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Original Research Article

Wound Healing Activity of Phyllanthin-rich Sub-fractions Ointment: Isolated from Meniran (*Phyllanthus niruri* L.) Leaf in Experimental Rats Using Hydroxyproline as Biochemical Marker

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ABSTRACT

Effective healing of skin injury involves complex simultaneous processes. Extracts of meniran (*Phyllanthus niruri* L.), a plant medicine found in almost all parts of Indonesia, have been essential in traditional wound healing treatments. Phyllanthin is the major therapeutically active lignan present in *Phyllanthus* species. This study investigated the wound-healing activity. of ointments prepared from a phyllanthin-rich subfraction of meniran leaf in an animal model. Two parameters were measured, namely, hydroxyproline concentration and wound healing percentage. Hydroxyproline is a biochemical marker representing collagen production in wound healing. Seventy-two rats were divided into four groups of 18, including a control group and three groups administered 5, 10, and 15% phyllanthin-rich fraction ointment. Each of the four groups was subdivided according to the treatment duration of 5, 10, and 15 days. The hydroxyproline concentration and wound healing percentage were analyzed by one-way ANOVA followed by Duncan's post-hoc test. The increase in hydroxyproline concentration and wound healing percentage showed that the effect of each ointment concentration was significantly different (p <0.05) compared to the control group. The optimal ointment concentration was 10%, with optimal results after 15 days. The study revealed that the phyllanthin-rich sub-fraction isolated from meniran (*Phyllanthus niruri* L.) had a wound healing activity on Adult male Wistar rats (*Rattus novergicus*).

***Keywords*:** *Phyllanthus niruri* L, phyllanthin, wound healing, biochemical marker, hydroxyproline, collagen

Introduction

Wounds are skin injuries defined by a discontinuity of the epithelial lining of the skin or mucosa that occurs when the skin is exposed to external or internal stimuli such as temperature, high or low pH, chemicals, friction, pressure trauma, or radiation. Wound healing is a complex, dynamic process supported by many cellular events that must be coordinated to repair damaged tissue efficiently.[1] In 2018, 31.4% of injuries in Indonesia occurred on the highway, of which 72.7% were suffered by motorists, and permanent physical disability resulting from injuries causing permanent scars that interfere with comfort has a prevalence of 9.2% and is increasing annually.[2]
The immune system is critical in wound healing, actively re-establishing homeostasis after tissue injury through several mechanisms.

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Wound healing is usually divided into four phases: homeostasis, inflammation, proliferation, and remodeling. Injured tissue is repaired, lost tissue is replaced, and the epithelium is restored.
Keratinocytes, fibroblasts, vascular endothelial cells, and immune cells all play critical roles in supporting inflammation, cell migration, and angiogenesis. One of the main functions of wound healing is to restore the epithelial barrier. Without these defenses, initial protection against infection is lost, leaving tissue vulnerable to outside pathogens and fluid loss.[3]
Physiologically, wound healing involves several concurrent phases, which begin immediately after the injury. The body responds with system homeostasis, releasing cytokines, including epidermal growth factor (EGF), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), and transforming growth factor beta (TGF-β), which play a role in neutrophil chemotaxis, and activation of macrophages, mast cells, endothelial cells, and fibroblast cells.[4]
Plant medicines can be safe, effective, easy to obtain, and relatively cheap. Many methods and procedures used in modern medicine differ from traditional wound treatment methods. While practitioners of modern Western medicine frequently refer to traditional medicine as alternative, indigenous, and complementary, traditional methods, which rely nearly exclusively on natural resources, including water, plants, animals, and minerals, are still respected around the globe.[5]
Many natural products can promote wound healing.[6] Meniran (*Phyllanthus niruri* L.) is a plant widespread on the plains of Asia, Africa, America, and Australia. Meniran can grow in rocky and humid places, like on the banks of rivers. It is used traditionally as a treatment for jaundice, fever, inflammation of the liver, and diarrhea

and helps heal abrasions or minor burns.[7] Extensive studies on *P. niruri* have confirmed its anti-inflammatory and anti-liver disease properties.[8,9] *P. niruri* has also been evaluated for diuretic, hypotensive, hypoglycaemic,[10] antioxidant,[11] antibacterial,[12] and analgesic activity.[13] Phytochemical studies have identified and characterized various compounds in *P. niruri*, including the lignans phyllanthin and hypophyllanthin, flavonoids, and tannins.[14] Phyllanthin is the major therapeutically active lignan present in various members of the *Phyllanthus* genus.[15] Studies have shown that phyllanthin exhibits various biological effects, including hepatoprotective,[16] antioxidant,[16,17,18] antidiabetic,[18] anti-inflammatory, and anticancer activities.[19]

In the healing process, collagen, which comprises the amino acid hydroxyproline, strengthens and supports extracellular tissue. Indeed, hydroxyproline is used as a biochemical marker for tissue collagen.[20] At low hydroxyproline levels, decreased collagen formation has been reported as a factor in delayed wound healing. Hydroxyproline is an amino acid formed upon hydrolysis of connective-tissue proteins such as collagen (comprising about 14% by weight) and elastin, but rarely from other proteins. It plays a vital role in the synthesis and stability of collagen. First isolated in 1902 from gelatine, a collagen breakdown product, hydroxyproline is a nonessential amino acid that can be biosynthesized from glutamic acid and is not required from dietary sources.[21] The use of hydroxyproline as a biochemical marker in wound healing activity studies is still rarely carried out. [20]

Previous research of ethnomedical uses of *P. niruri* to examine the wound healing activity only in extract form and the parameters that are limited only to wound healing percentage and histopathology.[6] The present study seeks the wound-healing activity of phyllanthin-rich subfraction of meniran leaf. We hypothesized that the higher concentration of phyllanthin the better the wound healing activity will be. The wound healing activity will be determined by hydroxyproline concentration and wound healing percentage. As one of the most abundant amino acids in collagen, hydroxyproline concentration reflects the concentration of collagen. The increasing amount of collagen synthesis will support the extracellular wound tissue repairing process, which is characterized by an increased amount of hydroxyproline.

Materials and Methods

Apparatus and Chemical Substances

Ethical Approval

This study was approved by Andalas University, Faculty of Medicine Research Ethics Commission, with approval number 543/UN.16.2/KEP-FK/2023. All efforts were made to mitigate harm to the study animals by administering aesthesia before incision wound modeling and during all experiments. Animals were kept in well-maintained cages in conditions to minimize or eliminate pain and distress, using inhalant agents before biopsy for hydroxyproline concentration measurements. After the incision wound modeling, the animals were given 5% lidocaine-prilocaine cream (2.5% lidocaine and 2.5% prilocaine) twice daily as an analgesic during the experiments. All the procedures are reported following the ethical roles, principles, and guidelines of Animal Research: Reporting of *In-Vivo* Experiments (ARRIVE), USDA Animal Care, and LIPI.[20,21]

Animals

Adult male Wistar rats used in this study weighed about 200−250 g, were three months old, met the inclusion criteria (healthy condition and active mobility), and showed no symptoms that met our exclusion criteria (behavioral alterations such as weak activity, lethargy, and excessive barbering). Stainless steel mice cages were used in a room at a constant temperature of 25 ± 2 °C, sufficiently ventilated at all times to minimize odors, ammonia levels, and moisture condensation, on a 12/12 h light/dark cycle, and the rats were provided with a pellet diet and tap water ad libitum. The rats were provided with a bedding of wood shavings that were changed once a week. Two to three rats were in each cage according to the size of the cage. The rats' clinical and behavioral state was evaluated daily during routine check-ups. During the trial, no rats were discovered dead, to have slowed their development or appetite, or to have changed their behavior. To ensure no animal suffered excessive pain, anesthesia was administered before incision wound modeling and during the experiments.[22,23]

Plant collection and identification

Fresh meniran leaves (18 kg) were collected in September 2023 from Jorong Talawi, Kenagarian Muaro Paiti, Kapur IX District, Lima Puluh Kota Regency, West Sumatra Province, Indonesia, with GPS location 0°14'36.0"N 100°32'23.0" E. The plant was identified and validated by Dr. Nurainas from Universitas Andalas Herbarium, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Andalas on 26 September 2023 with voucher specimen number 615/K-ID/ANDA/IX/2023. The voucher specimen is deposited in Andalas Herbarium, Department of Biology, Faculty of Mathematics and Natural Sciences.

Extraction and fractionation

Fresh *Phyllanthus niruri* leaves (18 Kg) were air-dried to remove water content and the dried leaves (5,2 Kg) were pulverized. In a dark brown glass bottle with a tight lid, the powder was extracted with 70% ethanol (1:10, 50 L) at room temperature 20 to 23 °C (68 to 73 ^{0}F) for 6 h with occasional stirring and for a further 24 hours without stirring. The macerate was separated and the extraction was repeated under the same conditions until the obtained macerate was colorless. The combined macerates were concentrated on a rotary evaporator (IKA RV-10) under reduced pressure at 40 ºC to remove ethanol and provide a viscous ethanol extract.

The viscous ethanol extract was fractionated into an n-hexane fraction (F1) and an ethyl acetate (EtOAc) fraction (F2).[24] The viscous extract was diluted with distilled water (1:5) and partitioned with hexane (2:1, Reagent Plus, ≥99%, Merck) in a separating funnel. The upper hexane layer was separated and concentrated to provide hexane fraction F1. The aqueous layer was then partitioned with EtOAc (2:1, ACS reagent, ≥99.5%, Merck). The upper EtOAc layer was separated and concentrated at 60 ºC to provide EtOAc fraction F2, which was sub-fractionated by column chromatography. To produce the phyllanthin-rich sub-fraction, a step gradient polarity chromatographic system was applied according to literature procedures.[18,25,26,27] Silica gel (700 g, 40–63 µm) as the stationary phase was added to the column and elution was carried out using n-hexane: EtOAc (1000 mL each of 2:1, 6:4, and 3:7 mixtures) to provide thirty 100 mL fractions. Monitoring was carried out by TLC using a silica gel 60 F_{254} plate and a mobile phase of n-hexane: EtOAc (3:7) using UV_{254} lamp detection. According to the R_f of the principle spots, the thirty fractions were divided into three sub-fractions A, B, and C (vials 22-30, R_f 0.63). Phyllanthin standard eluted with R_f 0.63 under the same conditions. After obtaining the phyllanthin-rich sub-fraction C, preliminary tests included an organoleptic test, a phytochemical study, and a pH measurement.

High-performance liquid chromatography

An Agilent 1220 Infinity II apparatus was used for HPLC analyses, with a C18 column (250 Å, 4.6 × 250 mm) at a detection wavelength of 230 nm and column temperature of 34 °C. The mobile phase was methanol at a flow rate of 1 mL/min and a method duration of 15 min. The sample injection volume was 10 µl.

Sample detection: A phyllanthin standard series was created with six concentration points (720, 540, 450, 180, 90, and 45 ppm) to provide a calibration curve and regression equation y = 17.203x + 82.147, with correlation coefficient (r) = 1, peak area y and concentration x. To detect phyllanthin in a sample, weigh 10−20 mg and dissolve in methanol to 5 mL in a 5 mL volumetric flask using an ultrasonic bath for 15 minutes. Take 0.5 mL, dilute with methanol to 2 mL, and filter through a 0.45 µm PTFE filter syringe into a 1.5 mL vial for injection into the HPLC system. Phyllanthin concentration was calculated using Equation 1

Equation 1:

$$\text{Phyllanthin concentration} = \frac{[Area - Intercept/Slope]}{\text{Sample Concentration } (\frac{\text{mg}}{\text{L}})} \text{ x } 100\%$$

Preparation of phyllanthin-rich sub-fraction ointment

Ointments (30 g) were prepared with three concentrations, containing 5, 10, and 15% of the phyllanthin-rich subfraction C. Precisely, 1.5, 3.0, and 4.5 g of subfraction C were added to vaseline flavum ointment base (28.5, 27.0, and 25.5 g, respectively) and combined in a mortar to provide a homogeneous paste.

Treatment of experimental animals

From the Federer equation [(4-1) (n-1) ≥15; (4-1) (n-1) >15; 3n-3 >15; 3n >18; n >6], the minimum number of rats was 6.[28]

Seventy-two rats were divided into three main groups of 24 according to the day of examination of the wound treatment (days 5, 10, and 15). Sub-groups of 6 rats received different treatments. The wounds of Group I (control) were treated with *vaseline flavum* ointment base, and the wounds of Groups II, III, and IV were treated with 5, 10, and 15% phyllanthin-rich sub-fraction C ointment, respectively. The grouping of experimental animals is shown in Table 1. Wound healing activity parameters were measured on days 5, 10, and 15.

Hydroxyproline content was determined at the maximum absorption wavelength of hydroxyproline (559 nm). A calibration curve provided regression equation y = 0.118 + 0.0089*x*, with correlation coefficient (r) = 0.999835. Hydroxyproline concentration, determined on days 5, 10, and 15 after wound incision, was calculated using Equation 2.

Equation 2:

$$\text{Hydroxyproline concentration} = \frac{\text{Neutral sample volume}}{\text{Volume of neutral sample to be pipetted}} \times \text{Whole sample volume} \times x$$

The percentage of wound healing was observed on days 5, 10, and 15 after the wound incision. A high percentage indicates a smaller wound size and better wound healing. The percentage of wound healing was calculated using Equation 3.

Equation 3:

$$\text{Wound healing \%} = \frac{(\text{Initial wound area} - \text{final wound area})}{\text{Initial wound area}} \times 100$$

For wound incision, the animals were anesthetized with isoflurane (2−4 mL) in a closed anesthesia chamber. The skin on the back of the rat was lifted with tweezers and a circular wound was made with surgical scissors.[29,30]

The general health status of the animals was continuously observed throughout the experiment and treatment. Before, during, and after treatment, body weight and wound diameter were measured. Humane endpoints of 15% weight loss and abnormal behaviors such as apathy or increased aggression were established to exclude the animal from the study.[31] Distress in the animals was indicated by clinical signs, such as changes in temperature, respiration, or feeding behavior.[29] At the end of the experiment, animals were sacrificed through 5% isoflurane euthanasia until one minute after the animals stopped breathing.[32]

Table 1: Grouping of experimental animals

	5 days	10 days	15 days
Group I (Control)	6 rats were given ointment base (*vaselin flavum*)	6 rats were given ointment base (*vaselin flavum*)	6 rats were given ointment base (*vaselin flavum*)
(Group II) Dose 5%	6 rats were given 5% phyllanthin-rich sub-fraction ointment from meniran (*Phyllanthus niruri* L.) leaves	6 rats were given 5% phyllanthin-rich sub-fraction ointment from meniran (*Phyllanthus niruri* L.) leaves	6 rats were given 5% phyllanthin-rich sub-fraction ointment from meniran (*Phyllanthus niruri* L.) leaves
(Group III) Dose 10%	6 rats were given 10% phyllanthin-rich sub-fraction ointment from meniran (*Phyllanthus niruri* L.) leaves	6 rats were given 10% phyllanthin-rich sub-fraction ointment from meniran (*Phyllanthus niruri* L.) leaves	6 rats were given 10% phyllanthin-rich sub-fraction ointment from meniran (*Phyllanthus niruri* L.) leaves
(Group IV) Dose 15%	6 rats were given 15% phyllanthin-rich sub-fraction ointment from meniran (*Phyllanthus niruri* L.) leaves	6 rats were given 15% phyllanthin-rich sub-fraction ointment from meniran (*Phyllanthus niruri* L.) leaves	6 rats were given 15% phyllanthin-rich sub-fraction ointment from meniran (*Phyllanthus niruri* L.) leaves

Determination of hydroxyproline levels in rat skin scar tissue

Six animals from each group were sacrificed on days 5, 10, and 15 to measure the hydroxyproline concentration in the wound scab. Skin scar samples were dried at 60 °C for 12 h, hydrolyzed with 6 N HCl for 24 h at 110 °C, and neutralized with a mixture of 2 mL NaOH, 1 mL buffer, and 1 mL aquabidest. A sample of hydrolysate (200 µL) was diluted with aquabidest to 1000 µL and further mixed with $CuSO_4$ (0.01 M, 1 mL), NaOH (2.5 N, 1 mL), and H_2O_2 (6%, 1 mL). The solution was then stirred and incubated at 80 °C for 5 minutes, cooled, and H_2SO_4 (3 N, 4 mL) and 4-dimethyl amino benzaldehyde (5%, 2 mL) were added to provide a total solution of 10 mL. The sample was incubated at 70 °C for 16 minutes and cooled to 20 °C. UV-Vis spectrophotometry (T92+ Spectrophotometer, PG Instruments Ltd.) at 559 nm provided the hydroxyproline concentration in the sample, calculated against a hydroxyproline standard curve.[33]

Data analysis

Hydroxyproline concentration and wound healing percentage were statistically analyzed using SPSS 27.0 statistical software, a one-way ANOVA, and Duncan's post-hoc test.

Results and Discussion

Ethanol extraction of powdered meniran leaf gave 585.6 g, 11.3% crude extract yield. After sequential partitions in n-hexane and ethyl acetate, the ethyl acetate fraction yield was 15.4% (F2), of which 40 g was separated by column chromatography to provide three sub-fractions A (0.41 g), B (3.52 g), and C (12.80 g). Phyllanthin-rich sub-fraction C was identified concerning phyllanthin standard by TLC (R_f 0.63)[18] and HPLC (retention time 3.23 min, 69.43% concentration), which provided a phyllanthin calibration curve (Figure 1) and regression equation y = 17.203x + 82.147, with correlation coefficient (r) = 1. The HPLC chromatogram of the phyllanthin standard is shown in Figure 2, and the HPLC chromatogram of phyllanthin in the sample is shown in Figure 3.

Organoleptic characterization of meniran leaf phyllanthin-rich sub-fraction C indicated a semisolid, white, and dark green mixed powder

with a characteristic odor. Phytochemical screening showed the presence of flavonoids and phenolic compounds and the absence of saponins, terpenoids/steroids, and alkaloids (Table 2). The ointments of 5, 10, and 15% sub-fraction C had a pH of 5, suitable for human skin with a pH of 4.5-6.5.

Table 2: Phytochemical examination of phyllanthin-rich sub-fraction ointment from meniran (*Phyllanthus niruri* L.) leaves

Chemical content	Result
Flavonoids	+
Phenolic	+
Saponins	-
Terpenoids/steroids	-
Alkaloids	-

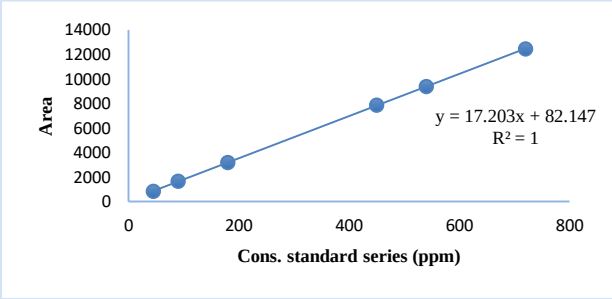
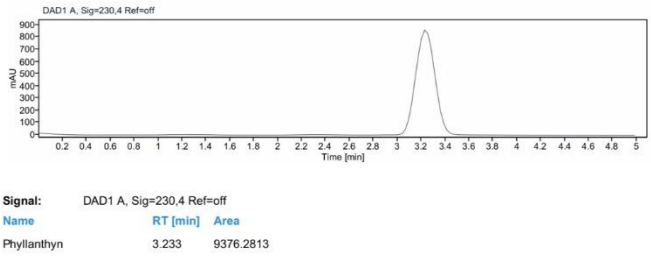


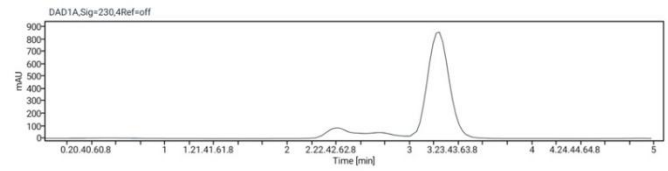
Fig 1: The calibration curve of standard phyllanthin



Signal: DAD1 A, Sig=230,4 Ref=off

Name	RT [min]	Area
Phyllanthyn	3.233	9376.2813

Fig 2: HPLC chromatogram of standard phyllanthin



Signal: DAD1A, Sig=230,4 Ref=off

Name	RT [min]	Area
Phyllanthin	3.231	10011.3662

Fig 3: HPLC chromatogram of phyllanthin-rich fraction from meniran leaf

The amount of collagen in the skin can be determined by measuring the hydroxyproline content. Hydroxyproline concentrations were determined on days 5, 10, and 15 after the wound incision, as this was within the proliferative phase when fibroblast formation occurred. Fibroblasts will synthesize collagen, the main element of the extracellular matrix, forming the strength of scar tissue in wounds. UV-Vis spectrophotometry in the 400-800 nm range showed the maximum absorption wavelength of hydroxyproline at 559 nm with an absorbance of 0.382 (Table 3). The regression equation obtained from the calibration curve (Figure 4) using a series of hydroxyproline standard solutions was y = 0.118 + 0.0089x, with a correlation coefficient (r) = 0.999835.

Administration of phyllanthin-rich sub-fraction C ointment affects the scar tissue hydroxyproline content, as shown in Table 4. Hydroxyproline content in Groups II-IV showed a significant difference ($p < 0.05$) compared with the control group, as shown in Table 5. The effect of 5, 10, and 15% phyllanthin-rich sub-fraction ointment was also determined by wound healing percentage on days 5, 10, and 15 after the rats were given incision wounds (Table 6). The descriptive statistics can be seen in Table 7. The average wound healing percentage of Groups II-IV showed a significant difference ($p < 0.05$) compared with control groups, as shown in Table 8.

The bar graph of hydroxyproline concentration based on the treatment group is shown in Figure 5. The estimated marginal means of hydroxyproline content based on the treatment groups shows that the optimal concentration of meniran leaf phyllanthin-rich subfraction was 10% because it has the highest value. The estimated marginal means of hydroxyproline content based on the day groups shows that the optimal concentration of meniran leaf phyllanthin-rich subfraction was on day 15 (Figure 6). The bar graph of wound healing percentage based on treatment group is shown in Figure 7. The estimated marginal means of wound healing percentage based on the treatment groups shows that the optimal concentration of meniran leaf phyllanthin-rich subfraction was 10% because it has the same value as 15%. Instead of using the higher concentration, we prefer the smaller one because it gives the same effect. The estimated marginal means of wound healing percentage based on the day groups shows that the optimal concentration of meniran leaf phyllanthin-rich subfraction was on day 15 (Figure 8). Overall, the optimal concentration of meniran leaf phyllanthin-rich sub-fraction

Table 3: The result of hydroxyproline absorption measurement standard at λ = 559 nm

No	Consentration (µg/mL)	Absorbance
1	10	0.209
2	20	0.296
3	30	0.382
4	40	0.472
5	50	0.566

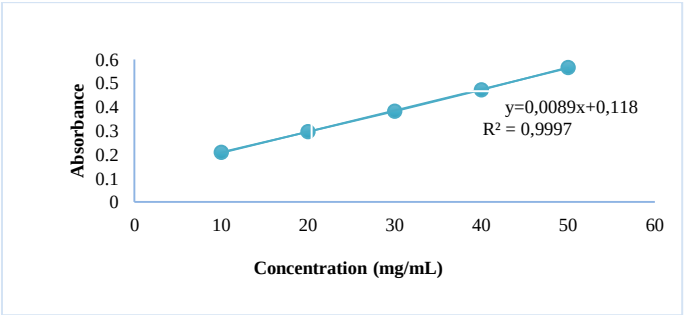


Fig 4: The calibration curve of hydroxyproline solution at λ = 559 nm

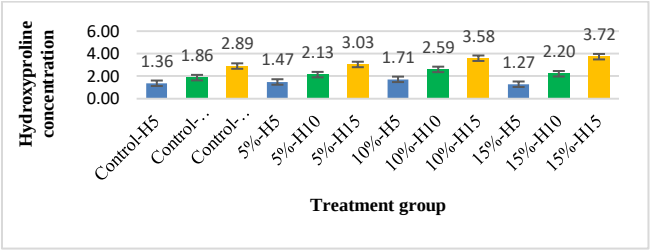


Fig 5: The hydroxyproline concentration bar graph based on the treatment group.

Table 4: The average hydroxyproline concentration on days 5, 10, and 15 of the control, 5%, 10%, and 15% phyllanthin-rich

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sub-fraction ointment from meniran (*Phyllanthus niruri* L.) leaves

DAY	Group	No	Absorbance	% Hydroxyproline
Day 5	Control	1	0.260	0.87
		2	0.274	0.96
		3	0.330	1.31
		4	0.476	2.20
		5	0.293	1.08
		6	0.400	1.74
	5% Sub-fraction	1	0.334	1.33
		2	0.335	1.34
		3	0.335	1.34
		4	0.382	1.63
		5	0.383	1.63
		6	0.376	1.59
	10% Sub-fraction	1	0.351	1.43
		2	0.476	2.20
		3	0.354	1.45
		4	0.446	2.02
		5	0.363	1.51
		6	0.382	1.63
	15% Sub-fraction	1	0.350	1.43
		2	0.285	1.03
		3	0.229	0.68
		4	0.377	1.59
		5	0.349	1.42
		6	0.358	1.48
Day 10	Control	1	0.430	1.90
		2	0.350	1.43
		3	0.350	1.43
		4	0.490	2.30
		5	0.380	1.62
		6	0.52	2.49
	5% Sub-fraction	1	0.447	2.03
		2	0.456	2.08
		3	0.479	2.22
		4	0.464	2.13
		5	0.465	2.14
		6	0.469	2.16
	10% Sub-fraction	1	0.603	2.99
		2	0.483	2.25
		3	0.522	2.49
		4	0.415	1.83
		5	0.612	3.04
		6	0.597	2.95
	15% Sub-fraction	1	0.403	1.75
		2	0.478	2.22
		3	0.477	2.21
		4	0.473	2.19
		5	0.511	2.42
		6	0.514	2.44
Day 15	Control	1	0.585	2.88
		2	0.600	2.97
		3	0.603	2.99
		4	0.566	2.76
		5	0.579	2.84
		6	0.588	2.89
	5% Sub-fraction	1	0.664	3.36
		2	0.665	3.37
		3	0.611	3.04
		4	0.550	2.66
		5	0.578	2.83
		6	0.591	2.91
	10% Sub-fraction	1	0.711	3.65
		2	0.703	3.60
		3	0.758	3.94
		4	0.689	3.52
		5	0.690	3.52
		6	0.648	3.26
	15% Sub-fraction	1	0.798	4.19
		2	0.799	4.19
		3	0.670	3.40
		4	0.680	3.46
		5	0.658	3.32
		6	0.727	3.75

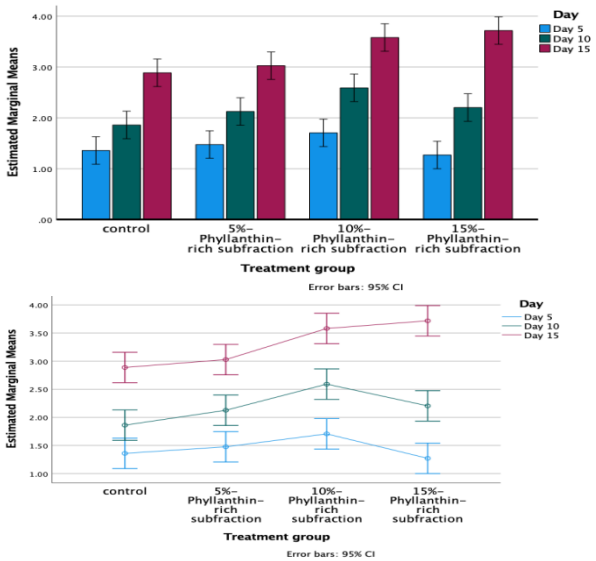

Fig 6: The estimated marginal means of hydroxyproline percentage based on the treatment group.
C ointment was 10% on day 15.

Constant observation of animal well-being during our study revealed no adverse effects, such as infection of the incised region, decreased

appetite, or behavioral changes after the wounding or during the recovery period. During the study period, no rats died by accident.

Wound healing is a complex biological process that results in the restoration of tissue integrity. Physiologically, it is divided into four phases: homeostasis, inflammation, proliferation, and tissue remodeling. Many factors adversely affect healing, including malnutrition, hypoxia, immunosuppression, chronic disease, and surgery.[34,35,36] The inflammatory phase of wound healing will persist as long as necessary, ensuring that excess bacteria and debris from the wound are cleared. However, protracted inflammation can lead to extensive tissue damage and delayed proliferation and result in the formation of a chronic wound. Multiple factors, including lipoxins and the products of arachidonic acid metabolism, are thought to have anti-inflammatory properties that dampen the immune response and allow the next phase of wound healing to occur.[37] Inflammation is necessary for effective defenses against pathogens and to set in motion tissue repair following injury.[38]

According to this study, 10% phyllanthin-rich sub-fraction C ointment was the most effective concentration for wound healing in a rat model regarding hydroxyproline concentration and wound healing percentage. This result is in line with the study conducted by Martin in 1996, which showed that topical application of *P. niruri* extracts could significantly enhance the rate of wound healing. *P. niruri* extract plays a significant role in wound healing, protecting tissues from oxidation by stimulating the production of antioxidants at the wound site and providing a favorable environment for tissue healing.[39,40,41] This corroborates research by Shukla in 1999, who found that wound healing effects may be due to the up-regulation of human collagen I expression, and by Bonte in 1994 who found that the administration of *P. niruri* extract can increase the tensile strength of a wound.[42,43]

The wound-healing activity of *P. niruri* has been evaluated by oral and topical administration. Proven to play a significant role in wound contraction and epithelialization,[44] it can also increase epithelial cell proliferation and angiogenesis, providing oxygen and necessary nutrients for healing.[45,46,47] Its extracts also showed inhibition of membrane lipid peroxidation and potent free radical scavenging.[48]

Phytochemical analysis of *P. niruri* extract showed the presence of several bioactive molecules, such as lignans, phyllanthin, hypophyllanthin, flavonoids, glycosides, and tannins.[49,50] These constituents may be responsible for wound healing activity individually or in synergy. This study focused on one bioactive compound, phyllanthin.[26] We measured hydroxyproline concentration and wound healing percentage to assess wound healing activity. As one of the most abundant amino acids in collagen, hydroxyproline concentration reflects the concentration of collagen[51] and is a good marker for wound healing assessment. A high concentration of hydroxyproline indicates a faster rate of wound healing.[52] In this study, using 5, 10, and 15% meniran leaf phyllanthin-rich sub-fraction ointment resulted in higher hydroxyproline concentrations than the control group, and, from statistical analysis, the optimal concentration was 10%. The higher hydroxyproline concentration reflects increased cellular proliferation and increased collagen synthesis. The result of this study is supported by data from Khaled *et al*., revealing that the application of *P. niruri* leaf extracts resulted in markedly fewer inflammatory cells, more fibroblast proliferation, less scarring at the wound enclosure, more mature and densely-packed collagen fibers, and more blood capillaries (angiogenesis) than wounds dressed with sterile deionized water or a blank placebo. These results strongly support the beneficial effects of meniran leaf phyllanthin-rich sub-fraction ointment on the acceleration of wound healing in rats.[53,54,55]

Conclusion

This study showed that an ointment containing 5-15% phyllanthin-rich subfraction of a meniran leaf ethanol extract has wound-healing activity, with increased hydroxyproline concentration in skin scar tissue and increased wound healing percentage. From Duncan's post-hoc test, the most effective concentration was 10% phyllanthin-rich subfraction on day 15. We can conclude that the phyllanthin-rich ointment has the potential as a wound-healing agent.

Table 5: Tests of the between-subjects effect of the average hydroxyproline concentration on days 5, 10, and 15 of the control, 5%, 10%, and 15% phyllanthin-rich sub-fraction ointment from meniran (*Phyllanthus niruri* L.) leaves

Tests of Between-Subjects Effects

Dependent Variable: hydroxyproline concentration

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	46.886[a]	11	4.262	38.791	.000
Intercept	386.883	1	386.883	3520.999	.000
Concentration	3.464	3	1.155	10.507	.000
Day	41.623	2	20.811	189.402	.000
Concentration * Day	1.800	6	.300	2.730	.021
Error	6.593	60	.110		
Total	440.362	72			
Corrected Total	53.479	71			

a. R Squared = .877 (Adjusted R Squared = .854)

Table 6: The wound healing percentage on days 5, 10, and 15 of the control, 5%, 10%, and 15% phyllanthin-rich sub-fraction ointment from meniran (*Phyllanthus niruri* L.) leaves

Day	Group	No	Initial Diameter (cm)	Final Diameter (cm)	Initial Wound Healing Area (cm^2)	Final Wound Healing Area (cm^2)	Wound Healing %	Average ± Dev.Standard
		1	2.15	2.05	14.51	13.20	9.09	
Day-5	Control	2	2.20	2.10	15.20	13.85	8.88	11.86
		3	2.20	2.05	15.20	13.20	13.17	±
		4	2.20	2.10	15.20	13.85	8.88	3.492
		5	2.10	1.95	13.85	11.94	13.78	

		6	2.20	2.00	15.20	12.56	17.36	
		1	2.13	1.80	14.25	10.17	28.59	
	5% ethyl	2	2.26	1.86	16.04	10.86	32.27	32.37
	acetate	3	2.13	1.80	14.25	10.17	28.59	±
	subfraction	4	2.16	1.73	14.65	9.40	35.85	3.350
		5	2.23	1.83	15.61	10.52	32.66	
		6	2.33	1.86	17.05	10.86	36.27	
		1	2.26	1.73	16.04	9.40	41.40	
	10% ethyl	2	2.06	1.56	13.32	7.64	42.65	43.65
	acetate	3	2.36	1.73	17.49	9.40	46.26	±
	subfraction	4	2.06	1.56	13.32	7.64	42.65	2.828
		5	2.26	1.63	16.04	8.34	47.98	
		6	2.16	1.66	14.65	8.65	40.94	
		1	2.13	1.74	14.25	9.51	33.27	
	15% ethyl	2	2.33	1.60	17.05	8.04	52.84	45.36
	acetate	3	2.26	1.70	16.04	9.07	43.42	±
	subfraction	4	2.26	1.62	16.04	8.24	48.62	6.733
		5	2.23	1.60	15.61	8.04	48.52	
		6	2.33	1.72	17.05	9.29	45.51	
		1	2.15	1.75	14.51	9.62	33.75	
Day-	Control	2	2.20	1.65	15.20	8.55	43.75	42.20
10		3	2.20	1.55	15.20	7.54	50.36	±
		4	2.20	1.6	15.20	8.04	47.11	6.734
		5	2.10	1.7	13.85	9.07	34.47	
		6	2.20	1.65	15.20	8.55	43.75	
		1	2.13	0.96	14.25	2.89	79.69	
	5% ethyl	2	2.26	1.06	16.04	3.53	78.00	82.96
	acetate	3	2.13	0.83	14.25	2.16	84.82	±
	subfraction	4	2.16	0.73	14.65	1.67	88.58	3.786
		5	2.23	0.93	15.61	2.72	82.61	
		6	2.33	0.93	17.05	2.72	84.07	
		1	2.26	0.73	16.04	1.67	89.57	
	10% ethyl	2	2.06	0.63	13.32	1.25	90.65	89.91
	acetate	3	2.36	0.76	17.49	1.81	89.63	±
	subfraction	4	2.06	0.63	13.32	1.25	90.65	0.813
		5	2.26	0.70	16.04	1.54	90.41	
		6	2.16	0.73	14.65	1.67	88.58	
		1	2.13	0.75	14.25	1.77	87.60	
	15% ethyl	2	2.33	0.72	17.05	1.63	90.45	89.43
	acetate	3	2.26	0.68	16.04	1.45	90.95	±

	subfraction	4	2.26	0.77	16.04	1.86	88.39	1.266
		5	2.23	0.73	15.61	1.67	89.28	
		6	2.33	0.74	17.05	1.72	89.91	
Day-15	Control	1	2.15	0.85	14.51	2.27	84.37	
		2	2.20	0.95	15.20	2.83	81.35	82.47
		3	2.20	1.00	15.20	3.14	79.34	±
		4	2.20	0.80	15.20	2.01	86.78	2.652
		5	2.10	0.90	13.85	2.54	81.63	
		6	2.20	0.95	15.20	2.83	81.35	
		1	2.13	0.53	14.25	0.88	93.81	
	5% ethyl	2	2.26	0.7	16.04	1.54	90.41	93.34
	acetate	3	2.13	0.63	14.25	1.25	91.25	±
	subfraction	4	2.16	0.46	14.65	0.66	95.46	2.100
		5	2.23	0.56	15.61	0.98	93.69	
		6	2.33	0.5	17.05	0.79	95.40	
		1	2.26	0.43	16.04	0.58	96.38	
	10% ethyl	2	2.06	0.36	13.32	0.41	96.95	96.56
	acetate	3	2.36	0.46	17.49	0.66	96.20	±
	subfraction	4	2.06	0.36	13.32	0.41	96.95	0.407
		5	2.26	0.40	16.04	0.50	96.87	
		6	2.16	0.43	14.65	0.58	96.04	
		1	2.13	0.45	14.25	0.64	95.54	
	15% ethyl	2	2.33	0.50	17.05	0.79	95.40	96.27
	acetate	3	2.26	0.38	16.04	0.45	97.17	±
	subfraction	4	2.26	0.45	16.04	0.64	96.04	0.751
		5	2.23	0.42	15.61	0.55	96.45	
		6	2.33	0.4	17.05	0.50	97.05	

Table 7: The mean wound healing percentage on days 5, 10, and 15 of the control, 5%, 10%, and 15% phyllanthin-rich sub-fraction ointment from meniran (*Phyllanthus niruri* L.) leaves

Dependent Variable: Wound Healing Percentage

Treatments Group	Day	Mean ± Standard Deviation	N
Control	Day 5	11.860 ± 3.49	6
	Day 10	42.198 ± 6.70	6
	Day 15	82.470 ± 2.65	6
	Total	45.509 ± 30.07	18
5% Sub-fraction	Day 5	32.371 ± 3.34	6

	Day 10	82.961 ± 3.78	6
	Day 15	93.336 ± 2.09	6
	Total	69.556 ± 27.56	18
10% Sub-fraction	Day 5	43.646 ± 2.82	6
	Day 10	89.915 ± .81	6
	Day 15	96.565 ± .40	6
	Total	76.708 ± 24.20	18
15% Sub-fraction	Day 5	45.363 ± 6.73	6
	Day 10	89.430 ± 1.267	6
	Day 15	96.275 ± .747	6
	Total	77.022 ± 23.51	18
Total	Day 5	33.310 ± 14.23	24
	Day 10	76.126 ± 20.53	24
	Day 15	92.161 ± 6.081	24
	Total	67.199 ± 28.98	72

Table 8: Tests of the between-subjects effect of the average wound healing percentage on days 5, 10, and 15 of the control, 5%, 10%, and 15% phyllanthin-rich sub-fraction ointment from meniran (*Phyllanthus niruri* L.) leaves

Tests of Between-Subjects Effects
Dependent Variable: wound healing percentage

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	57089.034[a]	11	5189.912	507.605	.000
Intercept	333613.793	1	333613.793	32629.489	.000
Concentration	14694.633	3	4898.211	479.075	.000
Day	40281.327	2	20140.664	1969.881	.000
Concentration * Day	2113.074	6	352.179	34.445	.000
Error	613.458	60	10.224		
Total	391316.285	72			
Corrected Total	57702.492	71			

a. R Squared = .989 (Adjusted R Squared = .987)

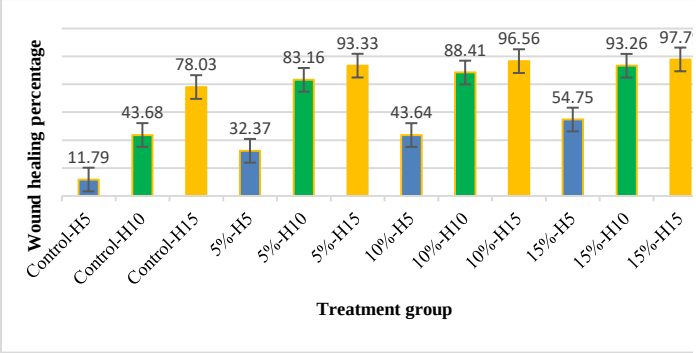

Fig 7: The wound healing percentage bar graph based on the treatment group.

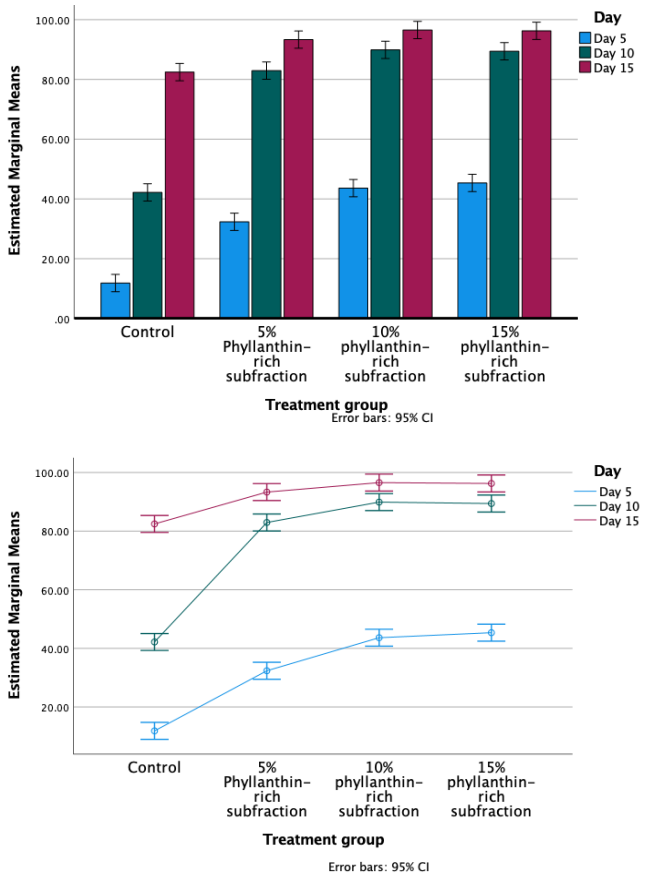

Fig 8: The estimated marginal means of wound healing percentage based on the treatment group

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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