtner GC, Werner S, Barrandon Y. Wound repair and regeneration. Nature, 2008; 453(7193): 314–321.
28. WHO. Traditional Medicine Strategy: 2014–2023. ISBN: 9789241506090, [Online]. Available: http://www.who.int/traditional-complementary-integrative-medicine/en/
29. Miguel OG *et al*. Chemical and preliminary analgesic evaluation of geranin and furosin isolated from *Phyllanthus sellowianus*. Plant Med, 1996; 62(2): 148–198.
30. Hakim MD, Kamruzzaman and Obydul Hoq MD. A review on ethno medicinal, phytochemical and pharmacological properties of *Phyllanthus niruri* L. J Med Plants Studies, 2016; 4(6): 173-180.
31. Badan POM RI. Monografi Ekstrak Tumbuhan Obat Indonesia Vol. 1. Direktorat Standarisasi Obat Tradisional, Kosmetik dan Produk Komplemen, Jakarta, 2010.
32. Federer W. Experimental Design Theory and Application, Oxford: Oxford and Lbh Publish Hinco, 1963.
33. Nathan C. Points of control in inflammation. Nature, 2002; 420: 846-852.
34. Gushiken LFS, Beserra FP, Bastos JK, Jackson CJ, Pellizzon CH. Cutaneous Wound Healing: An Update from Physiopathology to Current Therapies. Life (Basel), 2021; 11(7): 665. DOI: 10.3390/life11070665. PMID: 34357037, PMCID: PMC8307436.
35. Martin A. The use of antioxidants in wound healing. Dermatol. Surg, 1996; 22: 156-160.
36. Shukla AAM, Rasik BN and Dhawan BN. Asiaticoside-induced elevation of antioxidant levels in healing wounds. Phytother. Res, 1999; 13(1): 50-54.
37. Trabucchi EF, Preis-Baruffaldi C, Baratti and Montorsi W. Topical treatment of experimental skin lesions in rats: macroscopic, microscopic and scanning electron-microscopic evaluation of the healing process. Int. J. Tissue React, 1986; 8: 533-544.
38. Tara S, Arul A, Smita S, Sudhakar. Effect of *Phyllanthus niruri*. Linn on burn wounds in rats. Asian Pacific Journal of Tropical Medicine, 2010; 105-108.
39. Grambow E, Sorg H, Sorg CGG, Strüder D. Experimental Models to Study Skin Wound Healing with a Focus on Angiogenesis. Medical Sciences, 2021; 9(3): 55. [Online]. Available: https://doi.org/10.3390/medsci9030055.
40. Radha P and Saranya J. In vitro antioxidant activity of *Phyllanthus niruri* leaf extracts. Journal of Pharmacognosy and Phytochemistry, 2020; 9(3): 198-201. [Online]. Available: http://www.phytojournal.com.
41. Nisar MF and Junwei H, Ahmed A, Yang Y, Li M and Wan C. Chemical Components and Biological Activities of the Genus Phyllanthus: A Review of the Recent Literature, Molecules. A Journal of Synthetic Chemistry and Natural Product Chemistry, 2018; 23. [Online]. Available: https://api.semanticscholar.org/CorpusID:52937056.
42. Manoj KS and Ahmad MS. Phytochemical Profile of *Phyllanthus niruri* L and evaluation of its Potent Bioactive Compounds. Biosc.Biotech.res.Comm, 2020; 13(3): 1545-1551. [Online]. Available: http://dx.doi.org/10.21786/bbrc/13.3/82.

43. Khaled AA, Mahmood and Fahmi MW. Wound Healing Potential of *Phyllanthus niruri* L Leaf Extract in Experimental Rats. Middle East Journal of Scientific Research, 2012; 11: 1614-1618.
44. Departemen Kesehatan Republik, Indonesia. Indonesian Pharmacopoeia IV. Republic of Indonesia Ministry of Health. Jakarta, 1995.
45. Harbone JB. Physicochemical Methods Guiding Modern Ways of Analyzing Plants. Second Issue. ITB Bandung, 1987.
46. Cahaya, Herson H, Pramono, Dwi A. Pharmacological Test of Thick Extract of Meniran Leaves (*Phyllanthus niruri* L) to Help heal Cut Wounds in Male White Rats. Journal of Pharmamedics, 2017; 2(1): 25–31.
47. Amanda N. The Effect of Topical Administration of Ethanol Extract of Piladang Leaves (*Solenostemonscutellarioides* (L.) Codd) on Healing Excision Wounds in Male White Rats. Thesis. Padang: STIFI, 2017.
48. Nasution AI. Biomolecular Textbook for Basic Medicine. Syiah Kuala University Press. Banda Aceh, 2016.
49. Ozaki Y, Sekita S, Soedigdo S, HaradaM. Antiinflammatory effect of *Graptophyllumpictum* (L.) Griff., Chem Pharm. Bul.l (Tokyo), 1989; 37(10): 2799-802.
50. Samuelsson, Gunnar. Drugs of Natural Origin A Textbook of Pharmacognosy. Swedish Pharmaceutical Press, Sweden, 1999.
51. AriaM, Arel A & Monika. Testing the Anti-inflammatory Effect of Piladang Leaf Fractions (*Solenostemonscutellarioides* (L.) Codd) on Female White Mice, Scientia Journal, 2015; 5(2): 84-91.
52. Kurahasi T and Fujii J. Role of antioxidative enzymes in wound healing. Journal of Developmental Biology, 2015; 3: 57-70.
53. Kumar V, Cotran RS, and Robbins SL. Pathology Textbook 7th Edition. EGC Medical Book Publishers. Jakarta, 2007.
54. Supit IA, Pagemanan DHC and Marunduh SR. Tumor Necrosis Factor (TNF-α) Profile Based on Body Mass Index (BMI) in Unsrat Medical Faculty Students Class of eBiomedik Journal, 2014; 3(2): 640-643.
55. Navarro-González JF, Mora-Fernández C. The role of inflammatory cytokines in diabetic nephropathy. J Am SocNephrol, 2008 Mar; 19(3): 433-42.doi: 10.1681/ASN.2007091048. Epub 2008 Feb 6. PMID: 18256353.