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# *Binahong leaf extract activity in the 8th day of wound healing infected with Staphylococcus aureus towards collagen tissue*

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**Abstract:**
Infectious wound treatment that isn't dosed properly might have negative effects including bacterial resistance, hence plant-based solutions like binahong (Anredera cordifolia (Ten.) Steenis) leaf extract is needed. Alkaloids, saponins, and flavonoids found in binahong leaf act as anti-inflammatory, antiseptic, increase fibroblast cells, and increase collagen production throughout the healing process and scar tissue creation. The goal of the study was to determine the thickness of collagen tissue using Masson's Trichrome staining on the 8th day of wound healing infected with S. aureus after administering binahong leaf extract. The research method used a completely randomized design with 4 research groups, normal control, negative control, treatment 25% (P1), and 50% (P2) concentrations of binahong leaf extract. The thickness was measured in 5 fields of view at 400x magnification with a score of 0-4. The results showed that the P2 group had the same average collagen thickness as the normal control group, which was 50% in each field of view (score 3). While the average thickness of the P1 group was 25% in each field of view (score 2). These results indicate that the administration of binahong leaf extract at 25% and 50% concentrations can stimulate the formation of collagen on S. aureus-infected wounds.

**Keywords:** Collagen thickness, Wound healing, S. aureus infection, Masson's Trichrome.

## INTRODUCTION

Due to the general morphology of the skin on the outside of the body, it is frequently subjected to skin friction, so that it often suffers from injuries, whether caused by disease, wounds, or physical trauma[1]. Wounds are caused by sharp objects such as knives that damage the anatomy of the skin tissue which is characterized by the edges of the wound in the form of straight and regular lines. Wounds that are not treated can cause effects such as loss of tissue substance, bacterial contamination, and lead to complications such as infection[2]. Infection can occur when microorganisms enter the body and cause trauma or damage[3]. *Staphylococcus aureus* which has alpha hemolysis and a toxin that can cause necrosis of the skin is one of the bacteria that caused the infection[4]. Infection by *S. aureus* is characterized by tissue damage with purulent abscess[5].

Chemical medications are commonly used to treat infectious wounds, however incorrect dosages potentially lead to negative effects such as bacterial

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resistance[5]. Consuming medications derived from plants, such as the binahong plant, becomes an option to prevent the negative effects of chemical drugs. The Binahong leaf extract is known to stimulate fibroblast cells and collagen formation which can accelerate the wound healing process[6,7]. Alkaloids, saponins, and flavonoids are contained in binahong leaves. Flavonoid compounds have anti-inflammatory activity and potentially prevent oxidation. Himawan shows that the combination of basil leaf and binahong leaf (ratio of 2:1) on ethanol extract has forceful antioxidant activity[8]. Saponin compounds are used as cleaning agents and antiseptics in wounds to kill or prevent the growth of bacteria[9,10]. Based on the research of Amerta, et.al. (2012) binahong was able to inhibit the growth of *S. aureus*[11]. Saponins also have the benefit of increasing fibroblast cells and stimulating the formation of collagen[12]. According to Miladiyah and Prabowo's (2012) research, Binahong leaf extract has the potential for wound healing in guinea pigs[13].

Wound healing is a transition process that involves a series of responses and complex interactions between cells and mediators. It is one of the most complex processes in physiology. The wound healing process is limited to local regeneration processes and is also strongly influenced by endogenous factors such as age, nutrition, immunology, use of drugs, metabolic conditions, bacterial infection, and location of the wounds[13,14]. The wound healing process is classified into three phases: inflammatory, proliferative, and remodeling[15]. The proliferative phase lasts from days 3 to 14 and is designed to strike a balance between scar tissue production and tissue regeneration[14]. According to Landénet et al. (2016), macrophages are granulocytes, endothelial cells, and collagen that form the extracellular and neovascular matrix[16]. The construction of new blood vessels from pre-existing blood vessels is known as angiogenesis[17].

Collagen is needed in the process of wound healing and scar tissue formation. Collagen begins to form at the proliferative stage of the injury which occurs on the 3rd day following the physical injury and keeps increasing until the 3rd week. The proliferative phase occurs at 3-14 days, characterized by the formation of granulation in the wound. In the form of fibronectin and cytokines, the extracellular matrix, wil lead fibroblast cells to proliferate. Proliferating fibroblast cells then will migrate to the wound surface, where fibrin threads had previously clotted the wound[18,19].

Fibroblast cells slowly develop on the wound surface and produce new collagen fibers during the proliferative phase[9,20]. Collagen fibers that have an irregular shape due to injury will be destroyed and replaced with new collagen. However, the amount of collagen produced is determined by the amount of collagen needed for the wound area. Collagen fibers that are formed will cover the wound surface and be strengthened by fibronectin's presence[21]. Collagen formation can be observed microscopically with Masson's Trichrome staining, which will be shown in blue on the preparation. This study is the first to examine the thickness of collagen tissue with Masson's Trichrome staining on day 8 of wound healing infected with S. aureus and treatment with binahong leaf extract.

## MATERIAL AND METHOD

This research has been approved by the Research Ethics Commission of the Faculty of Public Health, University Muhammadiyah Semarang number 553/KEKP-FKM/UNIMUS/2021. This type of experimental research used a completely randomized design (CRD) with 4 research groups (2 control groups and 2 treatment groups). Table 1 shows the study group structure. The study population used white rats (Rattus norvegicus) aged ± 2.5 months with a weight of ± 200-250 gram. Research samples were obtained from rat skin tissue from each research group, with 3 replicate, and each replication was made into a tissue block and then each block made 5 slides.

Table 1. Research Group Design

| Group | Descriptions |
|---|---|
| Normal Control Group (KN) | normal rats without any treatment |
| Negative Control Group (K-) | Rats treated incision and suspension of *S. aureus* |
| Treatment group 1 (P1) | Rats treated incision and suspension of *S. aureus*, and 25% binahong leaf extract. |
| Treatment group 2 (P2) | Rats treated incision and suspension of *S. aureus*, and 50% binahong leaf extract. |

The experiment was started by culturing and preparing a suspension of *S. aureus* from the pure culture which was inoculated in a liquid BHI medium and then incubated at 37°C for 3-6 hours. Then *S. aureus* was inoculated on MC media (Mac Conkey) and incubated for ± 24 hours at 37°C. *S. aureus* colonies were grown, then injected into BHIA media, cultured for 24 hours at 37°C, then suspended in NaCl 0.9% using the standard Mc Farland 0.5. The next step is to make binahong leaf extract in a 96 percent alcohol solution using the maceration process. The resulting macerate was then evaporated using a rotary evaporator at 37-39oC to obtain a thick extract. The produced Simplicia was diluted according to the treatment group (25%, and 50%).

Experimental animal acclimation was carried out for 7 days at the Unimus Experimental Animal Laboratory. The wound region was administered a 20 L suspension of *S. aureus* after making an incision on the back skin of rats (2 cm length and 3 mm depth). Binahong leaf extract (up to 50 L) was applied to the wound area every morning and evening for 8 days, then flattened with a cotton bud. The paraffin method was used to prepare skin tissue slides, starting with the excision of the biopsy in the wound area (2x1cm and 3mm depth), followed by a fixation on 10% NBF. Dehydration using graded alcohol, clearing using xylol, and embedding using paraffin. The skin tissue was cut with a thickness of 5 micrometers, then the slides were stained with Masson's Trichrome (SkyTec Laboratories). Identification and measurement of collagen tissue thickness with a magnification of 400x as much as 5 fields of view on each preparation and then given a score according to Table 2. Collagen thickness data were analyzed using the difference test between groups with Maan Whitney.

Table 2. Criteria for Collagen Thickness Measurement Score[22,23,24]

| Score | Description |
|---|---|
| 0 | Very low collagen thickness, 0% of collagen thickness in the wound area |
| 1 | Low collagen thickness, ≤25% of collagen thickness (marked in blue color) in the wound area |
| 2 | Medium collagen thickness, 25% of collagen thickness (marked in blue color) in the wound area |
| 3 | Thick collagen thickness, 50% of collagen thickness (marked in blue color) in the wound area |
| 4 | The thickness of collagen is very thick, 75% of collagen thickness (marked in blue color) in the wound area |

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## RESULTS AND DISCUSSION

Identification of collagen tissue in skin tissue preparations by microscopic observation at 400x magnification indicated by the blue-colored section on Masson's Trichrome staining is presented in Figure 1. The results of the measurement of collagen thickness in each group are presented in Table 3. The P2 group had the same mean collagen thickness as the normal control group (KN), which had a score of 3 with 50% collagen thickness in the wound area. These results demonstrate that the administration of 50% concentration of

binahong extract for 8 days can increase the growth of collagen in the skin tissue S. aureus-infected wounds so that it has a thickness of collagen as in normal skin.

The collagen thickness score of the P1 group, which received a 25% concentration of binahong extract, was 2. These results showed that administration of 25% binahong extract for 8 days could increase collagen growth by 25% in the skin tissue S. aureus-infected wounds. While the negative control group, which treated incision and suspension of *S. aureus* on the 8$^{th}$ day, had not formed collagen. The results of the Maan Whitney test showed that there was no significant difference in collagen thickness in the normal control group and the P2 group administration 50% binahong leaf extract. Meanwhile, between KN groups with K- and P1, K- with P1 and P2 as well as between P1 and P2, there are differences. The results of these statistical tests are by the results of collagen thickness measurements in Table 3.

The average thickness of collagen in the negative control group (K-) was measured at 0%, which can be caused by *Staphylococcus aureus* infection. Bacterial infection can prolong the inflammatory period, impairing wound healing and reducing collagen activation in the wound area[5]. Meanwhile, the P1 and P2 groups showed a collagen thickness of 25% and 50% respectively in the wound area. The administration of binahong leaf extract at 25% and 50% concentrations led to an increase in collagen production. This is due to the presence of secondary metabolites in binahong leaf extract that can be utilized as medicine. Secondary metabolites found in binahong plants include flavonoids, saponins, terpenoids, alkaloids as well as tannins, and ascorbic acid[25].

The flavonoid content of the binahong extract, namely flavor, has been shown to enhance vascularization and decrease edema. Flavonoids also have anti-inflammatory and antioxidant activities, which can help to eliminate or alter free radicals. Free radicals can inhibit inflammatory processes as well as the contraction of the formed collagen tissue, interfering with the wound healing process[5]. The flavonoid content is also believed to help in wound repair[26].

Saponins have antibacterial, analgesic, and anti-inflammatory activities, as well as the ability to stimulate collagen formation[27]. Saponins can enhance wounds heal faster by stimulating fibroblast proliferation and myofibroblast differentiation. Saponins play a role in wound healing by stimulating the production of type I collagen, which is necessary during wound closure[28]. Saponins are also known to enhance the membrane's ability to activate cell hemolysis. Bacteria lyse when saponins interacted with them. Saponins, which increase the number of macrophages and release growth factors in the production of fibroblasts, and the synthesis of collagen for the wound area, can increase monocyte proliferation. Saponins can also help accelerate the migration of keratinocytes, which play an important role in the wound resurfacing process[6].

Ascorbic acid (vitamin C) is needed to stimulate prolyl-hydroxylase and lysyl hydroxylase enzymes in the process of forming hydrogen bonds as a molecular framework and stabilizing polypeptide interactions to produce procollagen. Furthermore, procollagen will be converted into collagen molecules by the enzyme procollagen peptidase[29,30]. In wound healing, ascorbic acid has an important role as an antioxidant, as demonstrated by cell proliferation, inflammatory suppression, and collagen tissue contraction[13].

## CONCLUSION

The administration of 25% and 50% binahong leaf extract stimulated the formation of collagen in wound healing in rats infected with S. aureus on the 8th day; the thickness of collagen in the group with 50% binahong leaf extract was the same as in the normal rat group.

## AUTHORS' CONTRIBUTIONS

All authors contributed equally to this work.


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## DATA AVAILABILITY STATEMENT

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

## DISCLOSURE STATEMENT

The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors. The data is the result of the author's research and has never been published in other journals.

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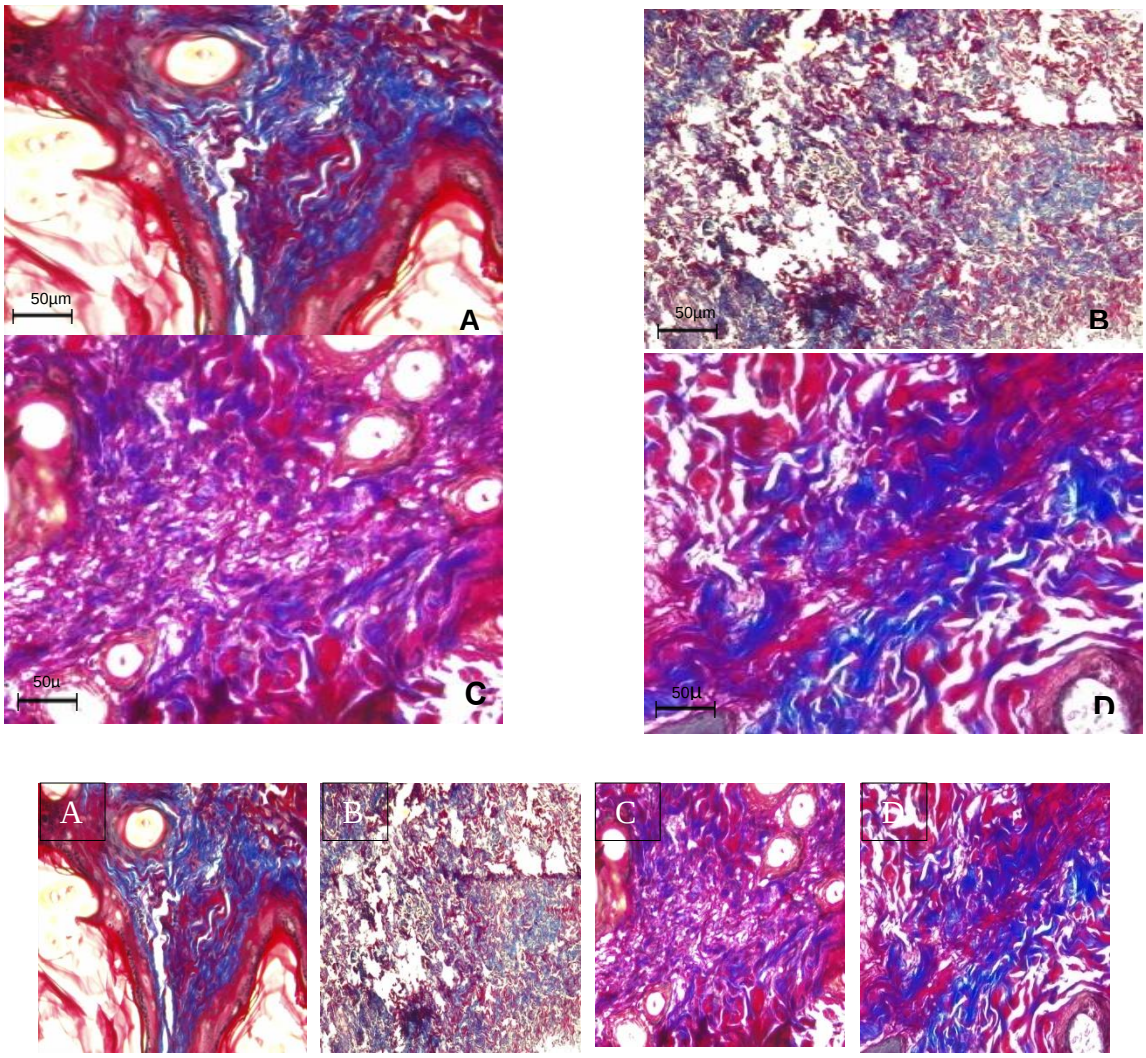


Figure 1. Identification of collagen (blue color) by Masson's Trichrome staining of skin tissue in the KN group with a score of 3 (A); K- score 0 (B); P1 score 2 (C); P2 score 3 (D) (400x)

Table 3. Average Score of Collagen Thickness Measurements in Each Treatment Group

| **Treatment Group** | Collagen Thickness Mean Score |
|---|---|
| Normal Control Group (KN) | 3,00[a] |
| Negative Control Group (K-) | 0,00[b] |
| Treatment Group 1 (P1) | 2,00[c] |
| Treatment Group 2 (P2) | 3,00[a] |

Different letters in each value in the same column indicate a significant difference (P<0.05) in the Maan Whitney test.