J Herbmed Pharmacol. 2023; 12(3): 388-398.

http://www.herbmedpharmacol.com doi: 10.34172/jhp.2023.42

Journal of Herbmed Pharmacology

Enhanced wound healing effect of *Areca catechu* L. ointment via antibacterial activity and anti-inflammatory process at grade IIA burns in rats

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ARTICLE INFO

Article Type:
Original Article

Article History:
Received: 2 January 2023
Accepted: 30 March 2023

Keywords:
Herbal medicine
Areca
Wound healing
Burns
Anti-bacterial remedy

ABSTRACT

Introduction: *Areca catechu* L. seeds contain flavonoids, tannins, saponins, and alkaloids that have antibacterial properties, can prevent skin infections, and have been used empirically for wound healing. This study aimed to determine the antibacterial activity and effectiveness of *A. catechu* ointment in wound healing at grade IIA burns in rats.

Methods: *A. catechu* seed extract was formulated into an ointment and then tested for its antibacterial activity using the agar diffusion method. Wound healing testing was conducted by dividing the rats into four groups: negative control, positive control, Formula I (F 1) ointment, and Formula II (F II) ointment. Grade IIA burns were made on the back skin of rats and treatment was performed for 14 days. The wound tissue was taken for histopathological observations.

Results: In this study, F II ointment had better antibacterial activity than F I, as indicated by a wider diameter of inhibition against bacteria *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Escherichia coli*, *Streptococcus pyogenes*, and *Staphylococcus aureus*. The scab formed on F II was faster on day 3, the wound diameter was reduced on day 7, and there was a decrease in inflammatory cell infiltration and coagulative necrosis and an increase in neovascularization and collagen formation on the 7th day ($P < 0.05$) compared to the negative control and F I.

Conclusion: *A. catechu* seed extract ointment with a concentration of 5.0% (F II) had a better effect on wound healing regarding the antibacterial and anti-inflammatory activity than that with a concentration of 2.5% (F I).

Implication for health policy/practice/research/medical education:
Areca catechu seeds extract ointment accelerates the healing process of grade IIA burns due to antibacterial activity, a decrease in inflammatory cell infiltration and coagulative necrosis, and an increase in neovascularization and collagen formation leading to the formation of scabs and reducing the diameter of the burn.

Please cite this paper as: Sandhiutami NMD, Fahleni F, Miftahurrohmah N, Widhiyasari NKA, Azalia A, Amalia I. Enhanced wound healing effect of *Areca catechu* L. ointment via antibacterial activity and anti-inflammatory process at grade IIA burns in rats. J Herbmed Pharmacol. 2023;12(3):388-398. doi: 10.34172/jhp.2023.42.

Introduction

The skin is the outermost protective body covering the body surface. Wounds on the skin can cause damage to the epidermis, dermis, and subcutaneous tissue. The severity of the wound depends on the causative factor and the length of time the skin is in contact with the heat source. The skin is a vast reserve of stem cells to rejuvenate the body's surface and repair wounds (1). Burn is defined as destruction found in the epidermal, dermal, or deeper tissue primarily due to contact with the heat source, such as contact with thermal, hot water, electric agents, or chemicals. Burns can affect metabolism and disrupt body homeostasis, most especially if they are very severe (2). The first treatment for burn patients is to provide a topical preparation in the form of an ointment that is applied to the wound area. In the formulation, the selection of the base

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is very important to consider increasing the effectiveness of the active substances it contains. A hydrocarbon base is one of the bases often used in ointment formulations due to its emollient texture; thus, it can prolong the contact time of the active substance with the skin. This type of base is also not easy to dry (3). In the treatment of burns, antibiotic ointment preparations are used, which are already widely circulated in the market. However, herbal medicines are also used by the community because they have been empirically proven to be useful and are inexpensive, relatively nontoxic, and easy to obtain (4,5).

Areca catechu L. is one of the plants that can be used as traditional medicine in curing a disease. People often use *A. catechu* seeds to heal burns. Decoction of *A. catechu* seeds can be used to clean wounds in order to prevent infection (6-9). *A. catechu* seeds have several main components in the form of carbohydrates, fats, fiber, polyphenols, including tannins, flavonoids, and alkaloids (namely, arecaine, arecoline, arecaidine, guvacine, guvacoline, and choline), and minerals. Tannins, flavonoids, and alkaloids have anti-inflammatory and antibacterial properties, which are effective in healing burns (10). Saponins have the ability as cleansers and antiseptics that work to eradicate pathogens or stop the growth of microorganisms that often develop in wounds in order to prevent the wound from becoming seriously infected (11). *A. catechu* seeds can help the wound healing process on the skin due to the presence of antibacterial substances. The presence of these substances can suppress the growth of pathogenic bacteria and prevent infection in the wound so that wound healing can be accelerated (12). Flavonoids also play a role in the healing process of burns by inhibiting the inflammatory process. They are astringent and increase the speed of epithelialization. Phenolic compounds act as antioxidants that can counteract free radicals and reduce lipid peroxidation which can also help in the re-epithelialization process. A decrease in lipid peroxidation can prevent necrosis. Tannins also play a role in increasing the tensile strength of burns and shrinking skin pores (13). Based on the above explanation, this study was conducted to scientifically prove the wound-healing effect of *A. catechu* seed extracts in an ointment form through preclinical trials in rats.

Materials and Methods

Plants and materials

Areca catechu L. seeds, 70% ethanol, Vaseline album, Adeps lanae/lanolin, cetostearyl alcohol, paraffin wax, neomycin sulfate (NEBACETIN®)(PHAROS), Blood Agar Base (OXOID CM0055), Tryptone Soya Agar (OXOID CM0131), Nutrient Agar (Merck), Tryptone Soya Broth (HIMEDIA®), Gram-negative bacteria *Pseudomonas aeruginosa* ATCC 9027, *Acinetobacter baumannii* ATCC 19606, *Escherichia coli* ATCC 8739, and gram-positive bacteria *Streptococcus pyogenes* ATCC 19615, *Staphylococcus aureus* ATCC 6538, male white rat strain Sprague Dawley, ketamine hydrochloride injection, USP (Hospira, Inc., USA), xylazine (Interchemie, Holland), petri dishes, tubes and glassware (PYREX®), analytical balance (AND, GR-200), electric oven (Memmert), autoclave (All American), Laminar Air Flow (Biobase), incubator (Memmert), Bunsen, Vortex (Thermolyne), micropipette (Thermo Fisher Scientific), cotton and gauze (KASA HUSADA), Öse needle, pH meter (Hanna Instruments), water bath (Julabo TW20), vaporizer cup (RRC), cotton swab steril (ONEMED), ferrous metal, and infrared thermometer were used in this study.

Preparation of *Areca catechu* seeds extract

Areca catechu was obtained from the Research Institute for Spices and Medicinal Plants (Balittro). Plant authentication and herbarium preparation were carried out in Research Center for Plant Conservation and Botanic Gardens, Indonesian Institute of Science with the number: B-1501/IPH.3/XI/2020. The seeds were washed, then cut into pieces and put in the oven, at a temperature of 30-50°C. The dried seeds were ground and sieved. *A. catechu* seeds were macerated using 70% ethanol. The filtrate obtained was concentrated with a vacuum rotary evaporator at a temperature of ± 45°C, a pressure of 175 mm Hg, and a speed of 35 rpm, to obtain a concentrated extract.

Preparation of *Areca catechu* seeds extracts ointment

The preparation of *A. catechu* seeds ointment was performed at the Laboratory of Pharmaceutical Technology Formulation of Semisolida, Faculty of Pharmacy, Pancasila University. An ointment base consisting of Adeps lanae (5 g), paraffin wax (5 g), cetostearyl alcohol (5 g), and vaseline album (85 g) was put into a vaporizer and heated at a temperature of 65°C on a water bath until all the ingredients were melted. The mixture was then allowed to cool and homogenized with a homogenizer at 1500 rpm for 10-15 minutes. Then, 2.5% *A. catechu* seed extract was added for Formula I (F I) and 5% for Formula II (F II) and stirred until homogeneous. Ointment base without *A. catechu* seed extract (placebo) was used as a negative control.

Evaluation of *Areca catechu* seeds extracts ointment

The evaluation of *A. catechu* seeds extract ointment was conducted using organoleptic examination (shape, color, and odor) and homogeneity examination that was conducted visually at room temperature by applying the ointment to a slide that was covered with another slide and then observed whether the base was made homogeneous with other materials. The pH of *A. catechu* seeds extract ointment was also measured by weighing approximately 0.1 g of the ointment and dissolved in 100 mL of distilled water with pH 7.0 (1000 ppm) and measuring the pH of the ointment. Another evaluation parameter was spreadability, which was conducted after the ointment was made for 48 hours by measuring the diameter of the

spread of 1 g of ointment between two glass boards for 3 minutes. The ointment was applied to a Teflon ring with an outer diameter of 55 mm, an inner diameter of 15 cm, and a thickness of 3 mm. The inside of the Teflon filled with ointment was then leveled with a spatula until a flat surface was obtained without air bubbles. The Teflon ring was then carefully removed to obtain an ointment spread with a diameter of 15 mm and a thickness of 3 mm. The ointment preparation was then covered with a glass plate with a diameter of 8 cm with a weight of 20 g that was then pressed with a load of 20 g and allowed to stand for 30 minutes. After that, it was removed and the diameter of the widened surface of the ointment was measured with a caliper mm, then calculated by the following formula: $F = \pi \times r^2$; F = spreadability (mm^2), π = 3,14, and r = radius (mm^2). A viscosity examination was conducted using a Brookfield-type RV viscometer. The viscosity obtained from the measurement results is the value of the viscosity of the preparation being examined, while the flow properties show the standard curve between RPM and force. Viscosity measurement was done by changing the rpm so that the viscosity value is obtained at various rpm. The flow properties were obtained by making a curve between the shear speed (rpm) and force ($dyne/cm^2$) according to the data obtained and then plotted on graph paper between the force as the x-axis and shear speed as the y-axis and determining the flow properties.

Antibacterial activity test of *Areca catechu* seeds ointment

The antibacterial activity test was conducted in the Laboratory of Microbiology, Faculty of Pharmacy, Pancasila University. The antibacterial activity was tested by the agar well diffusion method. The bacterial isolates were conducted by inoculating 1 Öse the test microbial stock *Pseudomonas aeruginosa* ATCC 9027 and *Acinetobacter baumannii* ATCC 19606 onto NA agar and *Escherichia coli ATCC 8739* and *Staphylococcus aureus* ATCC 6538 onto the surface TSA agar and incubated at 37°C for 18–24 hours. From a 24-hour-old bacterial culture at 37°C, 1 Öse each test bacteria was put into 5 mL saline solution (0.9% NaCl) aseptically, and the level of turbidity of the suspension was compared with the standard 0.5 McFarland visually. The diameter of the inhibition zone test for *A. catechu* seeds extract ointment was conducted on Gram-negative bacteria *P. aeruginosa* ATCC 9027, *A. baumannii* ATCC 19606, *E. coli* ATCC 8739, Gram-positive bacteria *Streptococcus pyogenes* ATCC 19615, and *S. aureus* ATCC 6538.

The medium was poured into each sterile petri dish, waited for 15 minutes to solidify, and scraped the bacteria according to the media using a sterile cotton swab. Sterile disc papers were saturated with the samples, including ointment base as a negative control, neomycin sulfate ointment as a positive control, F I (2.5% extract concentration), and F II (5% extract concentration). The saturated sterile disc paper was placed on the surface of the agar medium and gently pressed with tweezers so that the disc paper was completely attached to the agar.

After being incubated for 18-24 hours at 37°C, the diameter of the inhibition zone formed was measured in millimeters (mm) using a caliper. The clear area around the disc indicated the presence of antibacterial activity in the *A. catechu* seeds extract ointment

The wound healing effect of *Areca catechu* seeds extract ointment

Animal experimental design

In this test, 8-week-old male Sprague–Dawley rats, obtained from the Institute of Research and Development, Ministry of Health of Republic Indonesia, were divided into four groups, i.e., the negative group given a placebo, the positive group given neomycin sulfate ointment, F I group given Formula I ointment, and F II group given Formula II ointment, each consisting of five rats. Rats underwent a week of acclimatization. They were fed with regular pellets and always allowed access to water. They were kept in a room with a temperature maintained at 23°C–27°C, a humidity of 60%–70%, air ventilation of 12 times/h, and lighting for 12 hours per day.

Rats were shaved on the back and then anesthetized using a combination of ketamine hydrochloride and xylazine. The back was cleaned with 70% ethanol, and then the skin was injured using a 1.0-cm diameter burn inducer at 100°C that was attached to the back skin for 3 seconds. Each rat had four burns on its back. In the first burn, a placebo ointment with ointment base alone was applied as a negative control. In the second burn, topical neomycin sulfate ointment was applied as a positive control. In the third burn F I ointment and in the fourth burn F II ointment were applied. The burns were covered with sterile gauze and plaster, respectively. They were observed, cleaned, and given placebo ointment, neomycin sulfate, F I, and F II for 14 days every day, and sterile gauze dressings were changed every day. The cure rate was observed in healing phases 1 and 2, namely, the inflammatory and proliferative phases. The formation of scabs and changes in wound diameter for 14 days was macroscopically observed. Rats were euthanized on days 1, 3, 7, and 14 for histopathological analysis by assessing the number of inflammatory cell infiltrates, the number of coagulative necrosis cells, and neovascularization and collagen formation using a scoring system.

Histopathological examination of the rat's skin

The rat's skin was washed thoroughly with physiological NaCl. The skin was put into the normal formalin buffer solution for 48 hours. According to the guidelines of the dissecting technique, the skin was sliced laterally at 0.5 cm carefully from representative areas and processed in the automated tissue. The selected tissue was processed with the principle of dehydration, clearing, and embedding on an automated machine. The tissue was embedded in

paraffin blocks and cut into 5-µm-thick sections using a rotary microtome. The slices were immersed in a 40°C–45°C incubator and then transferred to microscope slides and stained with hematoxylin and eosin (H&E). Light microscopy observations were made on sections stained with standard H&E staining.

Comparisons were made between the histological preparations of the skin from the treatment groups and from the negative control group. Assessment of inflammatory cell infiltration and coagulative necrosis was done by giving a score using a light microscope. Values were divided into the following levels based on the categorization of Ghosh et al (14): 0, no histopathological changes; 1, inflammatory cell infiltration <1/3 field of view; 2, inflammatory cell infiltration in 1/3 to 2/3 of the visual field; and 3, inflammatory cell infiltration in >2/3 of the visual field.

Assessment of neovascularization was done by giving a score using a light microscope. Values were divided into the following levels based on the categorization of Sorg et al (15): 0, no new vessels found; 1, 1–10 new vessels; 2, 11–30 new vessels; and 3, ≥ 31 new vessels.

Assessment of collagen formation was done by giving a score using a light microscope. Values were divided into the following levels based on the categorization of Subagja (16): 0, no collagen was found in the wound area; 1, the density of collagen in the wound area was low (25%); 2, the collagen density in the wound area was moderate (50%); 3, the density of collagen in wound areas was tight (75%); and 4, the density of collagen in the wound area was very tight (100%).

Statistical analysis

Data were analyzed using SPSS 20 program. Non-parametric statistical techniques were used to analyze the wound healing effect data, namely, the Kruskal–Wallis test, to determine whether there was a significant difference in the wound healing effect of the four test groups with P value < 0.05

Results

The results of the phytochemical screening showed that *A. catechu* seeds positively contained saponins, tannins, alkaloids, flavonoids, steroids, and triterpenoids. The content of water was 26.70%; the content of ash was 10.50%, and drying shrinkage was 33.33%. The ointment of *A. catechu* seeds extract was evaluated to determine whether the preparations made met the predetermined quality standards. The evaluation tests included organoleptic tests, homogeneity tests, pH tests, and dispersibility tests, which are shown in Table 1.

This study also tested the flow properties of *A. catechu* seeds extract ointment. The measurement results are shown in Figure 1.

Results of the antibacterial activity of *Areca catechu* seeds extract ointment

Table 2 shows the antibacterial activity of *A. catechu* seed extract ointment against bacteria that commonly infect skin and wounds.

Table 1. Organoleptic, homogeneity, pH, and spreadability of *Areca catechu* L. seeds extract ointment

Formula	Organoleptic			Homogeneity	pH	Spreadability (cm)
	Color	Odor	Consistency			
Placebo	Yellowish white	No	Semi-solid	Homogen	5.04	5.175
Neomycin sulfate	White	No	Semi-solid	Homogen	5.60	5.500
Formula I	Light brown	No	Semi-solid	Homogen	5.09	5.250
Formula II	Dark brown	No	Semi-solid	Homogen	5.12	5.475

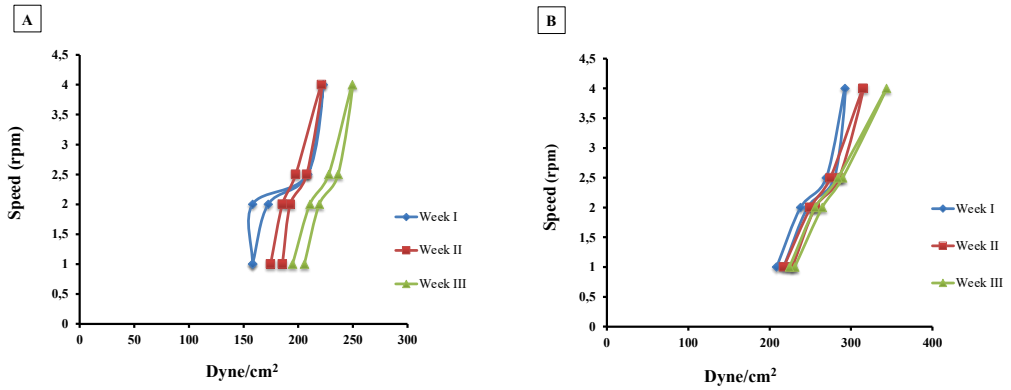

Figure 1. Flow properties curves of *Areca catechu* seeds extract ointment showed a thixotropic type of flow. (A) The formula I ointment (extract concentration of 2.5%); (B) Formula II ointment (extract concentration of 5%).

Table 2. The antibacterial activity test on *Areca catechu* L. ointment

Bacteria	Treatment	Inhibition zone diameter (mm)
Pseudomonas aeruginosa ATCC 9027	Negative control	0
	Positive control	16.56±0.20
	Formula I	8.93±0.32
	Formula II	9.40±0.26
Acinetobacter baumannii ATCC 19606	Negative control	0
	Positive control	11.43±0.25
	Formula I	7.13±0.40
	Formula II	8.13±0.35
Escherichia coli ATCC 8739	Negative control	0
	Positive control	22.53±0.35
	Formula I	13.53±0.20
	Formula II	14.23±0.15
Streptococcus pyogenes ATCC 19615	Negative control	0
	Positive control	16.36±0.30
	Formula I	11.43±0.35
	Formula II	13.06±0.25
Staphylococcus aureus ATCC 6538	Negative control	0
	Positive control	18.30±0.26
	Formula I	11.36±0.35
	Formula II	15.13±0.15

The ointment produced different zones of inhibition in each bacterium. The greatest inhibitory power was found at the concentration of 5% (Formula II) in *Staphylococcus aureus*, *Escherichia coli,* and *Streptococcus pyogenes*. Data are expressed as mean ± standard deviation.

The wound healing effect of *Areca catechu* seeds extract ointment

In this study, the determination of the wound healing effect with *A. catechu* seeds extract ointment was macroscopically conducted for 14 days to determine the rate of scab formation in grade IIA burns. At first, the wound still looked white. After being given treatment, changes in shape and color began to appear, marked by the formation of scabs. Table 3 shows the results of the scab formation by days in the wound healing, and Figure 2 shows the morphology of scab formation in the wound on the rat's back.

From the observations, the burns given to the backs of the rats changed every day, where there was blood accumulated and frozen over the wound that then formed a scab layer. In the negative control group, new scabs formed on the 5th day on average, while in the positive control, the scabs formed on the 3rd day on average. In the F I group, the average scab formation occurred on day 4, and in the F II group, the average scab formation occurred on day 3, the same as the positive control.

In the negative control group, the ointment preparation only contained an ointment base so that when a burn occurred it was only assisted by the body's ability to respond to the wound without any other assistance from the ointment ingredients. As for burns that were given F I and F II, the scabs formed more quickly. After a few days, the scab that has formed will dry up and then peel off. In the detached scab, it can be seen that the burn under the scab has decreased. The mean scab detachment occurred on days 11 and 6 for negative and positive controls, respectively. In the F I group, the new scab was released on the 8th day, while in the F II group, the scab was released on the 7th day. The difference in concentration between F

Table 3. Scab formation by groups and days in the wound healing

Groups	Scab formation														
	Days														
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Negative control	-	-	-	-	-	+	+	+	+	+	+	-	-	-	-
Positive control	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-
Formula I	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-
Formula II	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-

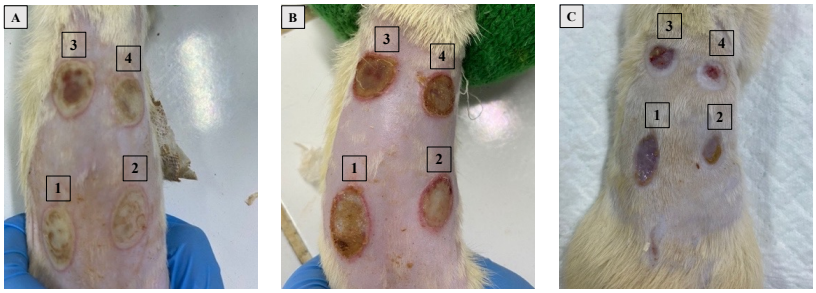

Figure 2. Scab formation in IIA-degree burns on rats in 14 days. Scab formation in the treatment group occurred on day 3 and in the negative control group, new scabs formed on day 5. (A) the 3rd day, (B) the 7th day, and (C) the 14th day. (1) negative control (ointment base), (2) positive control (neomycin sulfate ointment), (3) Formula I ointment (extract concentration of 2.5%), (4) Formula II ointment (extract concentration of 5%).

I and F II causes differences in effectiveness between the two.

The assessment of the healing effect of burns with *A. catechu* seed extract ointment was also conducted by measuring the diameter of the wound on the back skin of white rats on days 1, 3, 7, and 14 as shown in Figure 3.

The results of macroscopic observations for 14 days showed that wound care with the application of *A. catechu* extract ointment had an effect in reducing the diameter of the wound. Of the two formulas used, the one that was effective in reducing the diameter of the burn was in the F II group with an average wound diameter on day 14, which was 0.923 cm, while the F I group obtained an average of 1.062 cm wound diameter on day 14. In the treatment with the positive control, the average wound diameter was 0.555 cm, while in the treatment with negative control, the average wound diameter on day 14 was 1.038 cm.

Figure 3 shows that there was a decrease in wound diameter in the F II group, which was better than in the F I group. There was a significant difference ($P < 0.05$) in burn healing to wound diameter. The test results were obtained where the positive control was significantly different from F I and F II groups.

Histopathology of the rat skin by assessing inflammatory cell infiltration, coagulative necrosis, neovascularization, and collagen formation

The healing effect of burns with *A. catechu* seed extract ointment was assessed microscopically on days 1, 3, 7, and 14 to determine inflammatory cell infiltration, coagulative necrosis, neovascularization, and collagen formation.

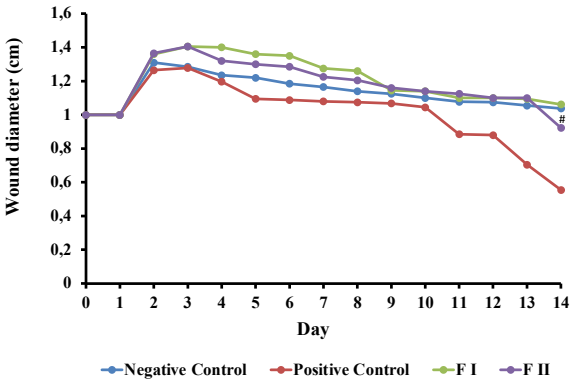

Figure 3. Wound diameter size by group in the wound healing process. #$P < 0.05$ vs. Formula I.

The histopathology of burns on the negative control on 7th day still showed damage to the epidermal layer of the skin and had started to form collagen with a low density of collagen. On the positive control on the 7th day, there was a formation of a new epithelial layer, the epidermal layer was formed and improved, and it looked like collagen with a medium density of collagen. On the F I on the 7th day, the epidermal layer was formed; it looked like collagen with a low density of collagen and medium spread. At F II on the 7th day, the epidermal layer was almost completely formed with granulation tissue, and the collagen and its density were moderate to densely spread (Figure 4).

The healing phase was seen in the proliferative and maturation phase between the days 7 to 21. In this study, the observations and assessments of inflammatory cell infiltration, coagulative necrosis, neovascularization, and collagen density were conducted until day 14 (Figure 5).

The results of the scoring analysis of inflammatory cell infiltration and coagulative necrosis (Figures 5A and 5B) on day 1 showed a significant difference ($P > 0.05$) in each group. The high score of inflammatory cells is due to the absence of changes in the skin tissue affected by burns. Immediately after the burn, neutrophils will phagocytose bacteria, foreign particles, and damaged tissue and prevent infection. On day 3, the results of the inflammatory cell scoring analysis still did not show any difference in each group ($P > 0.05$). There were still many inflammatory cells and coagulative necrosis; however, the average score decreased in the positive control, F I, and F II treatment groups. In the negative control group, the mean score of inflammatory cell infiltration and coagulative necrosis was still the same as on the first day. On the 7th day, there was a significant difference in each group ($P < 0.05$), so that histological changes were seen with a decrease in inflammatory cells and coagulative necrosis in each treatment group. The negative control had a different effect from the positive control, F I, and F II. F I and F II had almost the same effects.

The results of the analysis of neovascularization scoring and collagen formation (Figures 5C and 5D) on day 7

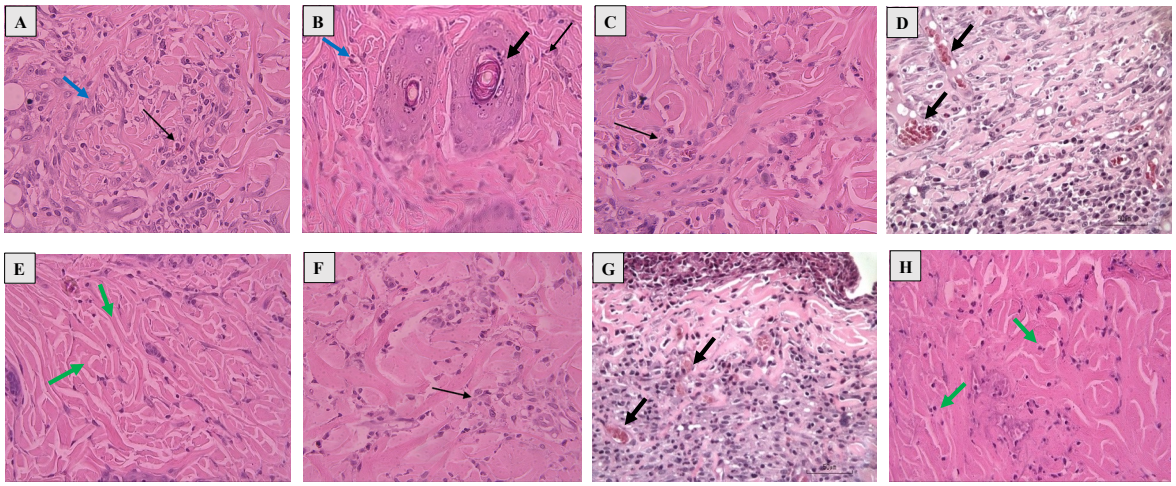

Figure 4. Representative histopathology profiles of rat skin (thin black arrow) inflammatory cell infiltration, (blue arrow) coagulative necrosis, (green arrow) neovascularization, and (thick black arrow) formation of collagen on 7th day. (A) Negative control: inflammatory cell infiltration and coagulative necrosis were abundant; (B) Positive control: decreased inflammatory cell infiltration and coagulative necrosis and increased neovascularization and collagen formation; (C), (D), (E) rats given Formula I ointment (extract concentration of 2.5%): decreased inflammatory cell infiltration and coagulative necrosis and increased neovascularization and collagen formation more than positive controls ($P < 0.05$); (F), (G), (H): rats given Formula II ointment (extract concentration of 5%): decreased inflammatory cell infiltration and coagulative necrosis and neovascularization and collagen formation were seen more than positive controls and Formula I. (H&E staining), magnification at 400x.

showed a significant difference ($P < 0.05$). Based on these statistical tests, there was a significant difference between F II and positive control or F I. Meanwhile, there was no significant difference between F I and positive control but had a difference with F II. This can occur because the concentration of *A. catechu* extract in F II was greater, namely 5%, so it was more effective than F I in the process of neovascularization and collagen formation.

Discussion

This study showed that *A. catechu* seed extract met the requirements for the quality of the thick extract material and the content of water (5%–30%). The higher the percentage content of water in an ingredient the easier it is for an extract to be damaged and decomposed due to bacterial growth. In this study, the drying shrinkage was 33.33%. The drying shrinkage test was intended to show

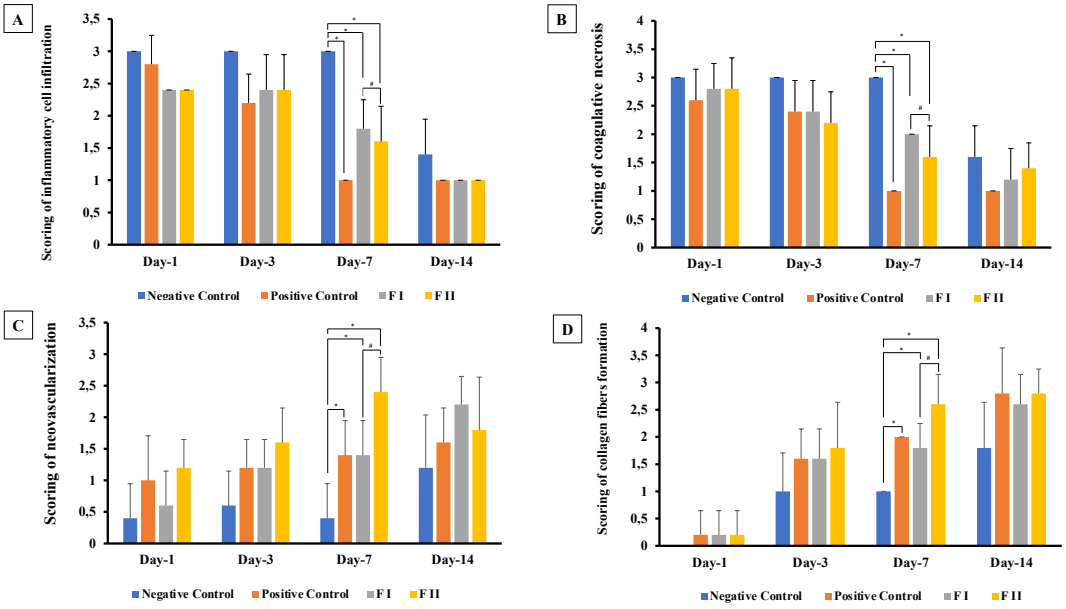

Figure 5. Scoring chart of the healing phase. On the 7th day, histological changes were seen with a decrease in inflammatory cells and coagulative necrosis in each treatment group (*$P < 0.05$ vs. negative). A. catechu extract in Formula II was more effective than Formula I in the process of neovascularization and collagen formation (#$P < 0.05$ vs. Formula I). (A): Inflammatory cell infiltration, (B): Coagulative necrosis, (C): Neovascularization, and (D): The density of collagen on days 1, 3, 7, and 14 between negative control (ointment base), positive control (neomycin sulfate ointment), Formula I ointment (F I; extract concentration of 2.5%), and Formula II ointment (F II; extract concentration of 5%). Values are expressed as mean ± SD. (n = 5).

how many compounds are contained in the extract and are lost or easily evaporated in the drying process. The drying shrinkage test is a parameter of an extract to maintain quality to avoid fungal growth (17,18).

The ointment was evaluated to meet the predetermined quality standards of preparations. Visually, each ointment showed different colors. The placebo ointment had a yellowish-white color because there was only an ointment base. The F I and F II ointments had a brown color due to the presence of *A. catechu* seed extract. All forms of ointment preparations did not produce odor and had a semisolid form following the requirements of the ointment. The placebo ointment showed homogeneous results. This means that all the ingredients used to make the base were mixed evenly. F I and F II also showed homogeneous results, where the ointment base was perfectly mixed with *A. catechu* seed extract. The pH test was conducted to determine the acidity level of the preparations made to suit the physiological pH of the skin so that there was no skin irritation. The physiological pH of the skin was within the range of 4.5–7.0. Both formulas met the requirements and were in the physiological pH range of the skin so they do not irritate.

In this study, the ability to spread the ointment on the skin was seen in the spreadability test, where the active substance had to be spread well to achieve the desired therapeutic effect. The requirement of good ointment spreadability was about 5–7 cm and both formulas had good spreadability. Good dispersion will cause the contact between the active substance and the skin to be wider, making it easier to use and stick to the skin affected by burns (19).

The next test of the ointment was a viscosity test. Viscosity is a statement of the resistance of a liquid to flow. The greater the resistance, the higher is the viscosity. A good viscosity requirement for semisolid preparations is 400–40 000 cPs (20). In this study, the flow curves of the two graphs of the ointment formulations showed a thixotropic type of flow. This could be seen from the shape of the descending curve to the left of the ascending curve. This also indicates that there is a breakdown of the structure that does not reform immediately if the stress is removed or reduced. The thixotropic flow belongs to the non-Newtonian type of flow. In this type, shearing rate (pressure velocity) and shearing stress (amount of pressure) do not have a linear relationship, and the viscosity changes depending on the amount of pressure applied. In pharmaceutical preparations, the thixotropic flow type is the most ideal flow type for semisolid preparations. The criteria for this type of flow are as follows: it does not settle immediately in the container, will become liquid when shaken, and will stay long enough during use (21).

In the antibacterial test, *A. catechu* seed extract ointment inhibited the growth of bacteria *P. aeruginosa* ATCC 9027, *A. baumannii* ATCC 19606, *E. coli* ATCC 8739, *S. pyogenes* ATCC 19615, and *S. aureus* ATCC 6538, indicated by the formation of a clear zone around the paper disc, indicating the presence of antibacterial activity. *A. catechu* seed extract ointment produced a different zone of inhibition in each bacterium. The greatest inhibitory power was found in *E. coli*, *S. pyogenes*, and *S. aureus*, which were included in the category of strong inhibition (10–20 mm), while the antibacterial activities of *P. aeruginosa* and *A. baumannii* were included in the category of moderate inhibition (5–10 mm) (22). This study is also linear with other studies, which reported that areca seed extract concentrations of 1.5%, 3%, and 4.5% showed antibacterial activity against *S. mutans* and *S. aureus*. Areca seed extract concentration of 4.5% resulted in diameter inhibitions of 11.37 mm for *S. mutans* and 20.03 mm for *S. aureus* (23). *A. catechu* seed extract ointment produced the greatest inhibition at a concentration of 5% (F II) with an average inhibition zone of 15.13 ± 0.15 mm in *S. aureus* bacteria whose inhibition value was close to the positive control. Hence, it could be concluded that *A. catechu* ointment has the potential as an antibacterial against bacteria that commonly infect skin and wounds. Another study stated that betel nut extract in the form of toothpaste had antibacterial activity against *S. mutans* and *S. aureus* bacteria. The effective toothpaste formula as antibacterial against test bacteria was a formula with a concentration of 4.5% areca nut extract that resulted in an inhibitory diameter of 11.37 mm for *S. mutans* and 20.03 mm for *S. aureus* (24).

The presence of this antibacterial activity was because of the ethanol extract of *A. catechu* seeds that contains alkaloids, saponins, flavonoids, and tannins (25). Since it was believed that tannins had a similar effect to phenolic compounds, they are thought to have antibacterial potency via precipitating protein (26). Tannins have antibacterial activities by interacting with cell membranes, deactivating enzymes, and destroying or impairing the function of genetic material. Flavonoids function as antibacterial agents by forming complex compounds against extracellular proteins that disrupt the integrity of the bacterial cell membrane (27). Additionally, by interfering with the peptidoglycan constituent parts in bacterial cells, flavonoids and alkaloids can also have the ability as antibacterial agents that kill bacteria by preventing the formation of cell layer. Saponins have the ability as disinfectants and antiseptics that work to eradicate pathogens or stop the growth of microorganisms that often develop in wounds to prevent the wound from becoming infected (28). *A. catechu* seeds can help the wound healing process due to the presence of antibacterial substances that can suppress the growth of pathogenic bacteria and prevent infection in the wound so that wound healing can be accelerated (29).

In this study, the determination of the healing effect of burns with *A. catechu* seed extract ointment was macroscopically conducted for 14 days to determine the rate of scab formation and diameter reduction in grade IIA burns. Scab formation is a part of the burn wound

healing phase, namely, the proliferative phase. This stage aims to reduce the area of tissue affected by the lesion. This stage is responsible for masking the lesion itself, which includes angiogenesis, fibrosis, and re-epithelialization (30). In burns that were given F I and F II, the scab was formed more quickly due to the help of compounds contained in *A. catechu* seeds. The results of the identification of ethanol extract compounds from *A. catechu* seeds state that areca seeds contain chemical compounds that can assist in the process of healing burns. Each of the *A. catechu* content has its ability in the process of healing burns. In the positive control group, scabs were formed faster than other treatment groups. Neomycin sulfate was used as a comparison product, and had stronger antibacterial activity so it could accelerate the healing process. Each *A. catechu* content had the ability in the process of healing burns (31). The scab that forms on each wound is useful for keeping the wound clean and free from microorganisms. The scab that acts as a protector also limits the outer area of the wound with its inner area, so that scabs that are formed quickly will cause the wound process to run faster and to heal faster (32).

The results of the study on day 0, when the injury first occurred, showed that there was a widening of the wound caused by an inflammatory reaction. Widening of the wound diameter can occur due to blood clots caused by the contraction of smooth muscle walls of injured blood vessels and blood clotting by thrombin and fibrin (33). At the end of the inflammatory phase, reddish and soft granulation tissue is formed, which supports the wound healing process. The next process enters the proliferative phase where this phase is an active fibroblast phase moving into the tissue around the wound area and forming collagen that acts as granulation tissue in building new tissue in the injured area (34). During fibroblastic proliferation, neovascularization occurs, which is the process of forming new blood vessels crucial for the process of healing wounds. The blood vessels formed are followed by an increase in the number of fibroblasts to produce collagen. Fibroblasts produce large amounts of collagen that are a part of a triple-chain glycoprotein useful for building strength in scar tissue. Fibroblasts produced collagen on the 4th day after injury and increased in the 1st to 3rd weeks and continued for 2 to 4 weeks. Fibroblasts change their structure into myofibroblasts that can contract the tissue. This happens to help form new tissue in closing the wound (35,36).

In this study, the treatment given to the injured rats' backs every day showed that the diameter of the wound slowly decreased and the scab was formed, which prevented the oxidation of the wound so that bacteria and foreign substances from the external environment did not infect the wound on the skin causing the wound healing process run well and fast.

Areca catechu seed extract ointment could reduce wound diameter because there were secondary metabolites acting as antibacterial and contributing to the healing process of burns by inhibiting the inflammatory process, reducing inflammatory cell infiltration by being astringent, and increasing the speed of epithelialization. Phenolic compounds act as antioxidants that can counteract free radicals and reduce lipid peroxidation. The decrease in lipid peroxidation can prevent necrosis of the re-epithelialization process.

Tannins play a role in preventing the infection of wounds due to their strong antiseptic properties and increasing tensile strength in burns and shrinking skin pores (13,37). Flavonoids have anti-inflammatory activity by inhibiting arachidonic acid metabolism through the cyclooxygenase and lipoxygenase pathways resulting in a decrease in the levels of neutrophil cells in the area of inflammation. Meanwhile, the inhibition of lipoxygenase pathway will affect the production of leukotrienes, which play a role in stimulating neutrophil aggregation and chemotaxis. Inhibition of leukotriene production can prevent the accumulation of excess neutrophils so that it can suppress the inflammatory process. The inflammatory phase is crucial to the healing process of the wound and lasts for 0–4 days after the injury. During the inflammatory phase, foreign objects, especially bacteria, can cause persistent inflammation that can slow down the wound-healing process (38). The saponins contained in *A. catechu* seeds were known to stimulate vascular endothelial growth factor (VEGF); therefore, they can accelerate the inflammatory phase and the wound healing process (39).

Macrophages are a transition from the inflammatory phase to the proliferative phase where macrophages produce platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β), and VEGF that stimulate the formation of new blood vessels and granulation tissue. The formation of new blood vessels due to tissue damage by burns plays a role in maintaining the continuity of the function of various affected tissues and organs, especially the skin. This occurs through the formation of new blood vessels to replace damaged blood vessels (40).

In this study, the formation of blood vessels could be seen on day 7 and had entered the proliferative phase. This might be due to the ability of the metabolites contained in *A. catechu* seed extract to increase the number and activity of macrophages so that proliferation could occur earlier, and the inflammatory phase was short. Necrotizing cells also began to decrease, but there was already formed collagen and in F II the density of collagen was already tight. The presence of collagen tissue is caused by the increasing number of fibroblasts that migrate to the wound area, which indicates that the wound is already in a proliferative stage. The extract of *A. catechu* seeds also contains saponins that play a role in wound healing because they can stimulate fibronectin by fibroblasts and change the expression of the TGF-β receptor. Fibronectin is a large and multifunctional glycoprotein

that contains areas to bind to macromolecules, one of which is collagen. Fibronectin is found in the early phase of wound healing and induces fibroblast migration (41). The density of collagen reached a peak on days 5–7 by appearing periodically on day 3 with a low density of collagen and reached a peak on day 7 with moderate to densely spread collagen density, and the formation of angiogenesis occurred simultaneously with fibroplasia. The more fibroblasts there are in the wound area, the more collagen synthesized by fibroblasts will make the collagen somewhat thicker and the wound healing process will be faster.

In this research F II ointment with 5% concentration of *A. catechu* seed extract showed a potential effect in healing burns and strong antibacterial activity, so it is a natural ingredient that can be explored further by isolating active compounds.

Conclusion

Areca catechu L. seed extract ointment has antibacterial activity against bacteria that commonly infect skin and wounds. The administration of *A. catechu* seed extract ointment with concentrations of 2.5% and 5.0% could accelerate the healing process of grade IIA burns with the formation of scabs and reducing the diameter of the burn. Histologically, there is a decrease in inflammatory cell infiltration and coagulative necrosis and an increase in neovascularization and collagen formation. The preparation of *A. catechu* seed extract ointment with a concentration of 5.0% (F II) had a better effect than that with a concentration of 2.5% (F I).

Acknowledgment

We thank the Pharmaceutical Technology Formulation of Semisolida Laboratory, Microbiology Laboratory, Pharmacology Laboratory, Faculty of Pharmacy, Pancasila University for kindly supporting the technical issues in this study.

Authors' contribution

NMDS contributed to the conceptualization design and supervised the experiments, analysis, and writing original of the manuscript. F: supervised the formulation and analysis of ointment, writing-review and editing, NM: supervised the microbiological test, wrote, reviewed, and edited the manuscript, IA: conducted the ointment formulation; NKAW: conducted the microbiological test; AA: conducted the in vivo experiment. All authors have read and approved the final version of the manuscript.

Conflict of interests

The authors declare that they have no conflicts of interest

Ethical considerations

The in vivo wound healing test was conducted after obtaining ethical approval from the Ethics Committee of the Faculty of Medicine, Universitas Indonesia - Cipto Mangunkusumo Hospital with the ethic number: KET-675/UN2.F1/ETIK/PPM.00.02/2021. All the actions were taken by minimizing pain and suffering in experimental animals.

Funding/Support

None.

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