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# *Adolescent vaginal hygiene and Trichomonas vaginalis: A Focused study in Balongbendo village, Sidoarjo, East Java, Indonesia*

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**Abstract:** Trichomonas vaginalis *is a protozoan parasite responsible for leucorrhoea, transmitted primarily through sexual contact, and commonly affecting the lower urogenital tract. According to the World Health Organization (WHO), the global incidence of trichomoniasis is estimated at 180 million cases annually. In the United States, the infection affects around 2-3 million women annually, with the organism also found in 30-40% of male sexual partners. This study aimed to examine the presence of* Trichomonas vaginalis *in adolescent urine samples and assess their vaginal hygiene practices in Balongbendo Village, Sidoarjo, East Java, Indonesia.* Trichomonas vaginalis *is widely prevalent across the globe, including both rural and urban areas. This focused study utilized both experimental and questionnaire-based approaches. Urine samples from 25 adolescents were analyzed directly for Trichomonas vaginalis presence, while a questionnaire was administered to assess personal hygiene practices, particularly focusing on genital care. Data were analyzed using One-Way ANOVA and Chi-Square tests to determine statistical significance and percentage distributions. The study identified* Trichomonas vaginalis *in 5 out of 25 urine samples, indicating a 20% prevalence among the participants. The majority of respondents demonstrated a solid understanding and practice of vaginal hygiene. The analysis revealed a statistically significant relationship between genital hygiene practices and the presence of Trichomonas vaginalis, with a p-value of 0.001 ($p < 0.05$). The findings indicate that while* Trichomonas vaginalis *remains present among adolescents in Balongbendo Village, the majority exhibit good vaginal hygiene practices, highlighting the importance of continuous education on genital care in preventing such infections.*

***Keywords:*** *Trichomonas vaginalis; Urine; Adolescents; Hygiene*

## INTRODUCTION

*Trichomonas vaginalis* is a protozoan parasite that causes trichomoniasis, a sexually transmitted infection that frequently targets the lower urogenital tract[1]. In women, the infection is often asymptomatic, but when symptoms do occur, they typically manifest as profuse, greenish, frothy vaginal discharge [2]. The incidence of *Trichomonas vaginalis* infection is on the rise, with several factors contributing to this increase, including age, education level, genital hygiene, access to clean water, the frequency of ch anging sexual partners, routine health check-ups, the use of medications or vaginal cleaning products, and knowledge of trichomoniasis[3].

Taxonomically, *Trichomonas vaginalis* belongs to the Kingdom Animalia, Phylum Protozoa, Class Zoomastigophora, Order Mastigophora, Genus *Trichomonas*, and Species *Trichomonas vaginalis* [4]. Unlike many other protozoa, *Trichomonas vaginalis* does not have a cyst stage and exists only in the trophozoite

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stage [5] .The parasite is characterized by its oval or piriform shape, with four anterior flagella and a fifth flagellum that forms the axoneme and undulating membrane [6]. At its posterior end, the axoneme extends beyond the body, likely aiding in tissue attachment and causing irritation. The trophozoite has a single nucleus and an anterior cytostome for nutrient uptake, and it reproduces through binary fission. In females, *Trichomonas vaginalis* primarily resides in the vaginal mucosa, whereas in males, it is found in the urethra [7].

*Trichomonas vaginalis* is pathogenic and typically inhabits the genitourinary tract of infected individuals. The infection is transmitted through sexual contact and can lead to vaginitis in women and non-gonococcal urethritis in men. The parasite is widely distributed globally, affecting both rural and urban populations. In 2002, the World Health Organization (WHO) estimated the global incidence of trichomoniasis at 180 million cases. In the United States alone, the parasite infects approximately 2-3 million women annually and is present in 30-40% of their male sexual partners. According to WHO data from 2007, the prevalence of trichomoniasis ranged from 25% to 50%, bacterial vaginosis from 20% to 40%, and candidiasis from 5% to 15%. In Indonesia, approximately 75% of women have experienced vaginal discharge at least once in their lives, with 45% experiencing it twice or more [8]. Data from the Ministry of Health indicates that 5.2 million adolescent girls in Indonesia report symptoms post-menstruation, primarily due to poor hygiene practices. Additionally, of the 69.4 million people in Indonesia, 63 million adolescents reportedly engage in unhealthy hygiene behaviors, particularly regarding reproductive organ cleanliness during menstruation. [9]

This lack of hygiene is linked to increased cases of vaginal infections, including trichomoniasis. A study conducted in 2012 at MA Al-Hikmah Aengdake Bluto found a significant relationship between personal hygiene and the incidence of vaginal discharge. The study revealed that all respondents had experienced menarche, with 95% having suffered from vaginal discharge, and the majority demonstrating poor personal hygiene practices. Given the significant public health implications of trichomoniasis and the observed deficiencies in adolescent hygiene practices, this study aims to investigate the presence of *Trichomonas vaginalis* among adolescents in Balongbendo Village, Sidoarjo, East Java, and to assess the cleanliness of their intimate organs[10].

## MATERIAL AND METHOD

### Study Design

This research employed an observational design with both survey and laboratory components. The study was conducted in Balongbendo Village, Sidoarjo Regency, East Java, with urine examinations performed at the Anwar Medika University Integrated Biology Laboratory during April-May 2022.

### Study Population

The population for this study comprised all adolescents aged 11-25 years residing in Balongbendo Village. A purposive sampling technique was used to select 25 respondents based on specific criteria The inclusion criteria in this study are: Young women in Balongbendo Hamlet with an age range of 11-25 years, willing to fill out informed consent, willing to fill out the questionnaire, can hear and read well. Exclusion criteria are criteria outside the inclusion criteria, which are included in the exclusion criteria for this study are Adult women aged >25 years, woman aged <11 years, not willing to fill out informed consent, and Cannot hear and read well. determined by the researcher.

### Study Type and Data Collection Methods

This research is classified as observational analytic with a cross-sectional approach. Data collection involved distributing questionnaires to the respondents and collecting urine samples. The questionnaires focused on intimate organ hygiene, while the sample pots were used for urine collection. Both the questionnaires and urine samples were then analyzed.

**Research Procedures**

1. **Urine Sample Preparation:** The collected urine samples were transferred into centrifuge tubes and centrifuged at low speed for 5 minutes. The supernatant was then carefully removed, and the remaining precipitate was homogenized.
2. **Microscopic Examination:** A drop of the homogenized precipitate was placed on a microscope slide and covered with a cover slip. The slide was examined under a microscope at 10x magnification to identify potential organisms, followed by 40x magnification to confirm the presence of *Trichomonas vaginalis*.

**Data Processing and Analysis**

Data from the questionnaires and urine examinations were analyzed quantitatively. The results were presented in tables and graphs for interpretation. Data analysis using One-Way ANOVA Chi-Square the calculation of the amount and percentage.

## RESULTS AND DISCUSSION

The study involved 25 adolescent female respondents from Balongbendo Village, Sidoarjo Regency. The age distribution of the respondents is shown in Table 1.

Table 1: Age Distribution of Respondents in Balongbendo Village, Sidoarjo Regency

| Age Range | Number of Respondent | Percentage % |
|---|---|---|
| 16-20 | 4 | 16% |
| 21-25 | 21 | 84% |
| **Total** | **25** | **100%** |

From Table 1, it is evident that the majority of respondents (84%) were aged 21-25 years, while the remaining 16% were aged 16-20 years.

*Table 2: Prevalence of Trichomonas vaginalis in Urine Samples by Age Group*

| Age Range | Negative for Trichomonas vaginalis | Positive for Trichomonas vaginalis |
|---|---|---|
| 16-20 | 4 (16%) | 0 (0%) |
| 21-25 | 16 (64%) | 5 (20%) |
| Total | 20 (80%) | 5 (20%) |

In Table 2, out of the 25 urine samples analyzed, *Trichomonas vaginalis* was detected in 5 samples (20%), all within the 21-25 years age group. The remaining 20 samples (80%) tested negative for *Trichomonas vaginalis*.

**Vaginal Hygiene Questionnaire Results**

The study also included a questionnaire assessing various aspects of vaginal hygiene among the respondents. The key findings are summarized, **76%** of respondents recognized the importance of maintaining genital hygiene; **68%** changed their underwear at least twice a day; Only **32%** washed their vagina from front to back after urinating or defecating; **92%** changed sanitary pads after 4 hours of use; **84%** washed their hands after changing sanitary pads; **76%** maintained vaginal hygiene after sexual intercourse; **72%** dried the vagina before reapplying

pads; **80%** understood the relationship between genital health and overall female organ health; and **80%** changed their underwear twice a day, while only **12%** changed it only after bathing in the morning.

Table 3: Statistical Analysis of the Relationship Between Vaginal Hygiene Practices and Trichomonas vaginalis Presence

| NO | Statement | P-value |
|---|---|---|
| 1. | Genital hygiene is important to maintain | 0.001 |
| 2. | I change my underwear at least twice a day | 0.000 |
| 3. | I wash my female organs from the back to the front after defecating and urinating | 0.000 |
| 4. | I change sanitary napkins after 4 hours of use | 0.003 |
| 5. | I will wash my hands before and after changing pads | 0.102 |
| 6. | The vagina needs to be washed after sexual intercourse | 0.349 |
| 7. | The vagina must be dried before using pads again | 0.075 |
| 8. | Genital health is health related to the health of the female organ system according to its function | 0.000 |
| 9. | Good underwear is one that is changed 2 times a day | 0.012 |
| 10. | I change my underwear when I take a shower in the morning | 0.538 |

The study primarily focused on assessing the prevalence of *Trichomonas vaginalis* among adolescent females in Balongbendo Village, Sidoarjo Regency, and its association with vaginal hygiene practices. The findings indicate a noteworthy presence of *Trichomonas vaginalis* (20%) among the respondents, particularly in the 21-25 age group, highlighting the relevance of sexual health education in this demographic. The higher prevalence of *Trichomonas vaginalis* among respondents aged 21-25 years aligns with existing research indicating that sexually active individuals within this age range are at increased risk for trichomoniasis. This age group often represents a period of increased sexual activity, and the presence of the parasite can be linked to sexual transmission, which is the most common route of infection [11]. The absence of *Trichomonas vaginalis* in the 16-20 age group may suggest lower sexual activity or better hygiene practices in this subset, although this cannot be conclusively determined without additional data on sexual behavior [12].

The questionnaire results revealed varying levels of awareness and adherence to proper vaginal hygiene practices [13]. While a majority of respondents recognized the importance of maintaining genital hygiene (76%) and changing underwear regularly (68%), a significant gap was noted in specific practices that are critical for preventing infections. Only 32% of respondents were aware that washing the vagina from front to back is essential to prevent the introduction of fecal bacteria into the vaginal area, a practice that, when done incorrectly, can increase susceptibility to infections like trichomoniasis [14]..

The strong statistical associations observed between certain hygiene practices and the presence of *Trichomonas vaginalis* further underscore the importance of proper genital hygiene. For instance, the significant p-values obtained for statements related to genital hygiene ($p = 0.001$), regular changing of underwear ($p = 0.000$), and timely changing of sanitary pads ($p = 0.003$) suggest that these practices are closely linked to reducing the risk of infection. Conversely, practices such as washing the vagina from back to front ($p = 0.000$) were significantly associated with a higher risk, reinforcing the need for education on correct hygiene techniques [15].

The results of this study are consistent with findings from other studies that emphasize the importance of proper vaginal hygiene in preventing sexually transmitted infections (STIs) [16]. For instance, research conducted by [Hubaedah]

(2019) in [Bangkalan] found that inadequate genital hygiene was a significant risk factor for trichomoniasis, with similar age-related patterns in prevalence [17]. This study's finding that only 32% of respondents practiced correct vaginal washing mirrors the results of previous studies, such as [Zubaedah] (2021), which reported that a majority of women (73.5%) did not know the correct technique for cleaning their genital area [18].

Moreover, studies have shown that educational interventions focusing on personal hygiene can significantly reduce the incidence of genital infections. For example, [Pamuji] (2019) demonstrated that targeted health education in [SMA student] led to a marked improvement in hygiene practices and a corresponding decrease in infection rates [19]. This suggests that implementing similar educational programs in Balongbendo Village could potentially reduce the prevalence of *Trichomonas vaginalis* and other STIs among adolescent females [20].

The findings from this study highlight the need for increased awareness and education on proper vaginal hygiene, especially among adolescents in rural areas [21]. Given the significant relationship between hygiene practices and the presence of *Trichomonas vaginalis*, public health interventions should focus on correcting misconceptions and promoting accurate information regarding genital care [22]. Educational campaigns should emphasize the importance of practices such as washing the genital area from front to back, changing sanitary pads regularly, and maintaining overall cleanliness, particularly during menstruation and after sexual activity [23].

Furthermore, the study suggests that sexual health education should be integrated into broader public health strategies targeting young women in this community [24]. This could include workshops, informational sessions, and distribution of educational materials that address both general hygiene and specific practices to prevent STIs. Collaboration with local health authorities, schools, and community leaders could enhance the effectiveness of these initiatives [25].

While this study provides valuable insights into the relationship between vaginal hygiene practices and the prevalence of *Trichomonas vaginalis*, several limitations should be considered [26]. The sample size was relatively small (n=25), which may limit the generalizability of the findings to the broader population. Additionally, the study relied on self-reported data for hygiene practices, which may be subject to reporting bias or inaccuracies[27]. Future research should aim to include larger and more diverse samples to validate these findings [28]. Longitudinal studies could also provide more comprehensive data on how changes in hygiene practices over time influence the prevalence of trichomoniasis and other STIs [29]. Moreover, investigating the role of sexual behavior, socioeconomic factors, and access to healthcare in conjunction with hygiene practices could offer a more holistic understanding of the determinants of trichomoniasis in this population [30].

## CONCLUSION

In conclusion, this study underscores the critical role of proper vaginal hygiene in preventing *Trichomonas vaginalis* infections among adolescent females. The significant associations found between hygiene practices and the presence of *Trichomonas vaginalis* highlight the need for targeted educational interventions to improve hygiene behaviors. By addressing the gaps in knowledge and promoting correct hygiene practices, it may be possible to reduce the prevalence of trichomoniasis and improve the overall sexual health of young women in Balongbendo Village.

## AUTHORS' CONTRIBUTIONS

Acivrida Mega Charisma:Corresponding authors, prepared the samples, designed the protocols, executed the protocols. Farida Anwari: wrote the manuscript, submit and revisionand reviewthe manuscript. Bagus Nuzul Maulana: collection. data analytic and visualization statistically. All authors have read and approved the final manuscript

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## DATA AVAILABILITY STATEMENT
The utilized data to contribute in this research 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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Original Research

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# *Assessing the Therapeutic Potential of Arctium lappa L. (Burdock Root) Ethanol Extract in Wound Healing on Male Rattus norvegicus*

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**Abstract:** Burdock root (*Arctium lappa L.*) is known for its rich content of lignans, flavonoids, and phenolic acids, which exhibit anti-inflammatory, antioxidant, anti-cancer, and free radical-scavenging properties, making it a promising agent for wound healing. This study aims to evaluate the efficacy of *Arctium lappa L.* ethanol extract in promoting wound healing in *Rattus norvegicus*. A laboratory experiment was conducted using five groups of male rats: three treatment groups receiving burdock root extract at concentrations of 15%, 30%, and 45%, and two control groups—one treated with povidone iodine (positive control) and the other with a basic gel (negative control). Results demonstrated that 30% and 45% burdock root extract significantly improved wound healing compared to both control groups, with the 45% concentration showing the greatest efficacy. These findings suggest that *Arctium lappa L.* extract, particularly at higher concentrations, can enhance the wound healing process and may serve as a viable alternative to conventional treatments.

**Keywords:** Burdock root extract; extract; rats; wound healing.

## INTRODUCTION

Our skin is key to our survival, as the sense of touch, maintains physicochemical and termal homeostasis, acts as a reservoir of essential nutrients, provides passive and active defense, and responds to trauma and injury. The skin also has a role as a body protector from pathogenic agents.[1] Maintaining skin function requires strong and effective mechanisms to protect against trauma, disruption, repair and replacement of critical skin functions when damaged or lost.[2] The incidence of injuries is increasing every year, both acute and chronic wounds. A recent study showed the prevalence of patients with wounds is 3,50 per 1000 population. The majority of injuries in the world's population are wounds due to surgery/trauma (48%), leg ulcers (28%), and decubitus ulcers (21%).[3] Wounds are changes in tissue continuity in cellular and anatomical terms, which can occur in the skin and oral mucosa and continue in the wound healing process.[4] Skin wound healing is the process in which the skin repairs itself after injuries caused by surgery, trauma and burns.[2] Achieving faster healing with fewer side effects is still one of the most important medical goals because it can reduce the risk of infection, complications, and costs.[5] The use of traditional plants for wound healing is based on their ability as antiseptic, antiinflammatory, astringent and antibacterial.[6] The

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use of herbal plants has been accepted by almost all countries in the world. In developed countries, the use of certain herbal plants is very popular. [7] While in developing countries, where standardized health services are limited, herbal plants are usually preferred, as cheap and accessible treatments for disease management. In Indonesia, traditional plants are still widely used in society, both rural and urban. One of the plants that is not yet known but has many benefits is burdock *(Arctium lappa* L.). [8]

Burdock is a plant known as burdock that can be found all over the world which considered a weed.[9] This plant is traditionally used to treat infections such as sore throats, boils, rashes and various skin problems. Many studies have investigated the biological various parts of the burdock, such as anti-microbial, anti-pyretic, anti-inflammatory, anti-hepatotoxicity and antioxidant activity. Compounds found in burdock roots are classified into lignans, flavonoids, and phenolic acids. Two main lignans found in roots are arctigenin and arctiin which have the potential as anti-inflammatory. Arctigenin is a bioactive compound that acts as an anti-inflammatory agent by controlling cytokines and the NF-B signaling pathway. When injured, the body initiates an inflammatory process characterized by an increase in proinflammatory cytokines such as IL-1β, TNF-α, and IL-6. By inhibiting the activation of the NF-B signaling pathway, which is involved in the expression of inflammatory genes, arctigenin helps reduce inflammation caused by injury. [10] Arctiine is also anti-inflammatory, which stimulates and agitates as a powerful antioxidant. Arctiine increases the activity of antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) to reduce oxidative stress generated during the inflammatory process. By reducing the damage caused by free radicals, this antioxidant activity helps to accelerate the passage from the inflammatory phase to the proliferative phase. Arctiine also stimulates macrophages to increase phagocytosis, essential for the elimination of microorganisms and cellular debris during the inflammatory phase. [11] Other bioactives including Lignans, quercetin, chlorogenic acid, and inulin have anti-inflammatory and antioxidant properties, modulate immune responses, reduce oxidative stress, and support wound healing and immunity. [12] Flavonoids such as luteolin and quercetin can clean up free radicals and act as anti-inflammatories. Phenolic acids in burdock roots such as caffeoylquinic acid derivatives and chlorogenic acid show strong antioxidant activity. In addition, a fatty acid derivative called oleamide is also found in burdock roots as an anti-allergic agent by inhibiting the release of histamine and producing IL-4 and TNF-α.[13] burdock root is used as a blood purifier, and believed to clear toxins from the bloodstream.[14] Burdock root also contains other chemical compounds such as inulin, essential oils, tannins, resins, sugar, iron, and vitamin C.[5] Tannins function as an astringent which causes shrinkage of the skin pores, hardens the skin, and stops light bleeding.[15] Vitamin C has a role in forming and maintaining collagen during wound healing and can prevent bleeding from the vascular component of connective tissues.[16] Burdock plant studies are more on the stems and leaves. [17] The renewal of this research burdock examines the roots. Research studies roots contain rich protein, oligosaccharides, and other nutrients, and also contain polyphenols, and aldehydes.[18] The content of chemical compounds are tested phytochemically that burdock roots contain saponins, flavonoids, alkaloids, tannins, arctigenin and arctiin function as anti-inflammatory, antioxidants play a role in the wound healing phase.[19,20] This research aims to see the potential of burdock root as an alternative wound healing agent.

## MATERIAL AND METHOD

The research was an experimental laboratory conducted at the Pharmacy, Science and Technology Laboratory of Al-Irsyad University, Cilacap from March to June 2023. The protocol of animal research has been approved by The Medical

Research Ethics Committee Fakultas Kedokteran, Universitas Jenderal Soedirman (UNSOED), No. 070/KEPK/PE/V/2023. The materials used were burdock root, 96% ethanol, basic gel, normal saline solution (0.9% NaCl), ketamine, alcohol swabs, povidone iodine. Fresh burdock roots were washed and thinly sliced and then dried in an oven at 50°C for 2x24 hours. The dried simplicia was then crushed to form a powder. As much as 200 g of simplicia powder was put into the maceration container and 300 ml of 96% ethanol was added, stored at room temperature for 3x24 hours with stirring once per day.[21] After soaking for 3 days, the solution was filtered and evaporated using a water bath at 50°C to get the crude extract.

This study obtained burdock root ointment we have prepared it by referring to the dosage of the previous research formulation conducted by Widyawati et al. [22] while the use of gel-based preparations allows for the enhancement of bioavailability, controlled release, and optimal wound environment, ensuring that the bioactive components in the extract work effectively in the healing process. [23]

**Table 1. Ointment Formulation**

| Group Treatment | Burdock Root Extract (g) | Vaseline Album (g) | Ointment (g) |
|---|---|---|---|
| Negative Control (Basic gel) (%) | - | 15 | 15 |
| 15 | 2,25 | 12,75 | 15 |
| 30 | 4,5 | 10,5 | 15 |
| 45 | 6,75 | 8,25 | 15 |

Before making a wound on the back of the rat, the hair was shaved and disinfected using an alcohol swab. The rat was anesthetized using ketamine at a dose of 50 mg/kg BW intramuscularly, then an incision was made in the back area parallel to the vertebrae using a scalpel to form a 2 cm long wound with a depth of 0,25 cm. Wound care is done shortly after the wound occurs. The wound was cleaned using normal saline (NaCl 0,9%) and then performed according to the treatment group. Treatment is carried out every three days for 14 days until the wound shows signs of healing, such as granulation growth, no swelling (edema) in the wound area, redness around the wound and wound length in cm units. The application was completed twice daily for 14 days, in the morning and the evening. The length of the wound was measured on days 3, 6, 9, 12, and 14 before the ointment was reapplied to track the duration of wound healing using macroscopic observation criteria. On day 14, the rat skin was examined under a microscope. [24]

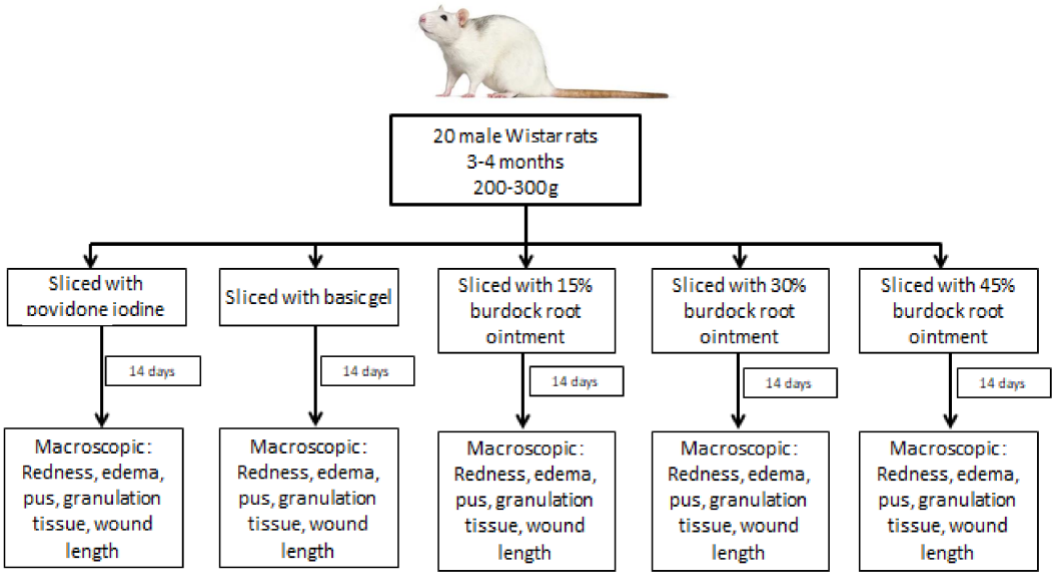


**Figure 1.** Scheme of animal experiments

Data analysis using the parametric test. The first step was to test the normality using Shapiro-Wilk because the number of samples was less than 50. Then, the data homogeneity test was carried out using the Test of Homogeneity of Variances. After fulfilling the requirements of the parametric test, a One-Way Anova test was carried out to find out whether there was a significant variance in the data significantly different or not. This test was significant if the p-value <0,05. After that, the LSD post hoc test was carried out to see a more meaningful treatment of wound healing in white rats. Data were not normally distributed, an alternative test was performed using the Kruskal-Wallis test and followed by the Mann-Whitney test to determine which treatment had the highest significant value for wound healing in white rats with a significance level of α<0,05. The Kruskal-Wallis Test is a non-parametric alternative to ANOVA that is most appropriate to use because it is the comparison of 4 groups of herbal dose formula groups on wound healing. This test examines whether there is a significant difference between the medians of two or more groups that are not normally distributed. The Mann-Whitney U Test (Wilcoxon Rank-Sum Test): If you only compare two groups (for example, a group with herbal doses and a control), then the Mann-Whitney can be used. This is an alternative to the t-test for data that is not normally distributed. [25]

## RESULTS AND DISCUSSION

The results of macroscopic observation of wound healing in male rats can be seen in the table.

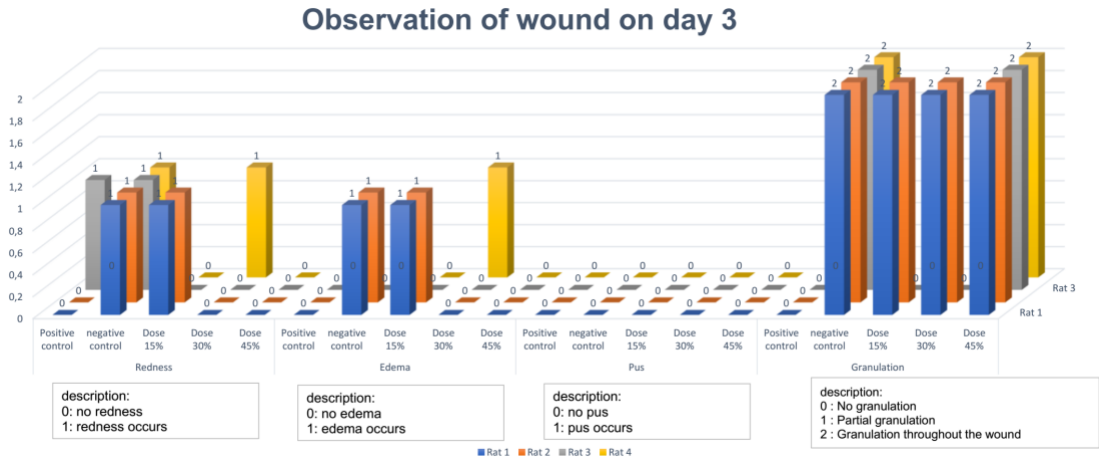


**Figure 2.** Observation of wound on day 3 includes observation of redness, pus, granulation in the positive control, negative control, dose 15%, dose 30% and dose 45% rat groups.

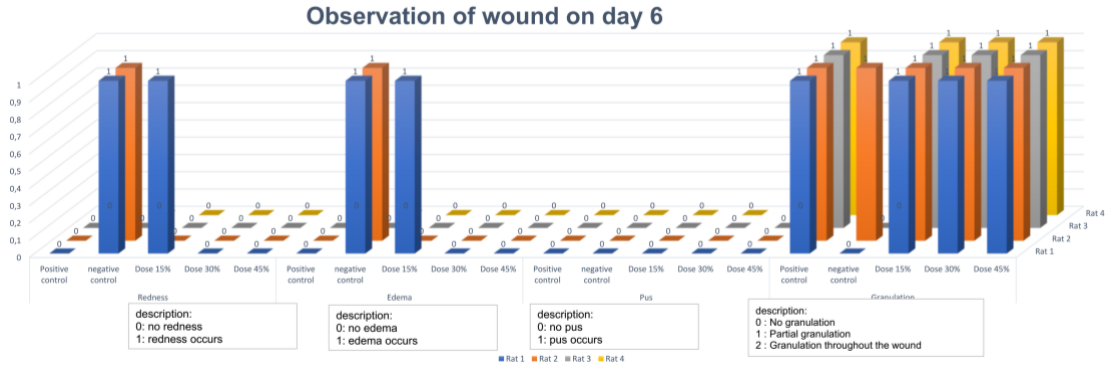


**Figure 3.** Observation of wound on day 6 includes observation of redness, pus, granulation in the positive control, negative control, dose 15%, dose 30% and dose 45% rat groups.

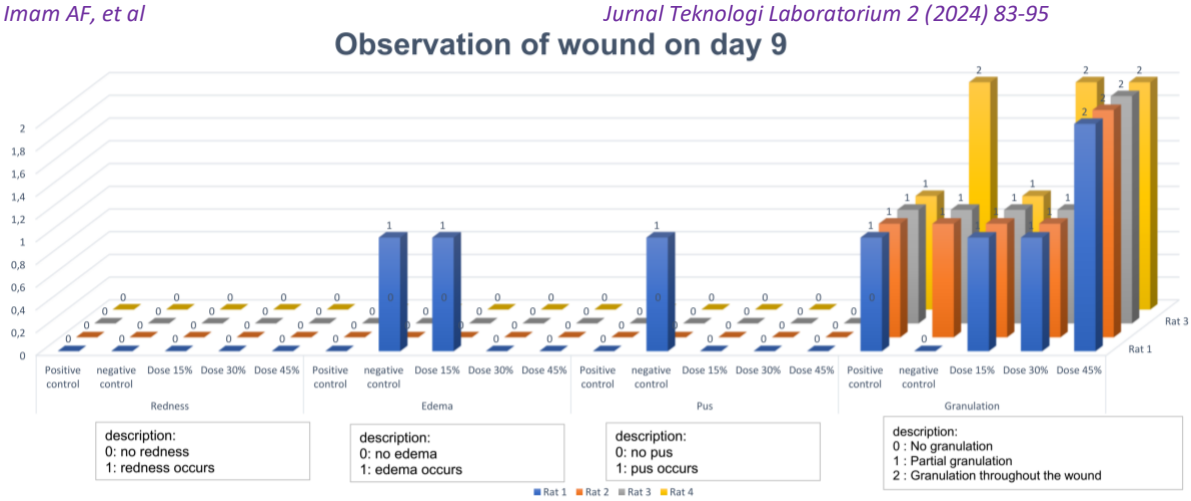


**Figure 4.** Observation of wound on day 9 includes observation of redness, pus, granulation in the positive control, negative control, dose 15%, dose 30% and dose 45% rat groups.

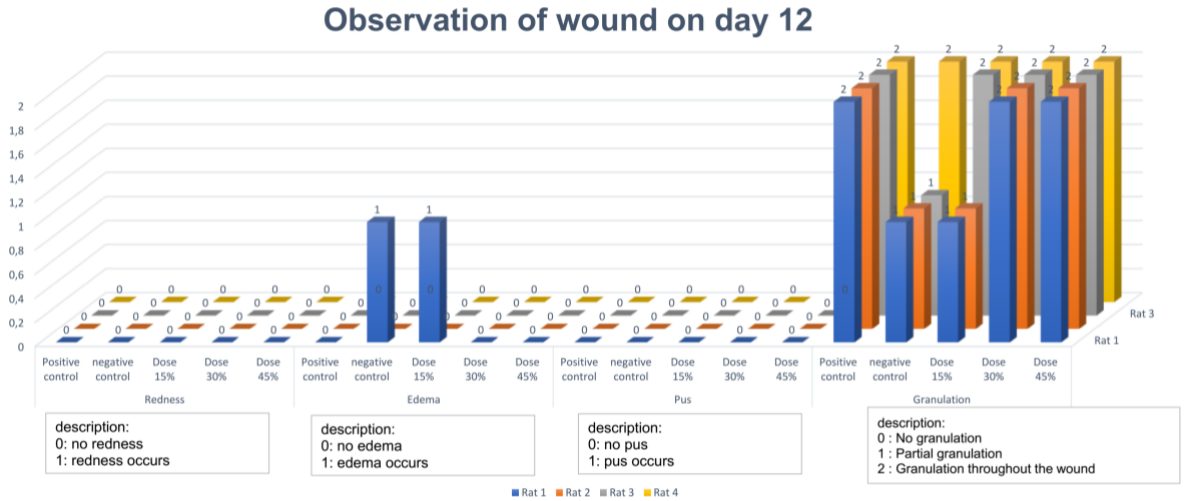


**Figure 5.** Observation of wound on day 12 includes observation of redness, pus, granulation in the positive control, negative control, dose 15%, dose 30% and dose 45% rat groups.

There are three phases in the wound healing process, namely the inflammatory phase, the proliferation phase, and the remodeling phase, which are mutually sustainable.[6]

1. The healing process in the inflammatory phase

In this section, we will discuss aspects that are observed macroscopically during the wound healing process in the inflammatory phase. Observations made included redness around the wound edema around the wound, and pus fluid in the incision.

a. Redness

Based on the results of the study on day 3, it was found that in the negative control group, redness occurred in all samples. The positive control group and test treatment group 2 (30%) had one sample with redness around the wound. Test 1 (15%) 2 samples had redness around the wound and in the test treatment group 3 (45%) there are four samples without redness around the wound.

When an injury occurs, vasoconstriction occurs in the arteries and capillaries to help stop bleeding. This process is mediated by epinephrine, norepinephrine, and prostaglandins released by injured cells. Blood vessels will enter stage vasodilation after 10 to 15 minutes after injury. Vasodilation of blood vessels is mediated by histamine, serotonin, prostaglandine, and kinins which cause increased blood flow to the injured area and increased capillary permeability.

Increased blood flow to the injured area causes the injured area to appear red and warm.[26]

The acceleration of redness around the wound in the treatment group is thought to be due to the effect of the active compounds derived from burdock root extract as anti-inflammatories and the presence of flavonoid compounds when applied to the skin inhibits bleeding. Flavonoids also function as antibacterials by forming complex compounds extracellular proteins that disrupt the integrity of bacterial cell membranes and tannin compounds are antibacterial by interfering with the permeability of bacterial cells as astringents.[27] Tannins interact with cell surface proteins to form complexes that reduce blood vessel permeability, helping to control fluid exudation during the inflammatory phase. They also have an astringent effect, which accelerates coagulation and helps prevent infection. Tannins are also known to reduce the activation of MMPs (matrix metalloproteinases) enzymes responsible for the degradation of the extracellular matrix during inflammation, thus maintaining tissue integrity during healing. [28] Astringent is a shrinking surface or substance that is protective against the mucosa and can agglomerate protein.[29] Flavonoids can reduce the intensity of redness in wounds by stopping bleeding.[30] Flavonoids act as powerful antioxidants that play a role in inhibiting the NF-κB pathway, which is a major pathway in the inflammatory response. By inhibiting NF-κB activation, flavonoids can reduce the production of pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α, which are important during the inflammatory phase. In addition, flavonoids also increase the expression of antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx), which reduce oxidative damage during inflammation and the transition to the proliferative phase. [31] In addition, the role of arctigenin has significant anti-inflammatory activity through inhibition of the JAK/STAT and MAPK pathways. This pathway is important for the production of inflammatory cytokines during the inflammatory phase. Arctigenin also decreases the production of nitric oxide (NO) and prostaglandin E2 (PGE2) through inhibition of the COX-2 and iNOS enzymes, which contribute to reduced inflammation. Arctiin also works by inhibiting NF-κB and MAPK pathways. This reduces the release of inflammatory mediators and helps reduce the infiltration of inflammatory cells such as macrophages and neutrophils into the wound area. Arctiin increases the phagocytic activity of macrophages, which is important in clearing cellular debris during the inflammatory phase and helping the transition to the proliferative phase. [32]

b. Edema

During the inflammatory phase, mast cells release biological substances, namely histamine. Histamine is a vasoactive amino that is released by mast cells after injury and plays an important role in vascular dilatation and permeability, causing plasma to move from the intravascular to the extravascular and cause edema. Vasodilation that occurs in the blood vessels helps inflammatory cells from the vessels to the wound area.[26]

Based on the results of observations of edema on day 3, in the negative control group and test group 1, there were two samples with edema. In the positive control, no edema was found in all samples. In the test group there was one sample with edema. Edema in the test treatment group 1 (15%) and test 2 (30%) have a gradual reduction in the area of edema seen on day 6, the samples that have edema became one, and others are test group 1 (15%) and observations on day 9 to day 14 edema in the control group negative and in the test treatment group 1 (15%) there was one sample each that still had edema.

Swelling in a wound that does not heal can be caused by a germ or bacterial infection that enters the wound which is left open [33] Edema that occurred in the negative control group and others are testing group 1 (15%) was suspected because the wound had an infection which resulted in the wound becoming swollen After all the white rat is a rodent and likes to dig holes, that is what makes the white

rat like to dig into the husks causing the husk dirt to enter the wound which can later cause the wound to become infected. Whereas the absence of edema around the wound in the positive control group and the treatment group, namely test 2 (30%) and test 3 (45%), is suspected to be due to the effect of the essential oil content derived from burdock root extract. Essential oil a function as a wound healing agent that prevents bacterial infection from entering the wound.[5]

Tannins contain anti-bacterial compounds with a mechanism of shrinking the cell wall so that it inhibits the permeability of bacteria to develop.[34] Flavonoids are anti-imflammatory so they can reduce inflammation and help reduce pain, if there is bleeding or swelling of the wound.[35]

c. Presence of pus

Based on the results of the research that has been done, it was found that the presence of pus only occurred on day 9, in the negative control group, was one sample that had pus. While all samples in the control group were positive, and the treatment group test 1 (15%), test 2 (30%), test 3 (45%) did not have pus on the wound.

The absence of pus in the treatment group was thought to be due to the effect of the content of tannins, saponins and essential oils derived from burdock root extract.[36] Saponins have the ability as cleansers and antiseptics which function to kill or prevent the growth of microorganisms and tannins are antibacterial by interfering with the permeability of bacterial cells and as an astringent which can cause closing of skin pores, hardening the skin, stopping exudate and light bleeding.[27]

The use of 10% povidone iodine for wound care in the positive control group also affected reducing the amount of wound fluid exudate in the study sample because povidone iodine was able to kill bacteria, germs, fungi, viruses, protozoa, and spores by working directly to quickly kill germs (bactericidal), not inhibiting the development of germs (bacteriostatic).[26]

2. The wound healing process in the proliferative phase

In the proliferative phase there is a decrease in the number of inflammatory cells, reduced signs of inflammation, the appearance of proliferating fibroblast cells, the formation of new blood vessels, epithelialization and wound contraction. fibroblasts will migrate to the wound area and begin to proliferate until their numbers are more dominant than inflammatory cells.[26] In the proliferation phase, tissue granulation and shortening of the wound length were observed.

a. Granulation tissue

Observations of granulation tissue on day 3 found that in the negative control group there was no granulation tissue. In the positive control group and the test treatment group 3 all samples had partial granulation. Test groups 1 and 2 contained three partially granulated samples. These results prove that the growth of granulation tissue in the three treatment groups that were given burdock root extract was faster than the negative control group that was given basic gel. The growth of wound granulation tissue in the treatment group is thought to be due to the effects of saponins, flavonoids, and tannins derived from burdock extract. Saponin compounds are steroids or triterpenoid glycosides that can stimulate vascular endothelial growth factor (VEGF) and increase the number of macrophages migrating to the wound area thereby increasing fibroblasts in the wound tissue.[26] Saponins also can stimulate the formation of collagen.[27] Tannins help the wound healing process by increasing the number of capillary blood vessels and fibroblast cells.[5] Flavonoids can help wound healing by increasing collagen formation, reducing macrophages and increasing the number of fibroblasts.[26] Tannins accelerate collagen synthesis by stimulating TGF-β and stimulating fibroblast activity. Tannins also induce angiogenesis by increasing VEGF expression, which is necessary for tissue healing. In addition, the antioxidant

properties of tannins protect fibroblast cells from oxidative stress, allowing the healing process to occur more quickly and efficiently. [28]

Based on the results of statistical tests, all treatment groups had a significant effect on the accelerated growth of granulation tissue in the wounds of white rats, but what was more influential was burdock root extract concentration of 45%, then 30% and 15% this was also seen from the results of macroscopic observations where on day 12 the treatment group test 2 (30%) and test 3 (45%) all samples have granulation in all wounds the same as compared to the positive control but not to the test treatment group 1 (15%) ). The treatment group test 1 (15%) experienced slow granulation growth as seen from the statistical test test 1 (15%) which was not significantly different from the negative control group. This was presumably because the 15% concentration contained a small amount of burdock root so ingredients such as saponins, flavonoids, and tannins derived from burdock extract were less effective in forming granulation tissue in test group 1 (15%). Flavonoids enhance fibroblast proliferation by increasing the expression of growth factors such as TGF-β and VEGF, which stimulate angiogenesis (formation of new blood vessels) and collagen synthesis by fibroblasts. Flavonoids' antioxidant activity also protects fibroblasts from oxidative stress, thereby enhancing their regenerative capacity during the proliferative phase. Arctigenin stimulates fibroblast proliferation and increases collagen synthesis through activation of the TGF-β/Smad pathway, which is important in the process of extracellular matrix formation. [37] Arctigenin is also known to stimulate angiogenesis by increasing the expression of VEGF and FGF (fibroblast growth factor), which increases blood supply to the wound area and accelerates healing. Arctigenin facilitates fibroblast proliferation and increases collagen deposition by stimulating the expression of COL1A1 and COL3A1, genes that encode the synthesis of type I and type III collagen. Arctigenin is also known to reduce the activity of MMPs, which prevents the degradation of collagen and extracellular matrix, thereby accelerating the recovery of tissue structure in the proliferative phase. [32]

b. Wound Length

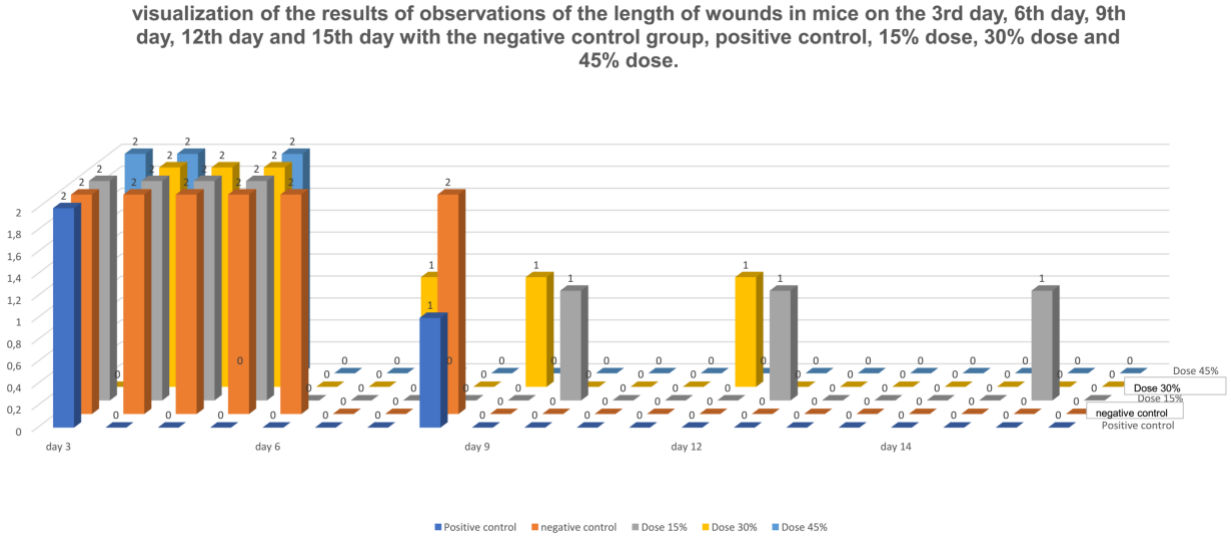


**Figure 6.** visualization of the results of observations of the length of wounds in mice on the 3rd day, 6th day, 9th day, 12th day and 15th day with the negative control group, positive control, 15% dose, 30% dose and 45% dose.

After granulation tissue is formed, epithelialization or growth of epithelial tissue will begin to occur. Epithelial cells that grow will move from the outside of the injured tissue to the inside of the tissue [38] The construction of wound tissue is the last stage of the reconstruction phase of wound healing. Construction will occur 6-12 days after the wound occurs and the wound will close.[39]

In this study the researchers observed the length of the wound starting on the 3rd day after making an incision with a length of 2 cm. The negative control

group ranged from 1.8 to 2 cm. Test group 1 ranged from 1.5 to 1.8 cm. Test group 2 ranged from 1 to 1.5 cm. Test group 3 ranged from 0.9 to 1.3 cm. Then there was a new significant difference that occurred on the 9th day which resulted in the positive group with wound lengths ranging from 0.6 to 0.8 cm. The negative control ranged from 1.4 to 1.5 cm. Test group 1 1 to 1.4 cm. Test 2 ranged from 0.6 to 1 cm. Test 3 ranged from 0.6 to 0.8 cm.

Saponins is a compound that act as antiseptic, stimulate the proliferation of epidermal cells and affect the rate of migration of keratinocytes to the wound area, thereby increasing wound epithelisation. Saponins can alson stimulate the production of type I collagen which plays a role in increasing epithelialization of sittues and wounf closure by inhibiting excessive tissue production.[40]

Based on the results of statistical tests, the treatment group using burdock root extract at a concentration of 15 % had no effect on the accelerated shortening of wound length in white rats. While the treatment group used burdock root extract with a concentration of 45% and 30% had a significant effect on accelerating the shortening of wound length in white rats, but the more influential was the 45% burdock root extract. Samples with a wound length of 0 cm (healed). Burdock root is effective in healing cuts in herbal medicine due to its anti-inflammatory, antioxidant, and fibroblast proliferation activities. In comparison, aloe vera accelerates epithelialization and angiogenesis through polysaccharides and glycoproteins and has strong anti-inflammatory effects.[41] Curcumin *(Curcuma longa)* in turmeric inhibits pro-inflammatory cytokines, increases collagen synthesis, and has antibacterial effects. Gotu Kola *(Centella asiatica)* supports wound healing by stimulating collagen synthesis, fibroblast proliferation, and tissue capillarization.[41] Comparison of burdock root to other herbs and povidone iodine shows that Burdock root contains arctigenin and arctiin which have been shown to have adequate anti-inflammatory and antioxidant properties to accelerate wound healing, especially through reducing inflammation and increasing collagen synthesis.[42] While Aloe vera has a rapid soothing effect and accelerates epithelialization, but does not have strong antioxidant potential like burdock root. Curcumin is superior in terms of inhibiting pro-inflammatory cytokines compared to burdock root, but burdock root is superior in terms of improving tissue remodeling. Comparison Povidone iodine is a conventional treatment that is widely used in wound healing, but its use is more intended to reduce the risk of infection, not to accelerate tissue regeneration like herbs.[43]

**Table 7. Kruskal-wallis test of wound length in male rats during 14 days.**

| Group Treatment | Average wound length (cm) | | | | |
|---|---|---|---|---|---|
| | Day 3 | Day 6 | Day 9 | Day 12 | Day 14 |
| Basic gel (negative control) | 2 | 1,90 | 1,50 | 1,12 | 0,72 |
| Povidone iodine (positive control) | 1,82 | 0,95 | 0,70 | 0,40 | 0 |
| 15% | 1,65 | 1,65 | 1,20 | 0,77 | 0,50 |
| 30% | 1,30 | 1,30 | 0,80 | 0,55 | 0,12 |
| 45% | 1,95 | 1,15 | 0,70 | 0,45 | 0,05 |
| **P-value* | 0,074 | 0,004 | 0,004 | 0,003 | 0,003 |

*Kruskal-wallis test

Based on the results of the Kruskal-wallis test above, it was found that the test dose and the positive control did not have a significant effect on the wound on day 3 (p-value>0,005). But it works on days 6 to 15 marked by narrowing of the incision area (p<0,005). [44]

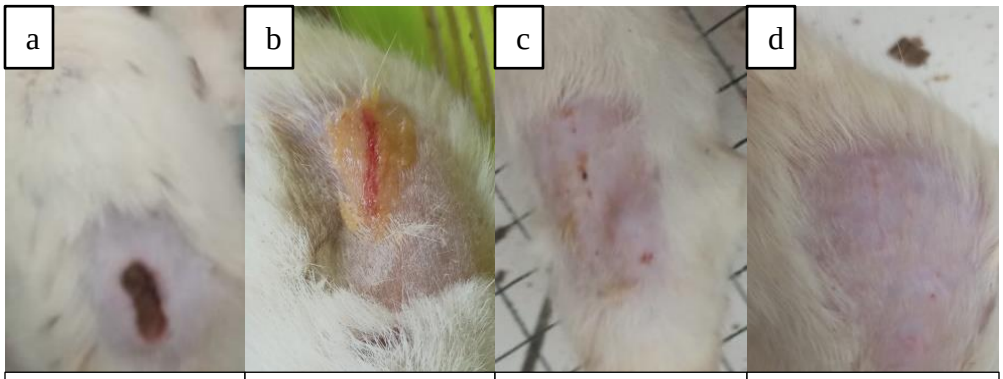


Figure 7. Edema and pus (a), redness (b) granulation (c), wound healed

Many studies examining the effectiveness of Burdock root in wound healing often involve small sample sizes, which can reduce the statistical power of the results. Studies with larger sample sizes are needed to ensure the reliability of the findings. Studies are often limited by short observation periods, usually only a few days to a few weeks. Wound healing is a process that requires long-term observation to assess full efficacy, especially during the tissue remodeling phase. Measurement of wound healing outcomes often relies on visual observation, which can be biased and subjective. The use of more objective techniques, such as histology or molecular measurements of growth factors or proteins, is needed to provide more precise data. Further studies are needed to test the effectiveness of Burdock root extract on different types of wounds, such as burns, diabetic wounds, or pressure ulcers. Combining Burdock root formulations with other treatments, such as co-application of silver nanoparticles or biomaterial scaffolds, may provide a synergistic effect that accelerates wound healing. This combination could also be tested on a larger scale to support the synergistic effect of the two methods.

## CONCLUSION

Administration of burdock root extract concentrations of 30 % and 45 % were effective in the wound healing process, both in the inflammatory phase and in the proliferative phase. Based on statistical tests and macroscopic observations there were differences in the effect of burdock root extract concentrations of 15%, 30% and 45% in the wound healing process. Burdock root extract with a concentration of 45 % is most effective in the healing process of cuts in rats.

## 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 upon 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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# *Therapeutic potential of Cinnamomum burmannii bark extract in reducing Malondialdehyde (MDA) levels in MSG-induced wistar rats: a preclinical study*

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**Abstract:** Increased consumption of monosodium glutamate (MSG) is associated with various health risks, including cardiovascular and neurological disorders. MSG triggers an increase in reactive oxygen species (ROS), the end product of lipid peroxidation produced when ROS increases is Malondialdehyde (MDA), On the other hand, interleukin 10 (IL-10) reduces the inflammatory effects in infectious conditions that can cause potential tissue damage. This study investigated the effect of cinnamon bark extract (*Cinnamomum burmannii*) on MDA and IL-10 levels in MSG-induced Wistar rats. Experiment with Post test only control group design was conducted. The number of samples was 24 male Wistar rats divided into 4 groups. KN healthy rats, K (+) rats were only induced by MSG, P1 rats were induced by MSG and given cinnamon bark extract at a dose of 100 mg, P2 rats were induced by MSG and given cinnamon bark extract at a dose of 200 mg. The average results showed a decrease in MDA levels after 14 days of treatment, one-way ANOVA test p = 0.001 (p <0.05) showed a significant difference in MDA levels KN 2.37 mg/ml ± 0.14, K+ 2.47 mg/ml ± 0.24, P1 2.32 mg/ml ± 0.20, and P2 0.84 mg/ml ± 1.07. Meanwhile, the average IL-10 levels showed no significant difference with one-way Anova test p = 0.127 (p <0.05) in the KN group, IL-10 levels were 93.25 pg / ml ± 25.01, K (+) 112.89 pg / ml ± 43.89, P1 69.48 pg / ml ± 12.83 and P2 93.29 pg / ml ± 12.11. Administration of cinnamon bark extract can reduce MDA levels in rats induced by MSG, but has no significant effect on IL-10 levels.

**Keywords:** Extract *Cinnamomum burmannii,* MDA Levels, IL-10 Levels, MSG.

## INTRODUCTION

Monosodium glutamate (MSG) is one of the most widely used food additives, known for enhancing the palatability of foods and stimulating appetite.[1,2] MSG is commonly added to various processed foods, with the average daily intake varying across regions. For instance, in industrialized European countries, the average intake ranges from 0.3 to 1.0 g/day, with 0.58 g/day reported in the UK and 10.0 g/day in Germany.[3] In Nigeria, the intake is estimated at 0.56–1.00 g/day, whereas in Asia, it is significantly higher, ranging from 1.1–1.6 g/day in Japan, 1.5–3.0 g/day in Taiwan, and 1.6–2.3 g/day in South Korea.[3] The Food and Drug Administration (FDA) classifies MSG as a safe substance; however, some animal studies have highlighted adverse effects associated with chronic MSG

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consumption.[4] In Indonesia, the 2013 Riskesdas survey reported MSG consumption at 77.3%, alongside other dietary risk factors such as high intake of sugary foods (53.1%), fatty foods (40.7%), and coffee (29.3%).[5]

Excessive MSG consumption has been linked to numerous health risks, including cardiovascular, gastrointestinal, muscular, and neurological disorders.[1] Chronic MSG exposure has also been associated with asthma, obesity, cancer, diabetes, and oxidative stress.[6] Furthermore, it has been implicated in the pathogenesis of neurodegenerative diseases such as Parkinson's and Alzheimer's, as well as conditions like addiction, anxiety, stroke, depression, and epilepsy.[6] The detrimental effects of MSG are partly attributed to its role in increasing reactive oxygen species (ROS) and disrupting redox homeostasis, which can lead to systemic damage.[7] Elevated levels of malondialdehyde (MDA), a lipid peroxidation byproduct, signal increased oxidative stress and indicate damage to cell membranes due to free radicals.[8] Conversely, interleukin-10 (IL-10) plays a critical role in counteracting inflammation by reducing the effects of pro-inflammatory cytokines, thereby protecting tissues from damage and maintaining immune homeostasis.[9] This anti-inflammatory cytokine is essential in preventing chronic inflammatory conditions, which are often precursors to non-communicable diseases.[9]

Mitigating the adverse effects of excessive MSG intake requires dietary interventions rich in antioxidants.[6] Natural compounds with high antioxidant activity have emerged as promising alternatives for neutralizing ROS, repairing cellular damage, and restoring hormonal balance.[7] cinnamon bark extract, known for their antioxidant properties, may help mitigate ROS-induced damage and support cellular recovery.[10,11] In addition, cinnamon bark extract has garnered attention for its potential as an antioxidant additive in food and pharmaceuticals due to its robust antioxidant activity.[12] Studies have shown that cinnamon bark extract, administered at doses of 300, 400, and 500 mg/kg body weight, exhibits significant anti-inflammatory and analgesic effects, as evidenced by its ability to inhibit carrageenan-induced edema and reduce writhing in acetic acid-induced pain models.[13] Moreover, research on *Cinnamomum burmannii* extract demonstrates its hepatoprotective effects against multi-walled carbon nanotube (MWCNT) exposure by enhancing antioxidant status, reducing stress markers, and downregulating pro-inflammatory cytokines such as IL-6, IL-1β, Cox-1, and TNF-α.[14] This study aims to investigate the effects of cinnamon bark extract on MDA and IL-10 levels in male rats subjected to MSG induction, thereby exploring its potential as a natural antioxidant intervention.

## MATERIAL AND METHOD

Laboratory experimental research on experimental animals with Post Test Only Control Group Design where this design was chosen to determine the effect of cinnamon bark extract on MDA and Il-10 levels analyzed after treatment. The subjects of the study used male white Wistar rats Rattus norvegicus aged 3-4 months with a body weight of 200-250 grams that met the inclusion and exclusion criteria. This study used 4 treatment groups, namely the normal group (KN) Wistar rats without MSG induction, the positive group (K +) namely Wistar rats induced by MSG, treatment group 1 (P1) Wistar rats induced by MSG and given cinnamon bark extract 100 mg / head, and treatment group 2 (P2) Wistar rats induced by MSG at a dose of 1g / head (200gr of rats) in 2ml aquadest and given cinnamon bark extract at a dose of 200 mg / head, all received oral treatment for 14 days. On the 15th day, blood was taken from all mice through the orbital sinus of the eye, then processed to obtain serum and MDA levels were measured by the Thiobarbituric acid assay (TBARS) method and IL-10 by the ELISA method.

### Making Cinnamon bark extract

Cinnamon bark is obtained in plantations in the Kerinci area, Jambi Province because many cinnamon bark plants thrive, the bark is crushed using a smoothing machine then 1000 grams of powder is put into a dark container, stirred until homogeneous, closed immediately then stored in a room that is protected from sunlight for 5 days and often shaken. The soak is filtered with a flannel cloth, the pulp is washed with a solvent to a volume of 750 mL. The results are concentrated with a vacuum evaporator until a thick extract is obtained.[15]

### Blood collection and serum collection

Blood collection is carried out through the vein of the eye using a capillary pipe. The blood is stored in a test tube and left for 15 minutes then in a centrifuge at 3500 rpm for 15 minutes, the serum that has been separated from the precipitate is then taken with a 100 µl pipette.

### Procedure for measuring MDA levels TBARS method

MDA measurement on the 15th day after administration of cinnamon bark extract was examined using the Thiobarbituric acid assay (TBARS) method by taking 1 ml of mouse blood through the orbital sinus and then putting it into a centrifuge tube. Furthermore, the blood sample was centrifuged at a speed of 3000 rpm for 30 minutes, 500 µl of supermate was taken and then put into a centrifuge tube. Add 500 µl of 20% TCA solution and add 1% TBA solution in ¬50% glacial CH3COOH. Then heated in a water bath at a temperature of 95°C for 45 minutes. Then cooled to room temperature. Centrifuge speed 3000 rpm for 30 minutes. Take 500 µl of Filtrate using a micropipette. The color intensity is read spectrophotometrically at a wavelength of 532 nm.[16]

### IL-10 level measurement procedure of elisa method

IL-10 measurement on the 15th day after administration of cinnamon bark extract according to each group. IL-10 level measurement was carried out using the Enzyme-Linked Immunosorbent Assay (ELISA) method, the stages are the standard solution and sample first at room temperature before use. The test is carried out at room temperature. Determine the number of strips needed for testing. Put the remaining strips into an aluminum zip for storage. Unused strips should be stored at 2-8°C. Add 50µl of standard to the standard well. Note: Do not add antibodies to the standard because the standard solution contains biotin-labeled antibodies. Add 40µl of sample to the sample well then add 10µl of IL-10 antibody to the sample label well, add 50µl of strettavidin-HRP to the sample well and standard well. Mix well. Cover the plate with a sealer. Incubate for 60 minutes at 37°C. Remove the sealer and wash the wells 5 times with a minimum of 0.3 ml wash buffer for 30 seconds to 1 minute for each wash. Add 50µl of substrate solution A to each well and then add 50µl of substrate solution B to each well. Incubate the sealed plate with fresh sealer for 10 minutes at 37°C in the dark. Add 50µl of Stop Solution to each well, the blue color will immediately change to yellow. Determine the Optical Density (OD) value of each well using a microplate reader set at 450 nm within 10 minutes after adding the stop solution.

## RESULTS AND DISCUSSION

### Analysis of the Effect of Giving Cinnamon Bark Extract on MDA Levels

The results of measuring MDA levels in each group are illustrated in table 1 and figure 1.

**Table 1. Descriptive test, normality and homogeneity of MDA levels between treatment groups**

| Group | KN | K+ | P1 | P2 | *p value* |
|---|---|---|---|---|---|
| Mean | 2.37 | 2.47 | 2.32 | 0.84 | |
| SD | ±0.14 | ±0.24 | ±0.20 | ±1.07 | |
| *Shapiro wilk* | 0.95* | 0.98* | 0.95* | 0.06* | |
| *Levene test* | | | | | 0.046 |

Based on table 1, the results of the analysis, the average MDA of the normal group (KN) was 2.37 mg/ml ± 0.14, the positive group (K+) was 2.47 mg/ml ± 0.24, the average of treatment group 1 (P1) was 2.32 mg/ml ± 0.20 and treatment group 2 (P2) was 0.84 mg/ml ± 1.07.

The MDA level data in the four groups were all normally distributed (p>0.05) and also had a non-homogeneous data variance with a value of 0.046 (p>0.05). The lowest average MDA level was in the treatment group (P2), in the positive group (K+) mice induced by MSG experienced an increase in MDA levels, in treatment group 1 (P1) mice induced by MSG and given sungkai leaf extract at a dose of 100 mg MDA levels decreased, while in treatment group 2 (P2) mice induced by MSG and given cinnamon bark extract 200 mg/mouse experienced a significant decrease in MDA levels.

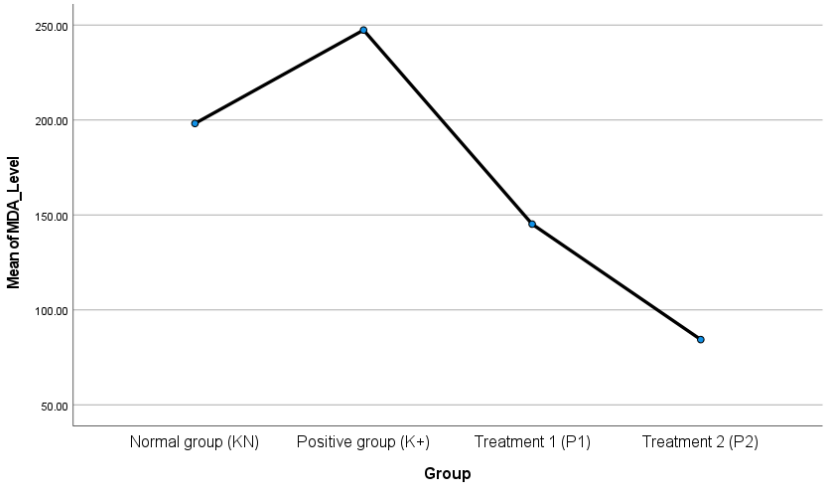


**Figure 1. Graph of the average value of MDA levels by group**

Based on the results of the one-way ANOVA test, a value of 0.001 (p<0.05) was obtained, indicating a statistically significant decrease in MDA levels among the treated groups. It was concluded that the administration of cinnamon bark extract could reduce MDA levels in MSG-induced mice. Determination of the dose of cinnamon bark extract that had the most effect on MDA levels was carried out.

**Table 2. Difference in average MDA levels by group**

| Group | KN | K+ | P1 | P2 |
|---|---|---|---|---|
| KN | - | 0.971 | 0.998 | 0.180 |
| K+ | 0.971 | - | 0.881 | 0.144 |
| P1 | 0.998 | 0.881 | - | 0.197 |
| P2 | 0.180 | 0.144 | 0.197 | - |

The results of the analysis of the average MDA levels of treatment group 2 (P2) which experienced a significant decrease in MDA levels compared to other treatment groups using the Tamhane post hoc test, obtained insignificant results when compared to KN, K+, and P1. It can be concluded that administration of 200 mg of cinnamon bark extract can reduce MDA levels in Wistar rats induced by MSG for 14 days.

## Analysis of the Effect of Giving Cinnamon Bark Extract on IL-10 Levels

The results of IL-10 level measurements in each group are illustrated in table 3 and figure 2

**Table 3. Descriptive tests, normality and homogeneity of MDA levels between treatment groups**

| Group | KN | K+ | P1 | P2 | *p value* |
|---|---|---|---|---|---|
| Mean | 93.25 | 112.89 | 69.48 | 93.29 | |
| SD | ±25.01 | ±43.89 | ±12.83 | ±12.11 | |
| *Shapiro wilk* | 0.877* | 0.151* | 0.955* | 0.570* | |
| *Levene test* | | | | | 0.050 |

Based on Table 3 and figure 2, the average IL-10 levels of the normal group (KN) were 93.25 pg/ml ± 25.01, the positive group (K+) 112.89 pg/ml± 43.89, treatment group 1 (P1) 69.48 pg/ml ± 12.83 and treatment group 2 (P2) 93.29 pg/ml ± 12.11.

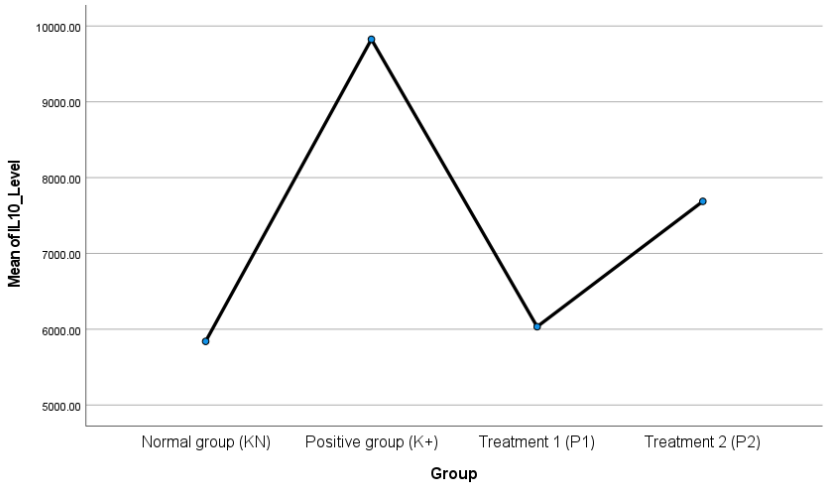


**Figure 2. Graph of the average value of IL-10 levels in each group**

The results of the analysis of the average IL-10 levels in the four groups were normally distributed (p>0.05), and had a non-homogeneous data variance with a value of 0.050 (p>0.05). The results of the one-way ANOVA test obtained a result of 0.127 (p<0.05) which means there is no difference in the average IL-10 levels between the four groups. It can be concluded that the administration of cinnamon bark extract has no effect on increasing IL-10 levels in MSG-induced Wistar rats.

Monosodium glutamate (MSG) is associated with various dangerous side effects. According to research, MSG has been linked to metabolic and digestive anomalies affecting the nervous, pulmonary, and circulatory systems. Damage to the hypothalamic nuclei, particularly the arcuate and ventromedial nuclei, was observed in newborn mice exposed to MSG. This damage resulted in increased body weight, fat deposition, reduced motor activity, and decreased growth hormone secretion.17,18 MSG shows a functional range at concentrations between 0.2-0.8%, with excessive amounts negatively affecting taste. The maximum tolerable dose for enhancing taste in humans is 60 mg/kg body weight (BW). The WHO estimates that 200,000 tonnes of MSG are produced annually, with daily consumption reaching up to 3 grams in some Asian countries.[19] These widespread consumption patterns underscore the need for further investigation into MSG's potential adverse effects on human organ systems.

The global increase in MSG consumption is driven by changing dietary habits, urbanization, improved living standards, and the expanding food processing industry in many Asian countries.[18] To counteract MSG's adverse effects, the role of antioxidants becomes critical. Antioxidants protect against free radicals and mitigate damage.[20] Cinnamon is a notable source of antioxidant compounds, including cinnamaldehyde, cinnamic alcohol, and cinnamic acid, which possess antioxidant, anti-inflammatory, and antibacterial properties. These compounds

have been used to treat diabetes and cardiovascular diseases.[21] An antioxidant-rich diet offers significant benefits, reducing oxidative stress and inflammation, both of which are linked to various metabolic disorders, including hyperglycemia.[10,22]

Research indicates that administering cinnamon bark extract at a dose of 200 mg/rat (treatment group P2) significantly reduces malondialdehyde (MDA) levels compared to other groups. This aligns with Handayani's (2023) findings that cinnamon bark ethanol extract improves lipid profiles due to its cinnamaldehyde and quercetin content, which inhibit HMG CoA reductase activity. Additionally, flavonoids, tannins, and other compounds in cinnamon reduce triglycerides and MDA levels.[23] Similarly, Perisnawati (2024) demonstrated the effectiveness of cinnamon bark extract in lowering MDA levels in hyperglycemic mice, with the best results at a dose of 100 mg/kg BW.[24] These reductions are attributed to the extract's antioxidant components, which combat oxidative stress, inhibit reactive oxygen species (ROS) production, and enhance antioxidant response pathways by activating nuclear factor erythroid 2–related factor 2 (NRF-2) and suppressing nuclear factor-kappa B (NF-KB).[25,26]

In treatment group P1, where cinnamon bark extract was administered at 100 mg/rat, no significant decrease in MDA levels was observed. This may be due to the high oxidative stress induced by excessive MSG, which overwhelmed the antioxidant capacity of the cinnamon extract. Inadequate antioxidants can fail to counteract free radicals, leading to oxidative stress and cellular damage.[27] MDA serves as a key marker for lipid peroxidation and cell membrane damage caused by oxidative stress. Elevated MDA levels indicate increased ROS activity, which can trigger cell death.[28]

The lack of significant differences in MDA levels among the healthy (KN), positive control (K+), and P1 groups could be influenced by various factors, including age, genetics, and environmental conditions, which impact the body's endogenous antioxidant levels.[29] Cinnamon bark extract has been shown to improve mitochondrial and endoplasmic reticulum inflammation caused by oxidative stress. Its active components enhance total antioxidant capacity and reduce lipid peroxidation, thereby mitigating tissue damage.[30] Oxidative stress can be countered through various mechanisms, with the antioxidant defence system being the most effective.[31]

Despite the observed benefits of cinnamon bark extract, no significant effect was seen on IL-10 levels in Wistar rats exposed to a toxic MSG dose of 1 gram/day for 14 days. IL-10 is an anti-inflammatory cytokine crucial for immune regulation and prevention of chronic inflammation.[32] The lack of significant change in IL-10 levels may be due to the acute phase of the experiment, as systemic treatment for 14 days might not have been sufficient to elicit noticeable effects on this parameter.[33]

This study did not conduct a detailed phytochemical analysis of the specific antioxidant components in the cinnamon bark extract, which could influence the MDA level analysis results. Baseline analyses prior to treatment would be necessary to compare pre- and post-treatment levels. Future studies could optimize MSG and cinnamon extract doses to enhance the observed effects. Adjusting MSG induction and increasing the cinnamon extract dose above 200 mg/rat may yield more pronounced outcomes.

## CONCLUSION

This study highlights the harmful effects of monosodium glutamate (MSG) on metabolic, digestive, and nervous systems, primarily through oxidative stress, as evidenced by elevated malondialdehyde (MDA) levels. Cinnamon bark extract shows promise as a natural antioxidant, significantly reducing MDA levels, indicating its ability to mitigate oxidative damage. However, at lower doses the effects were not significant, and no notable changes were observed in IL-10 levels,

likely due to the short duration of the study. These findings suggest that cinnamon bark extract could serve as a protective agent against MSG-induced oxidative stress. Future research should focus on optimizing doses, evaluating long-term effects, and conducting human trials to confirm its efficacy.

## AUTHORS' CONTRIBUTIONS

Dina Afrianti is the head of the TPP who coordinates with team members for the smooth running of the research process and writing of this scientific publication, Wahyudi is the research implementer at the IBL FK Unissula laboratory, Ririh Jatmi Wikandari is in charge of processing laboratory data, Rodhi Hartono is the journal reviewer and supervisor, Erisa Febriyani is the TPM chairman who designed the research flow, Egy Sunanda Putra as a TPM member whose job was to help the TPM chairman design the research flow. All authors have read and approved the final journal entry.

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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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# *Epitope-Driven Vaccine Development for Zika Virus: A Bioinformatics Approach*

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**Abstract:** Zika virus (ZIKV) has become a global concern in 2015-2016, which can infect adults and develop fetuses, has become a global concern. ZIKV is a member of the Flaviviridae family, which can spread through Aedes mosquitoes, sexual intercourse from mother to fetus, and blood transfusions. The genetic material is a single-stranded RNA with positive polarity. Conventional vaccine development requires a long time and significant resources; therefore, the bioinformatics approach is an efficient alternative for identifying B cell epitopes as vaccine candidates. This study used bioinformatics or *In silico* methods to identify B cell epitopes with the immune epitope database (IEDB) web server. This study showed that the peptide sequence of "EWFHDIPLPWHAGADTGTPHWNNKEA" (peptide 6E) in the envelope protein E of ZIKV is a potential vaccine candidate. This peptide is predicted to exhibit high antigenicity, non-allergenicity, and non-toxicity. This study concludes that peptide 6E is a promising vaccine candidate. Further studies are needed *in vitro* and *in vivo* to confirm that it can be used as a potential ZIKV vaccine candidate and applied in the future.
**Keywords:** Epitope; Medicine; Peptide; Vaccine; Zika Virus

## INTRODUCTION

Zika virus (ZIKV) is a member of the Flavivirus genus of the Flaviviridae family and is closely related to dengue (DENV), West Nile (WNV), Japanese Encephalitis (JEV), and Yellow Fever (YFV) viruses. ZIKV was first isolated from rhesus monkeys in Uganda, in 1947. Transmission mainly occurs through *Aedes* mosquitoes, particularly *Ae. aegypti* and *Ae. albopictus*, with humans as the primary host. ZIKV can be transmitted through sexual intercourse, vertical transmission from mother to fetus, and blood transfusion[1]. This statement was further strengthened by research by Pattnaik (2020), who stated that apart from mosquito bites, ZIKV can also spread through sexual intercourse, from mother to fetus, and through blood transfusions[2]. The ZIKV genome consists of positive single-stranded RNA that is translated into a single polyprotein and processed into three structural proteins (C, prM, and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The E protein is the main target of

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the neutralizing antibody response, with the most potent epitope located in domain III of the E protein. Effective antibodies bind strongly to and block areas of the E protein, which are crucial for the entry of the virus into host cells[3].

The Zika virus (ZIKV) has become a global concern since a significant outbreak in the Americas in 2015–2016, which revealed the potentially severe impact of this infection, especially on the developing fetus[1]. Although ZIKV cases have decreased significantly since then, the threat of future outbreaks remains, prompting ongoing efforts to develop effective and safe vaccines. Conventional vaccine development often requires a considerable amount of time and resources. Several researchers have previously attempted to design vaccines and identify the best candidates for T- and B-cell epitopes using immunoinformatics approaches[4]. The adaptive immune reaction against ZIKV is mediated by B cells, which produce antibodies in humoral immunity, and T cells, which produce antibodies in cellular immunity. The viral proteins E, prM, and NS1 are the main targets of the neutralizing antibody response caused by ZIKV in humoral immunity and, are very important for protection against viral infections. Several isolated human monoclonal antibodies (mAbs) have shown high specificity against all ZIKV strains and strong protective activity both *in vitro* and *in vivo*[5]. Bioinformatic approaches offer a more efficient and faster alternative. Bioinformatics enables the identification of potential B-cell epitopes as vaccine candidates through the analysis of genetic and protein data from ZIKV. B-cell epitopes are antigens that are recognized by antibodies to stimulate a specific and protective immune response against viruses. The E protein exhibits strong antigenicity, which means it can effectively stimulate both B cell and T cell responses. Some studies have indicated that targeting specific epitopes with in the E protein can elicit strong immune responses, so that making it a viable target for both subunit and epitope-based vaccines[6].

Vaccine development can be accelerated using immunoinformatics and computational methods, which are also cost-efficient ways to develop simulations and calculations in drug design. Epitope-based vaccine design has emerged as a promising approach in modern vaccine development, offering advantages in terms of selectivity, safety, and ability to stimulate focused immune responses[3]. This method involves the identification and selection of highly immunogenic viral epitopes through in-depth analysis of viral protein sequences. Protein- or peptide-based vaccines allow for more precise targeting of specific epitope regions in viral antigens, thereby inducing a strong and specific immune response. *In silico* approaches and modern bioinformatics techniques have accelerated the process of identifying potential epitopes, enabling the prediction of B- and T-cell epitopes with greater accuracy[7]. In addition, the use of bioinformatic methods in the design of B-cell epitope-based vaccines can also accelerate the development of ZIKV vaccines. Through *in silico* analysis, epitopes with the potential to stimulate a strong immune response can be identified with high precision.

Bioinformatics approaches in vaccine design have undergone significant development, offering greater time and cost efficiency, as well as flexibility in responding to the rapid evolution of pathogens such as ZIKV[8]. Recent machine learning and artificial intelligence algorithms have improved the accuracy of epitope and antigenicity predictions, enabling the rapid adaptation of vaccine designs to emerging virus variants[9]. Through this article, the author will explore bioinformatics methods in the design of the best ZIKV vaccine candidate in terms of the position of the B cell epitope, highlighting the advantages, challenges, and prospects of this approach in efforts to overcome ZIKV outbreaks and improve preparedness for similar infectious diseases in the future.

## MATERIAL AND METHOD

### Protein Preparation, Modelling, and Validation

The ZIKV protein used in this study was envelope protein E, which was obtained from the NCBI database (https://www.ncbi.nlm.nih.gov/) with accession number XBA21084.1, and the sample was downloaded in the FASTA format. The 3D structure of the ZIKV E protein was obtained from the results of homology modelling performed using the SWISS-MODEL web server (https://swissmodel.expasy.org/). This method aligns the query sequence to determine the 3D structure of the target protein[10,11]. The 3D model was validated using a Ramachandran plot with a score threshold of 90%[12,13].

### B-cell Epitope Mapping

B cell epitope mapping of the ZIKV E protein was predicted using The Immune Epitope Database (IEDB) web server (http://tools.iedb.org/bcell/) with the BepiPred-2.0 tool (https://services. healthtech. dtu. dk /service. php?BepiPred-2.0). BepiPred predicts the epitope region of a target protein or peptide candidate that can be identified based on the specific epitope position where the sequence will be input to The Immune Epitope Database (IEDB) with a threshold of 0.5[14,15].

### Identification Antigenicity and Allergenicity

Identification of peptide antigenicity using the VaxiJen v2.0, web server (https://www.ddgpharmfac.net/vaxijen/VaxiJen/VaxiJen.html), to trigger an immune response, which is predicted to have a score greater than the threshold value of 0.4[16]. Allergenicity prediction aims to determine the level of potential allergens and to ensure that the peptide is not an allergen as a vaccine candidate. This prediction uses AllerTOP v2.0 (https://www.ddgpharmfac.net/AllerTOP/)[17].

### Prediction of Toxicity Level and Physicochemical Properties

The toxicity level of peptide candidate vaccines must be determined using ToxinPred (https://www.crdd.osdd.net/raghava/toxinpred/) to determine whether the peptide candidate is not toxic with a threshold value of 0.1[18]. The physicochemical properties of the candidate peptides were predicted using the ProtParam web server (https://www.web.expasy.org/cgi-bin/protparam/protparam) to identify the average hydrophobicity score (GRAVY), aliphatic index, instability, molecular weight, and theoretical pI[19].

### Selection of Vaccine Candidate Peptides and 3D Visualization of Epitopes

The selection of peptides as vaccine candidates can be seen from sequences that have antigenicity, non-allergenicity and non-toxicity results or can be seen from the results of cell epitope potential graphs from The Immune Epitope Database (IEDB). The best peptide was visualized in 3D to determine the target position or region on the ZIKV E protein, which allowed it to be recognized or act as a B cell epitope using PyMol v.2.5.5 software through an academic license.

## RESULTS AND DISCUSSION

### Protein Modelling and Validation

In this study, ZIKV Envelope Protein E was used as a vaccine design target and the 3D structure was modeled using SWISS-MODEL, which consists of β-sheets, α-helices, and coil structures. The Ramachandran score plot shows no Bad Bonds (Bad Bonds 0/2311), 91.53% favorite bonds, 0.34% outliers, and GMEAN and QMEAN values of 0.81 and -3.74 respectively. The protein model identification score was 100%. Local quality estimation is related to the estimation of each model residual (x-axis) and the expected similarity value to the original structure (y-axis).

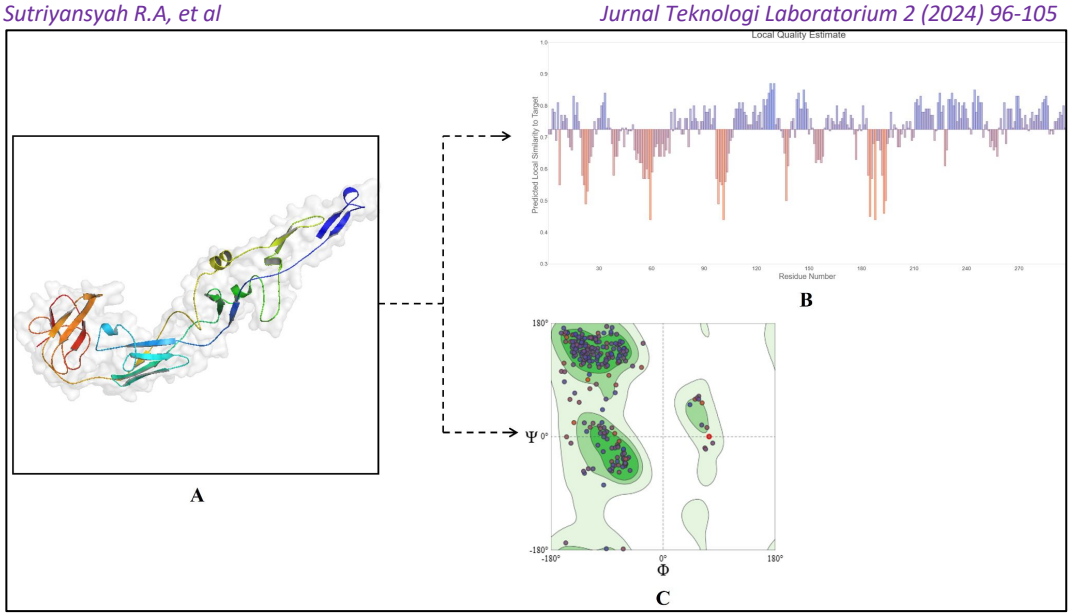


**Figure 1.** ZIKV Protein Visualization. (A) 3D Structure of ZIKV Envelope Protein E; (B) Graph of Estimated Local Quality of Envelope Protein E ZIKV; (C) Ramachandran Plot Results.

Fluctuating graph (Fig. 1B) shows similarity scores lower than 0.5 for some of the residues that form the model with the template. The weak bonds in the Ramachandran plot are weakly related to chemical interactions, such as van der Waals bonds that weakly affect protein structure, compared to the results of hydrogen bond interactions (Fig. 1C). However, the ZIKV E protein model was considered valid because it has a similarity value of 100%.

**B cell epitope mapping, antigenicity, allergenicity, toxicity, prediction of physicochemical properties**

B cells from the ZIKV E protein predicted using EIDB (The Immune Epitope Database) with the peptide naming ending in "E" obtained 11 epitopes, namely the longest 26-mer found at position 122–147, and the shortest 2-mer found at positions 80–81 and 256–257 (Table 1). To determine a good vaccine candidate, it is necessary to predict antigenicity, allergenicity, and toxicity. Of the 11 peptides in the ZIKV E protein predicted using VaxiJen, AllerTOP Server, and ToxinPred, peptide 6E was obtained (Table 2)., which was used for further analysis because it is thought to initiate the formation of a B cell immune response[20].

**Table 1.** Results of B-Cell Epitope Mapping.

| Peptide | Posisi | Length | Peptide Sequence |
|---|---|---|---|
| 1E | 6–9 | 4 | GWGN |
| 2E | 33–39 | 7 | GKSIQPE |
| 3E | 52–69 | 18 | SQHSGMIVNDTGHETDEN |
| 4E | 80–81 | 2 | PR |
| 5E | 100–103 | 4 | TGLD |
| 6E | 122–147 | 26 | EWFHDIPLPWHAGADTGTPHWNNKEA |
| 7E | 153–156 | 4 | DAHA |
| 8E | 219–228 | 10 | TFTKIPAETL |
| 9E | 241–243 | 3 | TDG |
| 10E | 256–257 | 2 | QT |
| 11E | 274–277 | 4 | STEN |

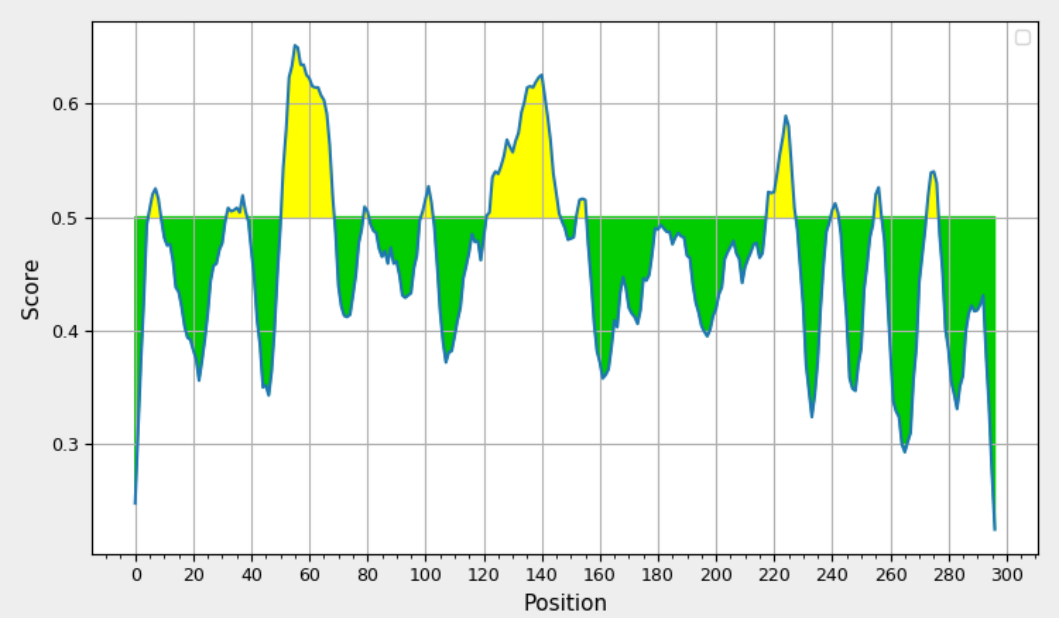


**Figure 2.** B-Cell Epitope Potential Graph.

Various methods have been used to analyze B-cell epitopes, such as the Kolaskar and Tangaonkar method, which is used to predict specific areas of proteins that bind to B cell receptors. These areas must be on the surface and be immunogenic[21]. The results of the B-cell mapping are displayed in a graphical form (Fig. 2) The yellow area shows sequences that have potential B-cell epitopes, while the green color shows sequences that do not have potential B-cell epitopes[15].

**Table 2.** Antigenicity, Allergenicity, and Toxicity Prediction Results.

| Peptide | Antigenicity | Allergenicity | Toxicity |
|---|---|---|---|
| 1E | - | Non-allergen | Non-toxin |
| 2E | Antigen | Allergen | Non-toxin |
| 3E | Non-antigen | Allergen | Non-toxin |
| 4E | - | Allergen | Non-toxin |
| 5E | - | Allergen | Non-toxin |
| **6E** | **Antigen** | **Non-allergen** | **Non-toxin** |
| 7E | - | Non-allergen | Non-toxin |
| 8E | Non-antigen | Non-allergen | Non-toxin |
| 9E | - | Allergen | Non-toxin |
| 10E | - | Allergen | Non-toxin |
| 11E | - | Allergen | Non-toxin |

**Table 3.** Prediction Results of Physicochemical Properties

| Peptide | Theoretical pI | MolecularWeight (Da) | GRAVY | Index | |
|---|---|---|---|---|---|
| | | | | Aliphatic | Instability |
| **6E** | 5.21 (antigen) | 3027.26 | -1.065 (non-allergen) | 41.54 | 6E |

The physicochemical properties of the peptide 6G were predicted using ProtParam (Table 3). Candidate vaccines have a molecular weight of 36 kDa. Apart from that, a GRAVY score is also needed, namely between -0.14 – -0.45, to allow natural hydrophilic interactions to form in the vaccine, but peptide 6E does

not meet this requirement. The aliphatic index score for peptide 6E was also low, between 64.13 and 80.42, indicating less stability at some temperatures. However, the instability index score for peptide 6E was low (<40) with a value of 12.04. This allows for stability when built and triggers the initiation of an immunogenic reaction[19].

**Selection of vaccine candidate peptides and 3D visualization of epitopes**

Epitope position selection was carried out on peptide 6E, namely the sequence "EWFHDIPLPWHAGADTGTPHWNNKEA, " at positions 122–147. Sequence display was perfomed by selecting or blocking the target position sequence using PyMol software.

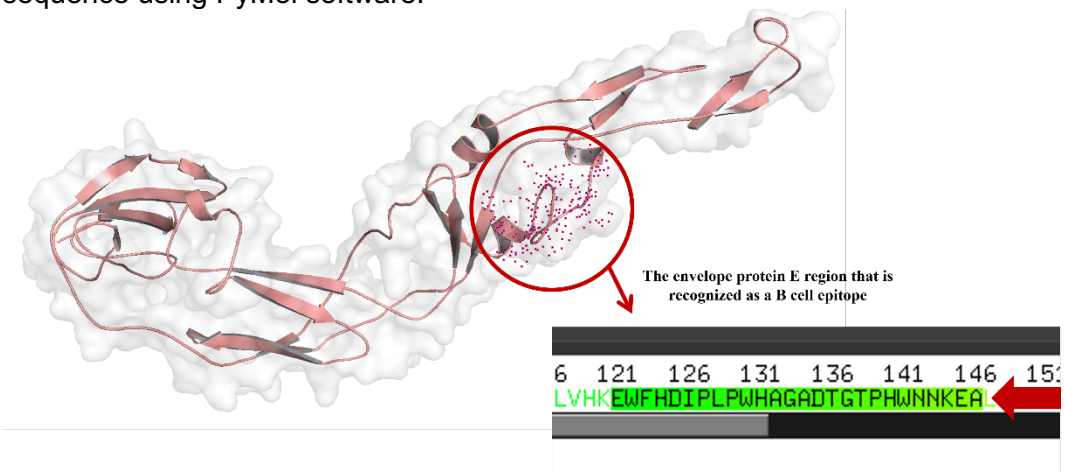


**Figure 3.** 3D Visualization of B-Cell Epitope Target Positions

3D visualization of the ZIKV E envelope protein region is needed to determine the position that allows it to be recognized or act as a B-cell epitope, as shown in (Fig. 3) above.

Bioinformatics has enabled rapid progress in the field of vaccinology, and vaccines developed using modern techniques are safer, more effective, and more cost-effective than traditional vaccines[20]. However, obtaining an appropriate immunological response requires a deep understanding of the pathogen, particularly genome analysis and epitope prediction[22]. The great potential of epitopes for vaccine development, disease prevention, diagnosis, and treatment has attracted significant research interest[23]. Using state-of-the-art technology, specific epitopes that can replace all pathogens in vaccines have been isolated. However, not all epitopes exhibit the same ability to produce antibodies[24].

*In silico* research on vaccine design based on epitopes has been carried out for various types of viruses, such as Zika virus (ZIKV)[22], and Dengue Virus (DENV)[25]. In this study, we aimed to create a vaccine based on ZIKV E protein of ZIKV based on ZIKV data from the NCBI reference sequence with accession number XBA21084.1, which has a genome size of 297 amino acids (aa) as a vaccine design target. The presented vaccine candidate showed significant potential through *in silico* analysis, but further *in vitro* and *in vivo* investigations are needed to confirm its effectiveness.

Generally, epitope reactions with antibodies occur on the protein surface; therefore, the amino acids that make up B-cell epitopes tend to be hydrophilic. Therefore, in this study, the hydrophilicity of amino acid residues was used as a criterion for predicting B cell epitopes. Most epitopes recognized by antibodies are discontinuous epitopes, namely in the form of a sequence of amino acids that are not sequential or discontinuous in their primary structure but are close together. Each has a folded three-dimensional (3D) structure[26,27]. However, the ZIKV E protein model produced through SWISS-MODEL was considered valid because it had a similarity value of 100%.

Results from B cell epitopes using The Immune Epitope Database (IEDB) to predict antigenicity, allergenicity, and toxicity on the ZKIV Virus E protein produced 11 peptides on the ZIKV E protein, which were predicted via VaxiJen, AllerTOP Server, and ToxinPred, and obtained peptide 6E, which met the requirements for vaccine candidates because it contains antigens, non-allergens, and non-toxicity. Peptide 6E contained a high antigen level (5.21). Antigenicity is one of the properties of antigens that triggers a B cell response to produce specific antibodies, which refers to immunogenicity[28]. The peptide in 6E has a high antigenicity value because the recommended vaccine has a minimum antigenicity of 0.5558, which allows the body to recognize it as an antigen[29].

Vaccine designs with B cell epitopes have been widely developed because B cells are part of the proteins that trigger the immune system or bind to antibodies. Vaccine production must meet safe levels by considering the non-allergenic and non-toxic design of vaccines. One peptide that meets this requirement is 6E, which is targeted for vaccine design because it is non-allergenic and non-toxic. The non-allergenic results obtained indicate that they do not cause allergic reactions in vaccine candidates because allergies can cause reactions that are dangerous to the owing to excessive activity that interferes with immunopathology[30]. In addition, vaccine design must be non-toxic because toxicity itself is a compound that is capable of causing toxic harmful effects on organisms, causing an imbalance in the vaccine administered to the candidate, which triggers the presence of toxins and excessively disrupts the body's working mechanisms[31].

Designing Zika virus (ZIKV) vaccine candidates based on B-cell epitopes has been a promising vaccine development approach because of its ability to target specific viral components that can trigger a strong immune response. B cell epitopes are part of the antigen that is recognized by antibodies, and by designing vaccines that target these epitopes, we can increase the effectiveness of the vaccine in protecting against ZIKV infection. This approach involves identifying and mapping the B-cell epitope of the ZIKV envelope protein (E), which is the main target of neutralizing antibodies. Recent studies have used bioinformatics to predict the potential B-cell epitopes of the ZIKV E protein. For example, Gupta (2023) used computational modelling techniques to identify B-cell epitopes that can trigger a protective immune response[32].

Additionally, epitope-based vaccines can be tested in various *in vitro* and *in vivo* models to evaluate their immunogenicity and protective efficacy. Zhang (2020) showed that a vaccine candidate based on B-cell epitopes can stimulate the production of antibodies capable of effectively neutralizing ZIKV in a mouse model[33]. These results demonstrate great potential for the development of an effective anti-ZIKV vaccine using a B-cell epitope-based approach. Therefore, the design of a B-cell epitope-based ZIKV vaccine offers an innovative and specific strategy to protect the public from ZIKV infection.

## CONCLUSION

From the results of this study, the "EWFHDIPLPWHAGADTGTPHWNNKEA" peptide (peptide 6E) is a good candidate for the ZIKV vaccine because it has high antigenicity, low toxicity, and does not trigger an autoimmune response. In addition, it can increase the immune response of B cells through activation and differentiation into plasma cells, formation of memory cells, and increase IgM or IgG antibody titers for virus neutralization. However, this needs to be further verified through *in vivo* and *in vitro* experiments to ensure the effectiveness and safety of peptide 6E as a vaccine candidate.

## AUTHORS' CONTRIBUTIONS

M. Hilmi Ihsanul Iman prepared the samples; Cahya Ajeng designed the research protocols; all authors executed the research protocols; Riska Ayu Sutriyansyah and Nelly Indira Kusuma Wardani wrote the manuscript. Arif Nur Muhammad Ansori reviewed and supervised the study. All the authors have read and approved the final manuscript.

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None.

## DATA AVAILABILITY STATEMENT

The data utilized in this investigation are available from the corresponding author up reasonable request.

## DISCLOSURE STATEMENT

The data are the results of the author's research and have never been published in other journals.

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# *Hematological profile of pulmonary tuberculosis patients before and after 1 month of taking anti-TB drugs*

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**Abstract:** Tuberculosis (TB) is an airborne infectious disease caused by *Mycobacterium tuberculosis*. *(MTB)* Indonesia ranks third in the number of global TB cases. Studies in Indonesia have shown reduced levels of Hgb (Hemoglobin), Hct (Hematocrit), MCV (Mean Corpuscular Volume), MCH (Mean Corpuscular Hemoglobin), and leukocytosis in newly diagnosed and untreated TB patients. Further investigation is needed to evaluate the combined use of hematological markers to assess the inflammatory response before and after treatment.This study was conducted in several publice health center (Puskesmas) in Semarang to determine the hematological profile of TB patients before and after 1 month of treatment. This study used a quasi-experimental design with one-group pre-test and post-test methods. The study population included all TB patients in the third area of the puskesmas. Non-probability sampling technique with quota sampling is used to select a sample, consisting of newly diagnosed TB patients (aged 20-60 years, both male and female, and not cases of retreatment). A total of 30 samples were taken (10 from each Puskesmas). Analysis of blood samples is performed using the automated hematology device Sysmex (KX21-N). Data were analyzed with descriptive statistics and paired t-test was used to compare hematologic profiles before and after treatment. The results showed that anemia often occurred in TB patients and the number of patients who experienced anemia after treatment decreased from 16 patients to 13 patients. The study also found significant changes in white blood cell ($p = 0.004$) and platelet ($p = 0.005$) counts in TB patients before and after treatment. The increase in white blood cell count after treatment shows clinical improvement, while the decrease in platelets may be due to the action of anti-TB Drugs. Normocytic normochromic anemia is the most common form of anemia in TB patients before treatment, while microcytic hypochromic anemia is more common after 1 month of treatment.

**Keywords:** Hematological; Tuberculosis; Anti TB Drugs; Intensive Phase; Anemia.

## INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by bacteria *Mycobacterium tuberculosis (MTB)*. TB is an airborne disease. Transmission is through airborne particles called droplet nuclei, with a size of 1-5 microns. TB is one of the oldest infectious diseases in the world.[1] About a quarter of the global population is estimated to have been infected with TB. About 90% of these cases are adults, with men having more cases than women.[2] Based on WHO data Indonesia ranks third in terms of the highest number of cases in the world.[3] The number of TB cases found and treated in Indonesia in 2020 was recorded at 393,323 cases, then increased in 2021 with 443,235 cases.[2]

Data from the 2021 Indonesian Health Profile states that TB in Central Java occupies the third position with the highest number of TB cases in Indonesia at

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54,640 cases.[4] Data obtained from the Semarang City Health Office, the number of new discoveries of TB cases as of early October 2022 is 1,989 cases. Kedungmundu District ranked first with the highest number of TB cases of 186 cases in the January-November 2022 period. The second highest ranking is Bangetayu District with a total of 158 cases. The success rate of TB treatment in Indonesia is 73% in 2021, not yet reaching the national target that should be achieved is 90%.[5] WHO recommends multi-drug first-line anti-TB drugsconsisting of Isoniazid, Rifampicin, Pyrazinamide, Ethambutol and Streptomycin.[6,7]

Infection MTB is characterized by a cellular response that includes various manifestations reflecting the interaction between the MTB bacillus and the main effector cells of the host's cellular defense defense mechanism. Host immune responses to infections are thought to play an important role in the pathophysiology of TB resulting in a wide variety of immunopathologies, ranging from asymptomatic infections to disseminated disease and ultimately patient death. Systemic inflammation facing pulmonary tuberculosis (PTB) and extrapulmonary tuberculosis (EPTB) and characterized by increased concentrations of various inflammatory markers in peripheral blood and the spectrum of proinflammatory cytokines, as well as chemokines.[8]

White blood cell count (WBC), platelet and various relative ratios of white blood cells, such as neutrophil/lymphocyte ratio (NLR), platelet/lymphocyte ratio (PLR) and monocyte/lymphocyte ratio (MLR), have been widely studied in chronic inflammatory diseases including TB. TB is associated with reversible changes in hematological parameters. Some changes occur also associated with anti-TB drugs.[9,10] Changes in hematological parameters include anemia, leukocytosis, neutrophilia, thrombocytosis, and increased erythrocyte sedimentation rate (ESR), lymphocytosis, thrombocytopenia or lymphopenia depending on severity and comorbidities.[7] In addition, other hematological parameters in chronic inflammatory diseases such as mean corpuscular volume (MCV), red blood cell distribution (RDW), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobinconcentration (MCHC), MPV, platelet distribution (PDW) and erythrocyte sedimentation rate (ESR) have been investigated.[11] Research in Indonesia on TB such as the Yuniza et all (2022) study on healthy patients and newly diagnosed TB patients who have not received treatment found a decrease in Hgb, Hct, MCV and MCH levels and leukocytosis, while Asa (2022) research on the erythrocyte index, found TB patients to have anemia by 60%.[12,13] However, the use of these combined hematologic markers that may reflect a systematic inflammatory response before starting treatment and after 1 month of treatment in TB patients has not been fully investigated. The aim of the research is to determine the morphological abnormalities of blood cells in pulmonary TB patients and to find out the type of anemia that most often occurs in pulmonary TB patients.

## MATERIAL AND METHOD

The research was conducted between January 2024 - Mei 2024 at 3 public health center (puskesmas) locations in Semarang City, namely Kedungmundu Health Center, Bangetayu Health Center and Tlogosari Wetan Health Center. Quasi experimental one-group pre-test and post-test to determine hematological profile in TB patients before and after administration of anti-TB drugs over a period of 1 month. The study population included all TB patients in the Kedungmundu Health Center, Bangetayu Health Center and Tlogosari Wetan Health Center, Semarang City. The sampling technique used is non-probability sampling with the type of quota sampling for determine the sample of the population that has certain characteristics up to the desired number (quota). Sampling. Quota sampling was used, including all patients who met the inclusion criteria: men and women aged 20–60 years, newly diagnosed with TB, and not undergoing retreatment. Researchers set 30 samples, with details of 10 patients from Kedungmundu Health

Center, 10 Bangetayu Health Center patients and 10 patients from Tlogosari Wetan Health Center. Approximately 5 ml of venous blood was collected aseptically using EDTA tubes from each selected study patient. after collection, EDTA tubes are labeled with a code number. Blood tests are performed on the same patient, the patient's blood sample is taken twice when the patient has not received treatment and after undergoing anti-TB drug therapy (OAT) for one month. The analysis was performed using the Sysmex automatic hematology tool (KX21-N) using fresh venous blood samples anti-coagulant EDTA, by: EDTA blood is mixed, placed on the sample probe. Press the start button. The device will perform an automatic analysis and display the results on the LCD screen. The analysis results displayed include WBC, RBC, HGB, HCT, MCV, MCH, MCHC, PLT, LYMP, NEUT, and MXD.[14] The reason for using automated blood counts is that automated blood counts can quickly produce accurate and precise blood counts. [15]

The data obtained were analyzed using stastical programme. Frequency and cross-tabulation are used for descriptive statistics. Descriptive analysis to describe the frequency and percentage of research variables such as sex, hemoglobin, hematocrit, leukocyte count, platelet count, erythrocyte count, differential count, MCV, MCH, MCHC. Criteria for anemia in men, HgB <13 g / dL and female HgB < 12 g / dL.[16] Normal Hct values Nonpregnant women age > 15 years.< 36% Men > 15 yrs.<39%. Normal leukocyte count 5000-10000/ µl. Leukocytosis is a condition characterized by a very high total number of circulating leukocytes (> 10000/ µl). Leukopenia is a condition characterized by a very low total number of circulating leukocytes (< 5000/ µl). Normal platelet count 150,0000-400,000/ µl. Thrombocytosis is a high platelet count in the blood (>400,000/ µl). Thrombocytopenia is a very low level of platelets in the blood (<150,0000/ µl). Normal erythrocyte count 4-5 x $10^6$ per $mm^3$. Erythrocytosis is a high number of erythrocytes in the blood 4-5 x $10^6$ per $mm^3$). Low erythrocyte count is < erythrocyte count of 4-5 x $10^6$ per $mm^3$. Normal lymphocytes 20-40%. Lymphocytosis is > 40% lymphocyte count. Lymphocytopeny is < 20% lymphocyte count. Monocytes 2-8%, monocytosis is the number of lymphocytes >8%, low lymphocytes < 2%. Normal neutrophils are 50-70%, neutrophils are the number of neutrophils >70%, neutropeni is the number of neutrophils <50%.[17] Anemia can be classified by morphology into Normochromic normocytic (MCV 76–96 fL, MCHC 30–35 gm/dL), Macrocytic (MCV >96, MCHC 30–35 gm/dL), Microcytic (MCV <76 fL, MCHC 30 gm/dL).[16] The paired t-test was used in the analysis to compare hematological profile values before and after 1-month TB treatment. A P value of < 0.05 is considered statistically significant, which means that there are differences in hematological profile values before and after treatment. This research has obtained ethical approval from the ethics committee of the Health Polytechnic of the Ministry of Health Semarang No. 0575/EA/KEPK/2024

## RESULTS AND DISCUSSION

TB is an infectious infectious disease caused by *Mycobacterium tuberculosis* (MTB). Tests that can be used in the diagnosis of TB include sputum examination for acid-resistant bacilli, chest CT scan, chest X-ray, tuberculin culture and skin test, PCR test used for TB diagnosis and blood tests.[18] Blood examination is one of the supporting methods carried out to examine patients suffering from TB. Assessment of hematological parameters/profiles plays an important role in designing treatment strategies and influencing patient prognosis. This is very important in planning treatment strategies and can have an impact on a patient's prognosis. Using hematological markers as a guide in treatment can not only improve treatment outcomes, but also improve patient survival and quality of life.[19]

Hematological profile is a parameter that has a normal range or value as a reference value to determine whether blood morphology (red blood cells, white blood cells, platelets) is normal or not. When infected with pulmonary TB, certain

changes occur in the blood due to bacterial infection secreting substances that cause certain effects.[18] Hematological parameters such as hemoglobin (HgB), Packed Cell Volume (PCV), red blood cell count (RBC), erytrocyte index, platelet count, white blood cell count (WBC), erythrocyte sedimentation rate (ESR) can be used for diagnosis, prognosis, and follow-up of TB patients.[20]

**Table 1. Distribution of TB cases by gender**

| Gender | Number of cases | Percentage (%) |
|---|---|---|
| Male | 16 | 53.33 |
| Female | 14 | 46.67 |
| Total | 30 | 100 |

In Table 1, the gender of male patients is 53.33% more than female. Our findings are in line with previous studies by the Ministry of Health (2023) and Situmorang (2020), which reported similar findings, showing that pulmonary TB cases are dominated by men.[21,22] Men mostly have smoking habits, smoking as a major risk factor for TB can inhibit the target of reducing the incidence and death from TB.[23] In addition, men's lifestyles such as drinking alcoholic beverages and doing a lot of activities outside the home are at risk of exposure to air that has been contaminated with bacteria so it is easy to contract TB.[24]

**Table 2. HgB, Hct and RBC levels before and after 1 month of TB treatment**

| Parameter | Before treatment Mean ± SD | After treatment Mean ± SD | p-value |
|---|---|---|---|
| HgB (g/dl) | 12,450 ± 2,30 | 12,343 ± 2,10 | 0.768 |
| Hct (%) | 37.865 ± 9,57 | 35.868 ± 8.58 | 0.195 |
| RBC (cel//µl) | 4,81 ± 0,21 | 4,63 ± 0,11 | 0.138 |

"HgB : hemoglobin, Hct : hematocrit, RBC : red blood cell count."

**Table 3. Proportion of TB patients who experience anemia before and after treatment**

| Parameter | Criteria | Before treatment | After treatment |
|---|---|---|---|
| HgB | Anemia | 16 | 13 |
| | No anemia | 14 | 17 |
| Erythrocyte index (MCV, MCH, MCHC) | Normocytic and normochromic | 17 | 14 |
| | Hypochromic microcytic | 13 | 16 |

The number of erythrocytes (RBC) is used as a determinant of the degree of anemia, along with hemoglobin (HgB), and hematocrit (Hct).[6,25] There was no statistically significant difference in hemoglobin levels before and after 1 month of treatment (p-value 0.768), indicating that a longer treatment period may be required for significant hematological improvement. The 1-month period is still within the time range of intensive phase treatment which should be completed within 2 months. Intensive TB treatment lasts for 2 months with the administration of 4 types of anti-TB Drugs, namely Isoniazid, Rifampicin, Pyrazinamid, and Ethambutol and consumed every day. Treatment at this stage aims to lower the number of bacteria *MTB* effectively and minimize bacteria *MTB* who may have been resistant from before treatment.[26]

Most TB patients before and after treatment have low hematocrit levels. According to Kassa (2016), the low hematocrit value is caused by a decrease in hemoglobin levels in erythrocyte cells, causing anemia.[6, 27] Anemia, a decrease in red blood cell mass, is also interpreted as a decrease in the concentration of hemoglobin and hematocrit. The criteria for anemia are based on hemoglobin levels, in men <13 g/dL and women <12 g/dL.[16] Anemia was identified in 16 patients before treatment and 13 patients after TB treatment (Table 3). In our study, the number of anemic TB patients decreased in the number of patients. The results

of this study are different from the results of Come's (2023) study, anemia in TB patients before and after administration of anti-TB drugs for 2 months has increased.[28] Anemia due to chronic diseases such as TB can be caused by inflammatory pathogenesis that causes a short life span of erythrocytes, poor binding of iron and erythrocytes and decreased sensitivity or supply of erythropoietin. Low food intake is one of the causes of iron deficiency anemia. Loss of appetite is thought to be one of the causes of reduced food intake. Malabsorption problems result in decreased iron absorption.[29]

The mechanism of occurrence of anemia in pulmonary TB patients is explained as bacterial invasion causes activation of T lymphocytes and macrophages, which induce the production of cytokines (IFN-ÿ), (TNF-α), Interlukin-1 (IL-1) and interlukin-6 (IL-6) which will cause iron diversion in the reticulo-endothelial system resulting in a decrease in iron concentration in plasma thereby limiting its availability to red blood cells for hemoglobin synthesis, inhibition of erythroid progenitor cell proliferation and erythropoietin production and activity.[6,27]

**Table 4. MCV, MCH and MCHC levels before and after 1 month of TB treatment**

| Parameter | Before treatment Mean ± SD | After treatment Mean ± SD | p-value |
|---|---|---|---|
| MCV (fl) | 80.97 ± 1.23 | 78.61 ± 1.10 | 0.144 |
| MCH (pg) | 25.82 ± 0.51 | 25.79 ± 0.81 | 0.968 |
| MCHC (g/dl) | 32.08 ± 0.70 | 33.73 ± 0.61 | 0.008 |

"MCV : mean corpuscular volume, MCH : mean corpuscular hemoglobin, MCHC : mean corpuscular hemoglobin concentration (MCHC)."

The erythrocyte index consists of mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobinconcentration (MCHC).[30] The results of our research showed that the MCV and MCH values before and after treatment did not change significantly, but there was a significant difference in the MCHC values before and after treatment (p-value 0.008). Average cell volume (MCV) is the most frequently used index. It measures the average volume of red blood cells by dividing the hematocrit by RBCs. MCV categorizes red blood cells based on size. Cells of normal size are called normocytic, smaller cells are called microcytics, and larger cells are called macrocytics. This size category is used to classify anemia.[25] The MCH value usually rises or falls as the MCV increases or decreases. MCHC categorize red blood cells based on their hemoglobin concentration. Cells with a normal hemoglobin concentration are called normochromic; Cells with a lower than normal concentration are called hypochromic.[25] Using accurately determined red blood cell counts, hematocrit, and hemoglobin values, the size and average hemoglobin content of red blood cells in a given blood sample can be calculated. The value obtained is an erythrocyte index that helps in the classification and study of anemia.

Normocytic and normochromic anemia were the most common categories of anemia identified in 17 (57%) TB patients prior to treatment. This is in line with Chavan et al's 2016 research found normocytic and normochromic anemia in new patients.[31] Microcytic hypochromic anemia was found in 16 anemic patients after 1 month of treatment. Mild to moderate anemia, which is common in patients with infectious, inflammatory, or neoplastic diseases and lasts more than 1 to 2 months, is called chronic disease anemia. Anemia in pulmonary TB patients has been reported in 16% to 94%. All chronic infections, including TB, can cause anemia. Anemia usually develops during the first month or two of the disease and does not develop afterwards.[18]

Before treatment, anemia was predominantly normochromic normocytic in 17 (57%) patients followed by hypochromic microcytic in 13 (44%) patients. After treatment 14 (44%) developed normochromic normocytic anemia and 16 (53%)

patients developed microcytic hypochromic anemia. This result is different from Kumar's (2017) study which showed the majority of TB patients (76%) had normochromic normocytic anemia. This may be due to the fact that after treatment with anti-TB drugs, anemia improves but is not completely corrected, reflecting the chronic disease TB anemia.[32]

**Table 5. WBC and Plt levels before and after 1 month of TB treatment**

| Parameter | Before treatment Mean ± SD | After treatment Mean ± SD | p-value |
|---|---|---|---|
| WBC (cel/µl) | 9.030 ± 701 | 6.873 ± 317 | 0.004 |
| Plt (cel/µl) | 352.300 ± 2.179 | 297.633 ± 18.695 | 0.005 |

"WBC : white blood cell count, Plt : platelet."

There was a significant difference in the number of WBC (p-value 0.004) and platelets (p-value 0.005) indicating a hematological effect of tuberculosis treatment (Table 4). Our findings on the number of white blood cells are in line with Sheetal's (2020) study which reported similar findings.[27] This study also found that normal leukocyte counts were found in 17 (57%) patients before treatment and 27 (90%) patients after the treatment phase. The results of our study are in line with previous studies by Reta (2023) and Sheetal (2020) which showed that before starting treatment, TB patients experienced leukocytosis, but after treatment, the leukocyte count decreased to normal. In the early stages of MTB infection, MTB moves and accumulates in lung lesions, increasing the number of white blood cells associated with the host's innate immune mechanism.[27,33]

The significant difference in platelet counts from our findings is in line with the study by Eyuel Kassa et al (2016) which showed similar results.[6] However, our findings differ from the study by Karwiti et al (2021) which stated that there was no difference in platelet counts in TB patients before and after taking anti-TB drugs (p-value.0.728).[34] Although platelet counts were within normal limits in most patients, thrombocytosis was observed in 9 (30%) patients before treatment and 4 (13%) patients after treatment (Table 4). The difference in platelet count in TB patients before and after taking anti-TB drugs can be due to the influence of anti-TB drugs, especially the type of Rifampicin. Rifampicin can cause a decrease in platelet count. This is because Rifampicin can be absorbed into platelets and cause platelets to be recognized as antigens by antibodies, resulting in a mechanism of platelet destruction by the immune system.[35] There were significant differences in white blood cell and platelet counts, indicating the hematological influence of tuberculosis treatment.

**Table 6. Proportion of hematology profiles with normal, high, and low values in TB patients before and after 1 month of treatment**

| Parameter | Criteria | Before treatment (%) | After treatment (%) |
|---|---|---|---|
| Hct | Normal | 11 (36%) | 10 (33%) |
| | Low | 19 (64%) | 20 (67%) |
| WBC | Normal | 17 (57%) | 27 (90%) |
| | High (Leukocytosis) | 10 (33%) | 2 (6.6%) |
| | Low (Leukopenia) | 3 (10%) | 1 (3.4%) |
| Plt | Normal | 21 (70%) | 25 (83%) |
| | High (Thrombocytosis) | 9 (30%) | 4 (17%) |
| | Low (Thrombocytopenia) | 0 | 1 (3.4%) |
| RBC | Normal | 18 (60%) | 15 (50%) |
| | High (Erythrocytosis) | 9 (30%) | 10 (33%) |
| | Low | 3 (10%) | 5 (27%) |
| Lymp | Normal | 20 (66%) | 24 (80%) |
| | High (Lymphocytosis) | 1 (3.4%) | 3 (10%) |
| | Low (Lymphopenia) | 9 (30%) | 3 (10%) |
| Monocytes | Normal | 19 (63.4%) | 15 (50%) |
| | High (Monocytosis) | 11 (36,6%) | 15 (50%) |

| | | | |
|---|---|---|---|
| Neut | Normal | 17 (56,6%) | 23 76,6%) |
| | High (Neutrophilia) | 12 (40%) | 5 (16,6%) |
| | Low Neutropenia) | 1 (3.4%) | 2 (6.8%) |

**Table 7. Lymphocytes, Monocyte and Neutrophils levels before and after 1 month of TB treatment**

| Parameter | Before treatment Mean ± SD | After treatment Mean ± SD | p-value |
|---|---|---|---|
| Lymp (%) | 24 ± 2 | 29 ± 2 | 0.021 |
| Monocytes (%) | 8.23 ± 0.49 | 8.18 ± 0.46 | 0.928 |
| Neut (%) | 65.95 ± 2.82 | 62.25 ±1.84 | 0.236 |

"Lymp : lymphocytes, Neut : neutrophils."

Our study results showed that the average lymphocyte count was different before (24 ± 2) and after TB treatment (29 ± 2). The results of statistical tests on lymphocytes also showed a significant difference between before and after treatment (p-value 0.021). Our findings are in line with the studies of Chedid (2020) and Sheetal (2020) which reported similar things. An increase in lymphocytes indicates general clinical improvement in response to treatment. TB treatment can increase the number of lymphocyte cells to be more or return to normal.[36,27]

Most patients had normal monocyte and neutrophil counts before and after TB treatment. However, statistically the data showed no significant difference in monocyte and neutrophil counts in TB patients before starting treatment and after completing 1 month of TB treatment. Monocytes are an important component of the innate immune response that acts as a link to the adaptive immune system through antigen presentation to lymphocytes. Monocytes are the dominant innate immune cells in the early stages of MTB infection as a host defence against intracellular pathogens. Therefore, any factor that interferes with the function or relative numbers of these cell types has the potential to influence an individual's response to infection.[18,37]

Before starting treatment, 12 (40%) TB patients had neutrophilia, but after treatment, the neutrophil count decreased to normal. The immune system's reaction to TB may be the cause of this increase. Neutrophilia and neutropenia are also found in TB patients but neutrophilia is more common than neutropenia.[38] Neutrophilia is a sign of recurrent and continuous inflammatory reactions and often turns into lymphocytosis when the inflammatory response becomes chronic.[39] Neutrophilia describes a high number of neutrophil granulocytes in the blood. Neutrophils are the primary white blood cells that respond to bacterial infection. Relative or absolute neutrophilia is documented in 29-57 percent of patients with tuberculosis.[40]

The study results underline the importance of routine blood parameter monitoring during TB treatment to assess the response to treatment, whether the patient's condition improves as treatment progresses, or worsens, requiring immediate action.

## CONCLUSION

TB treatment does not directly increase Hb levels in a short time, it affects several other aspects of the patient's hematological profile. This indicates that hematological monitoring during TB treatment remains important to detect changes that might affect the patient's condition, especially with regard to the type of anemia and changes in white blood cells and platelets. Overall, this study demonstrates the importance of hematologic profile monitoring in the treatment of TB, which can improve patient outcomes and quality of life. This research has several limitations, such as a relatively small sample size because it is a longitudinal study (the study was only conducted in Semarang) which may affect the generalization of the findings. Patient non-compliance with anti-TB treatment, resulting in some patients

not returning after 1 month of treatment. This research has not yet taken into account the nutritional status or health conditions of the patients that could affect the hematological profile.

## AUTHORS' CONTRIBUTIONS
Ririh Jatmi Wikandari and Surati conducted research and prepared research reports. Siti Nuryani and Sistiyono analyzed the data and interpreted the results.

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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.

## REFERENCE
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# *Histopathological evaluation of green betel leaf extract ointment on incision wounds infected with Staphylococcus aureus in wistar rats*

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**Abstract:** The skin, as the largest organ in both humans and animals, functions as a protective barrier against various external threats such as sharp objects, temperature fluctuations, chemicals, and physical trauma. Incision wounds are particularly vulnerable to infections, with *Staphylococcus aureus* being a common causative agent of secondary complications, including prolonged inflammation and delayed healing. This study investigates the efficacy of green betel leaf extract ointment in accelerating the healing process of incision wounds in Wistar rats infected with *Staphylococcus aureus*. An experimental, post-test-only controlled group design was employed, involving five groups of five rats each. The groups were treated with an ointment base (control), mupirocin ointment (positive control), and green betel leaf extract ointments at concentrations of 10%, 15%, and 20%. Data were analyzed using normality tests, homogeneity tests, and ANOVA. Phytochemical analysis revealed that green betel leaf extract contains alkaloids, terpenoids/steroids, saponins, tannins, and glycosides, all of which are secondary metabolites known for their antimicrobial and wound healing properties. Among the treatment groups, the 15% green betel leaf extract ointment exhibited the most rapid wound closure (average wound healing of 1.44 mm), approaching the effectiveness of mupirocin ointment. Histopathological observations further demonstrated a significant increase in epithelialization and fibroblast proliferation in treated groups compared to controls. In conclusion, green betel leaf extract ointment, particularly at a 15% concentration, shows promising potential as a topical agent for treating *Staphylococcus aureus*-infected incision wounds in Wistar rats.

**Keywords:** *Staphylococcus aureus*, Incision wound, Skin histopathology

## INTRODUCTION

Wound severity can occur due to bacterial infection with the presence of a number of microorganisms that attack the tissue in the wound area, especially in open wounds, causing worse consequences[1,2]. One of the bacteria that can cause wounds in a serious condition is *Staphylococcus aureus*, which is a round gram-positive bacterium that is pathogenic to humans causing infections and disorders of the skin, usually only acting as a carrier [3,4]. *Staphylococcus aureus* infection is one of the most serious problems in wound healing [5,6]. This bacterium can disrupt the healing process and cause serious complications due to its ability to form biofilms, a protective layer that shields the bacteria from antibiotics and the body's immune system, making treatment difficult and infection more durable[7]. *Staphylococcus aureus* has a variant known as Methicillin resistant *Staphylococcus aureus* (MRSA) that is resistant to many antibiotics[8]. This makes

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treatment of the infection more difficult and leads to excessive inflammation, tissue damage, and slows down the formation of new tissue[5].

According to research by Nadya et al. 2021 *Staphylococcus aureus* is one of the most common pathogenic bacteria found in humans and animals that can infect mild skin to more serious infections such as pneumonia, endocarditis, and sepsis. In this study, it was explained that the preparation of coriander seed extract ointment (*Coriandrum sativum* L.) provided good effectiveness on wound healing in mice infected with *Staphylococcus aureus* bacteria but not by knowing the histopathological picture of the skin of mice infected with *Staphylococcus aureus* bacteria[9].

Currently, synthetic and herbal medicines are widely used in wound healing[10]. One of the herbal plants in Indonesia used in traditional medicine is green betel leaf (*Piper betle Linn*) to help accelerate the wound healing process which is known to have a number of benefits and contain bioactive molecules that have the potential to support health, including in the wound healing process[11]. The content contained in green betel leaves tannins, alkoloids, saponins, and flavanoids function as antimicrobials and stimulate the growth of new cells in the wound[12,13]. According to research by Atika et al. 2021 green betel leaf extract (*Piper betle* L.) has an effect on wound healing in mice (*Mus musculus* L.) which contains alkoloid compounds as antibacterials[14].

The purpose of this study was to determine the effect of green betel leaf extract on wounds infected with *Staphylococcus aureus* and determine the extent to which the extract has antimicrobial properties and reduces inflammation, accelerating tissue regeneration in infected wounds[15]. In addition, this study also wanted to evaluate the potential of green betel leaf extract as an alternative or traditional treatment for infected wounds and determine the histopathological picture in rat skin by examining the number of fibrolas and re-epithelialization.

This study aims to know the effect of healing incision wounds infected with *Staphylococcus aureus* bacteria with green betel leaf extract ointment based on macroscopic, microscopic observations, the number of fibrolas and epithelialization in wistar strain rats.

## MATERIAL AND METHOD

This study is an experimental study with post test only controlled group design on male wistar strain rats. The research was conducted at the Pharmacology Laboratory of the Faculty of Pharmacy, University of North Sumatra and the Anatomical Pathology Laboratory of the Royal Prima Hospital Medan for more than 3 months from October 2023 to January 2024. The experimental animal research protocol was approved by the Prima Indonesia University Health Research Ethics Committee (041/KEPK/UNPRI/X/2023).

The animal test used male wistar rats consisting of five treatment groups with different concentrations[16]. In this study, five treatment groups were used to be able to evaluate the extent to which increasing the concentration of green betel leaf extract affects wound healing and infection control.

### Tools and Materials

The tools used in this research are scalpel and surgical scissors, microscoprotary evaporator, gloves, and Fourier Transform Infrared (FTIR) spectroscopy. The materials used in this study were green betel leaves, Nutrient Agar (NA), Staphylococcus aureus ATCC 25923 bacteria, mupirocin ointment, HE staining, 10% formalin, ketamine HCL-xylazine, xylol, PBS pH 7.4.

### Green Betel Leaf Extract

Green betel leaf extract was obtained through maceration method using 96% ethanol for 7x24 hours to separate solid-liquid mixture from green betel leaf

simplisia. Maceration is continued by evaporation using a vacuum rotary evaporator to produce a solvent-free thick extract[14,17].

To get green betel leaf extract ointment, the researcher made an absorption base ointment formulation in table 1 below[18]:

Table 1. Ointment formulation

| Concentration | Green betel leaf extract (g) | *Adeps lanae* (g) | Stearyl alcohol (g) | White Beeswax (g) | Vaseline yellow (g) | Total ointment (g) |
|---|---|---|---|---|---|---|
| 10% | 2 | 0.6 | 0.6 | 1.6 | 15.2 | 20 |
| 15% | 3 | 0.6 | 0.6 | 1.6 | 14.2 | 20 |
| 20% | 4 | 0.6 | 0.6 | 1.6 | 13.2 | 20 |
| Ointment base | - | 0.6 | 0.6 | 1.6 | 17.2 | 20 |

The ointment preparations to be made in this study have different concentrations of green betel leaf extract, namely 10%, 15% and 20%. The process of making betel leaf extract ointment uses an absorption base. Weighed each of the above ingredients. Stage I stearyl alcohol, white beeswax were melted by heating. Stage II added vaseline yellow and adeps lanae, then stirred until homogeneous, then cooled. Added green betel leaf extract and then crushed until homogeneous. After homogeneous, put it in a tube and label it[18].

**Phytochemical screening of green betel leaf extracts**

Phytochemical analysis was carried out to determine the type of secondary metabolites using alkaloid test, terpenoid/steroid test, flavonoid test, tannin test and saponin test[19,20].

**Fourier Transform Infrared (FTIR) Spectroscopy**

FTIR (Fourier Transform Infrared) is a method that uses infrared spectroscopy. In infrared spectroscopy, infrared radiation is passed through a sample. Part of the radiation will be absorbed by the sample and the other part will be passed or forwarded. FTIR can be used quantitatively because the energy absorbed at a particular wavelength is directly proportional to the amount of kinetic energy associated, so the higher the concentration of the analyte, the more energy is absorbed.How to use FTIR the first step is to turn on the FTIR spectrophotometer. Then the sample powder is placed on the ATR plate then the ATR lever is rotated until it presses the sample on the ATR plate, background measurements are taken every scanning, and has been connected to a computer that has been equipped with OPUS software which is used to control the work of the spectrophotometer in the range of 4000-600 cm-1 with a resolution of 4 cm-1 and scans 32 times. Spectra were stored as absorbance data in OPUS format with three replications[21].

**Bacterial Cultures**

*Staphylococcus aureus* ATCC 25923 bacteria were grown on Nutrient Agar (NA) medium by scratching bacteria from pure culture on the surface of a slanted agar after which it was incubated at 37ºC for 24 hours. Bacterial colonies were suspended in test tubes containing 10ml of Natrium Borth (NB) solution, the turbidity was measured with a visible spectrophotometer at a wavelength of 580 nm, until a transmittance of 25% was obtained[22,23].

**Animal and Experimental Design**

Wistar rats were placed in plastic cages lined with wire mesh covers with a base in the form of husks and consisted of 5 treatment groups with each group consisting of 5 animals in one cage[24]. All test animals were acclimatized for one week and fed ad libitum[25]. Before treatment, rats were anesthetized by injecting 0.5 cc ketamine in the muscle (intramuscular) to ensure that the rats were unconscious during the incision and infection process. An incision was made on the rat's back with a sterile slingshot with the length of 2cm and 0.5 mm wide and 200 uL of *Staphylococcus aureus* ATCC 25923 bacteria were applied to the rat wounds that had formed using a micropipette evenly[9]. The skin of the rat's back after applying the bacteria was observed for 2-5 days to see the occurrence of wounds and pus formed.

The incision wounds in group I (positive control) mupirocin ointment was applied, group II (negative control) was applied vaseline blank, group III was applied 10% concentration green betel leaf extract ointment, group IV was applied 15% concentration green betel leaf extract ointment and group V was applied 20% concentration green betel leaf extract ointment. The application was done twice a day in the morning and evening for 14 days. Macroscopic observation parameters observed the length of wound healing, measuring the length of the wound on days 1, 3, 6, 9, 12 and 14 before the ointment was reapplied. Microscopic observations were made by observing rat skin on day 14[14,26].

**Histopathological Examination**

Histopathological preparations were made using the paraffin method, to observe the presence of inflammatory cells, fibrolas and epithelial cells by taking samples of rat skin and performing Harris-hematoxylin eosin staining viewed in a binocular light microscope with 100x and 450 x magnification[10,27].

**Macroscopic and Microscopic Observations**

Macroscopic observations were made by observing the length of wound healing of incisions infected with *Staphylococcus aureus* ATCC 25923 bacteria. Observations were made of wound healing, the larger the diameter of the healing that occurs, the better the healing. Diameter measurements were taken on days 1, 3, 6, 9, 12 and 14 before the betel leaf extract ointment was reapplied.

Microscopic observation with the development of histological wound healing, namely by making microscopic observations in the epidermis, dermis and hypodermis by identifying the presence of inflammatory cells, fibroblasts, epithelium[28,29].

**Data Analysis**

Data analysis was performed macroscopically observing the disappearance of pus, erythema and the length of healing of incisions infected with *Staphylococcus aureus* ATCC 25923 bacteria and microscopically observing the development of wound healing in the epidermis, dermis and hypodermis by identifying the presence of inflammatory cells, fibrolas and epithelium through the ANOVA test[30].

The ANOVA test is used to compare the averages of populations represented by several sample groups together or to test the average difference in data from more than five groups. The basis for decision making is if the sig value < 0.05 then the data is not normally distributed, but if sig > 0.05 then the data is normally distributed[31].

## RESULTS AND DISCUSSION

**Phytochemical screening test results of Green Betel Leaf Extract**

The results of the phytochemical screening test observations on green betel leaf extract arepositive for alkaloid, terpenoid/steroid, saponin, tannin and

glycoside compounds contained secondary metabolite compounds can be seen from the reagents formed in the following table.

Table 2. Compounds contained in green betel leaf extracts

| Secondary Metabolite Compounds | Reagents | Result |
|---|---|---|
| Alkaloid | Bouchardart | - |
| | Maeyer | - |
| | Dragendroff | + |
| Terpenoids/steroids | Salkowsky | - |
| | Lieberman-Burchad | + |
| Saponins | Aquadest + 96% Alcohol | + |
| Flavonoids | $Mg_{(s)}+HCl_{(p)}$ | - |
| Tannin | $FeCL_3$ 1% | + |
| Glycosides | Mollish | + |

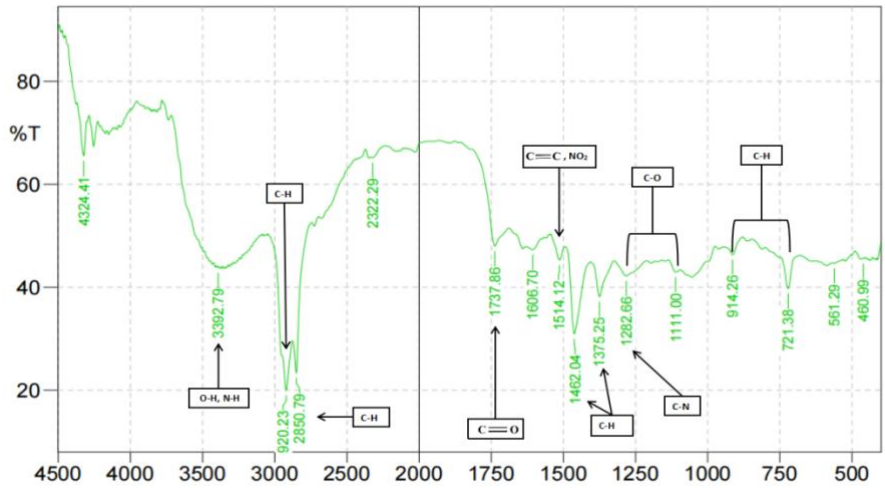

**Figure 1.** FTIR Test Analysis of Green Betel Leaf Extract Ointment

The percentage of transmission above 15 with a wave number of 3394.72 $cm^1$ indicates the presence of O-H functional groups in the form of phenols, nomoner alcohols, hydrogen bond alcohols that change, sometimes widening and N-H functional groups of amines, amides that are moderate. At wave number 2922.16 $cm^1$ shows a strong alkane C-H functional group. The wave number 1710.86 $cm^1$ indicates the presence of strong C=O aldehyde, ketone, carboxylic acid, ester functional groups. The wavelength of 1514.12 $cm^1$ shows the changing aromatic C=C functional group and strong NO2. The $NO^2$ functional group is also present at wave number 1367.53 $cm^2$ and in the range of wave numbers 1444.68 $cm^1$ and 1367.53 $cm^2$ shows a strong alkane C-H functional group. At wave number 1280.73 cm1 shows the presence of strong C-N amine, amide functional groups, and at wave numbers 1280.73 $cm^1$, 1112.93 $cm^1$, 1056.99 $cm^1$ shows the presence of strong C-O functional groups of alcohol compounds, ethers, carboxylic acids and esters. The alkane and aromatic C-H functional groups appear at wave numbers 912.33 cm1, 864.11 $cm^1$, 813.96 $cm^1$ and 758.02 $cm^1$.

**Duration of Incision Wound Healing**

Wound healing observations were made for 14 days, with measurements taken on days 1, 3, 6, 9, 12, and 14. The decrease in wound length is shown in Figure 2 below:

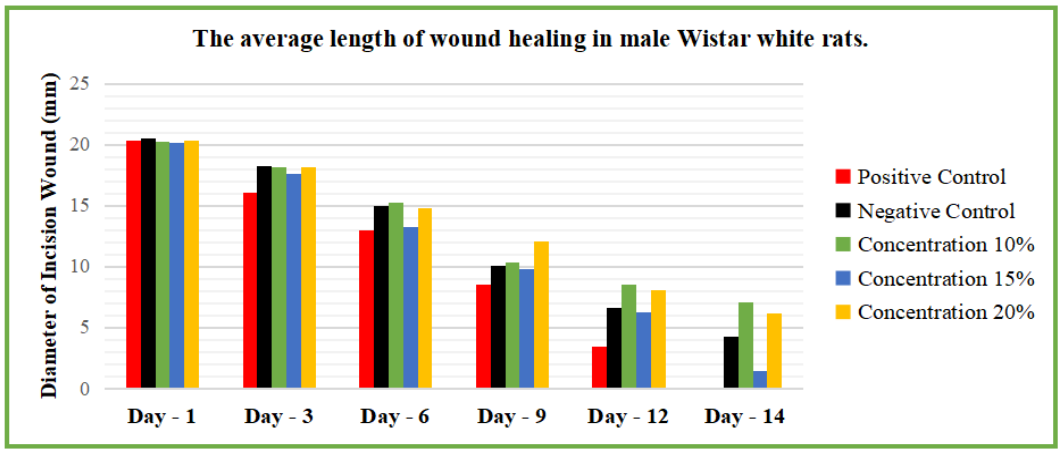


**Figure 2.** Visualization of the length of wound healing of incisions infected with *Staphylococcus Aureus* bacteria shows changes in the length of the wound in each different treatment group as thehealing time progresses.

The length of wound healing of incisions infected with *Staphylococcus aureus bacteria* on day 14, the negative control group was better than the average of the 10% concentration and 20% concentration groups, while the length of wound healing of the positive control incision was better than the length of wound healing in the negative control, 10% concentration, 15% concentration, and 20% concentration. The wound healing length of 15% concentration was significantly better than 10% concentration and 20% concentration. The graph depicts wound healing length on the vertical axis and group category on the horizontal axis.

Table 3. One-way Anova Test

| Group | Mean | Std. Deviation | P-Value |
|---|---|---|---|
| Positive Control | 10,23 | 7,713 | |
| Negative Control | 12,37 | 6,298 | |
| Concentration 10% | 13,27 | 5,415 | 0,917 |
| Concentration 15% | 11,49 | 7,275 | |
| Concentration 20% | 13,25 | 5,566 | |

Based on the One-Way ANOVA test results, a p value of 0,917 was obtained, which is greater than the predetermined significance level of 0.05. Thus, it can be concluded that there is not enough statistical evidence to reject the null hypothesis (H0). This analytical framework can accept the possibility that the null hypothesis is correct, indicating that there is no significant difference between the treatment groups.

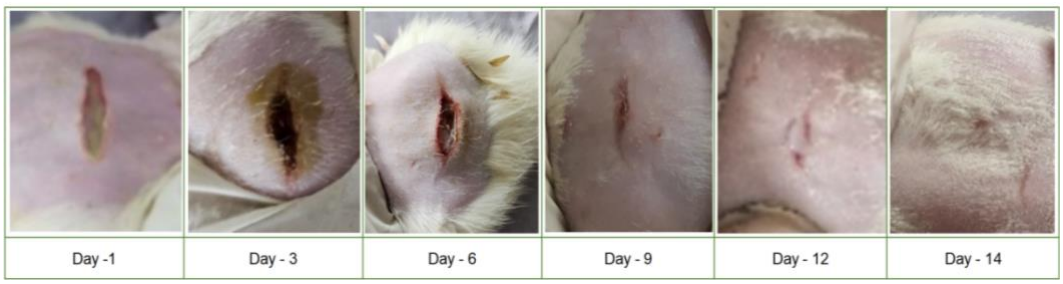


Figure 3. Wound healing with 15% concentration of Green Betel Leaf Extract Ointment Wound healing with 15% concentration of Green Betel Leaf Extract Ointment The use of green betel leaf extract at a concentration of 15% results in a healing time of 15% the fastest wound healing.

On day 14, the average length of wound healing in this concentration reached 1,44 mm.The average final wound size showed a significant difference compared to the other comparative concentrations.

## Skin Histopathology in Wistar Rats

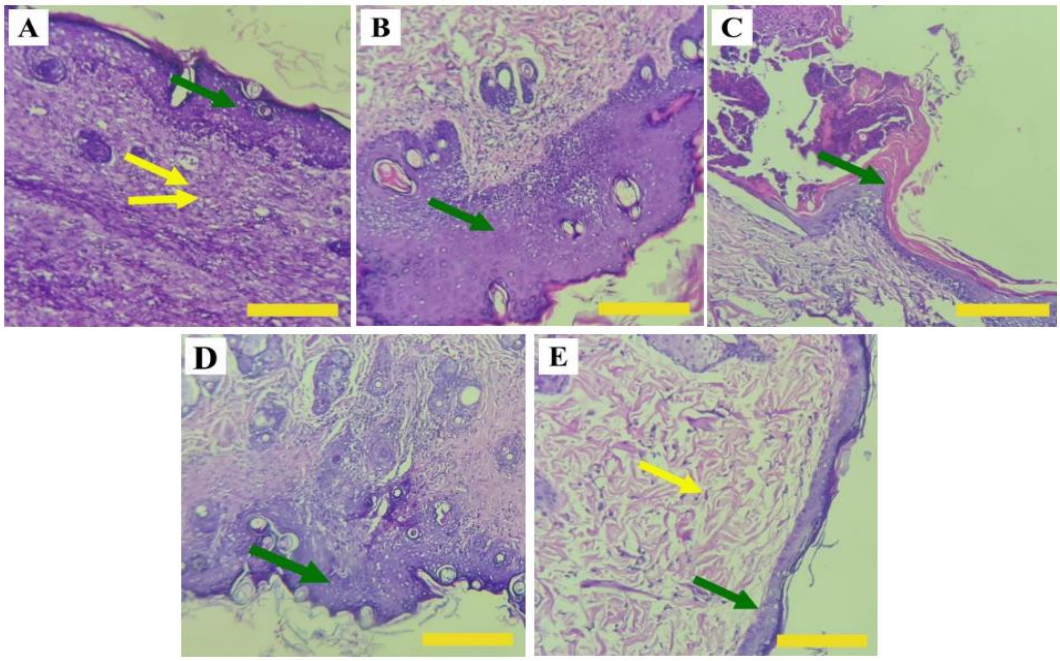


Figure 4. Positive control (A); negative control (B); 10% concentration of green betel leaf extract (C); 15% concentration of green betel leaf extract (D); 20% concentration of green betel leaf extract (E)

Microscopic picture of skin histopathology A: histopathology of skin with green betel leaf extract in the positive control group shows that the surface area of the tissue in the form of epithelium (green arrows) in the epidermis has been thickened and well formed and fibrolas cells (yellow arrows) in the dermis have been thickened, this indicates the process of epithelial cell migration in the normal skin layer has merged. B: Histopathology of the skin of the negative control group with green betel leaf extract shows that the surface area of the visible tissue has a barrier in the form of epithelium (green arrow) in the epidermis which has not yet fused with epithelial cells and is still in the inflammatory phase. Based on the description of the picture, it indicates that the wound is still in the process of tissue formation and is in the inflammatory phase. C: Histopathology of rat skin with 10% concentration of green betel leaf extract shows that the surface area of the tissue in the form of epithelium (green arrow) on the epidermis is still not well formed, this shows irregular epithelial cells and still does not show re-epithelialization. Based on the description of the picture, it indicates that the wound is still in the inflammatory phase. D: Histopathology of rat skin with 15% concentration of green betel leaf extract shows that the surface area of the tissue in the form of epithelium (green arrow) in the epidermis is thickened and epithelial cells begin to fuse in the normal skin layer, this indicates the process of re-epithelialization by epithelial cells that are almost complete and fibrolas cells in the dermis that begin to thicken and spread. Based on the description of the picture, it indicates that the wound is at the end of the inflammatory phase and then continues into the proliferation phase. E: Histopathology of rat skin with 20% concentration of green betel leaf extract shows that the surface area of the tissue in the form of epithelium (green arrow) on the epidermis is still not well formed and the epithelial cells have not fused with the normal skin layer, this indicates that the process of tissue formation has not been maximized. Fibrolas cells (yellow arrows) in the dermis are still scattered and look

tenuous. This description indicates that the wound is still in the early phase of tissue formation.

The results of phytochemical tests on green betel leaf extract contain alkaloid, steroid, and triterpenoid compounds. Alkaloid compounds are found in many plants and have various pharmacological properties, including antimicrobial, anti-inflammatory, and analgesic. Steroids can have anti-inflammatory and immunosuppressive effects, while triterpenoids are known as antioxidants and antimicrobials. Both groups of compounds may contribute to wound healing and antimicrobial effects. Saponins may play a role in inhibiting bacterial growth and supporting wound healing, and tannins have astringent and antimicrobial properties that may help reduce inflammation and wound healing by reducing infection and accelerating clotting[13,32].

The results showed that the p-value of each research group with concentrations of 10%, 15% and 20% were all <0.05, indicating that green betel leaf extract ointment has an effect on the healing of cuts infected with *Staphylococcus aureus* bacteria. In this study, it shows that betel leaf extract ointment with these concentrations has a significant effect on the healing of cuts infected with Staphylococcus aureus bacteria. Thus, the null hypothesis stating that there is no significant difference can be rejected, and we can conclude that the concentration of green betel leaf extract has a significant effect on wound healing. Based on the ANOVA output, it is known that green betel leaf extract ointment with a concentration of 15% has an average value of 11.49 with a standard deviation value of 7.275, providing evidence that this concentration is effective in accelerating wound healing infected with bacteria. The same results were also obtained from research conducted by Atika (2021) entitled The Effect of Green Betel Leaves (Pipet betle L.) Extract on Wounding Healing in mice (Mus musculus L.), where the results showed that green betel leaf extract can affect wound healing in mice (Mus musculus L.)[14].

That green betel leaf extract ointment has been proven to have a significant impact on the improvement of the macroscopic picture of the skin of rats that have incision wounds infected with *Staphylococcus aureus* bacteria. The macroscopic picture of the 10% green betel leaf extract ointment shows that the wound has not closed completely, the wound surface also still does not appear to have dried up. Green betel leaf extract with a concentration of 15% effectively heals wounds. The average increase in incision wound healing at a concentration of 15% is better when compared to the negative control group (K-) due to the content of active compounds in green betel leaf extract that act as anti-inflammatory and antibacterial. wound healing infected with Staphylococcus aureus bacteria in mice in the negative control group (K-) shows that the wound area is still in the inflammatory phase, which is characterized by the presence of reddish inflammatory characteristics (rubor), and has not dried completely. In contrast, in the positive control group (K+) the wound infected with *Staphylococcus aureus* bacteria had dried completely, and hair growth around the wound area began to increase. This is consistent with the statement of Athifah Royani Ma'sum (2018), which states that infection with *Staphylococcus aureus* can cause hair growth with a high number of bacteria in the wound can inhibit the wound closure and healing process. This study used three concentrations for three treatment groups with green betel leaf extract (Piper betle L.), namely with concentrations of 10%, 15%, and 20%, as well as one control group with measurements of wound diameter or 14 days [10], in line with Mawarti's research (2016) that there were differences in wound diameter observed even on the length of wound healing in mice (Mus musculus L.) day 14 of wound healing. This is done to determine the effective concentration for wound healing in mice (Mus musculus L.). The Anova test results showed that green betel leaf extract (Piper betle L.) was effective in healing wounds in mice. Betel leaf extract (Piper betle L.) at a concentration of 30% used by researchers is suitable as a wound healing drug. According to Akbar et al.

(2022), green betel leaves contain flavonoids, tannins, phenols, and saponins that play a role in the wound healing process because they have antimicrobial, anti-inflammatory, and antioxidant properties that affect the wound healing process and accelerate epithelialization[33].

## CONCLUSION

Green betel leaf extract ointment is effective on the healing process of cut wounds infected with *Staphylococcus aureus* bacteria in wistar strain rats for 14 days of observation, the treatment groups show significant differences based on macroscopic observations, it can be concluded that the three concentrations of green betel leaf extract have an influence in healing cut woundsinfected with *Staphylococcus aureus* bacteria. The administration of green betel leaf extract ointment at a concentration of 15% showed a higher significance compared to the concentrationsof 10% and 20% in the healing process of cut wounds in wistar strain rats infected with *Staphylococcus aureus bacteria*. In addition, the administration of green betel leaf extract ointment at a concentration of 15% was able to improve the skin histopathology picture in wistar strain rats infected with *Staphylococcus aureus* bacteria, approaching the level of positive control (K+).

## AUTHORS' CONTRIBUTIONS

Winda Irawati Zebua prepared the samples, designed the protocols, executed the protocols, and wrote the manuscript. Linda Chiuman and Edy Fachrial reviewed and supervised the manuscript. All authors have read and approved the final manuscript

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## DATA AVAILABILITY STATEMENT

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

## DISCLOSURE STATEMENT

There is no conflict of interest.

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Original Research

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# *Effectiveness of ethanol extract of Morinda citrifolia L. as an anti-inflammatory: A preclinical study*

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**Abstract:** The inflammatory response is triggered by infections or tissue damage, often requiring effective anti-inflammatory treatments. *Morinda citrifolia L*. (noni fruit) contains bioactive compounds such as flavonoids, steroids, and triterpenoids with potential anti-inflammatory properties. The aims of this research to evaluate the anti-inflammatory effects and optimal dosing of ethanol extract of noni fruit in reducing paw edema and improving histopathological conditions in male rats induced with Complete Freund's Adjuvant (CFA). This experimental study involved 30 male rats divided into six groups: normal control, negative control (CFA-only), positive control (triamcinolone 0.72 mg), and three treatment groups receiving noni fruit extract (150, 300, 600 mg/200 g BW). Edema volume was measured using a plethysmometer, and histopathological analysis of joint tissues was conducted. Noni fruit extract significantly reduced edema volume, with 600 mg/200 g BW being the most effective dose. However, histopathological analysis revealed persistent inflammatory cell infiltration and synovial hyperplasia in treated groups, indicating incomplete tissue repair compared to triamcinolone. Ethanol extract of noni fruit demonstrates significant anti-inflammatory effects, particularly at higher doses, but is less effective than triamcinolone in resolving tissue inflammation.

**Keywords:** Morinda citrifolia L, anti-inflammatory, histopathology

## INTRODUCTION

The body's inflammatory response is a critical defense mechanism triggered by infection or tissue damage. This response is characterized by heat, redness, swelling, discomfort, and functional impairment. Common causes of inflammation include microorganisms, mechanical injury, chemical exposure, and physical effects. The ultimate goal of inflammation is to attract plasma proteins and phagocytes to the site of injury or invasion, isolate or neutralize invaders, clear debris, and prepare tissues for healing processes. However, persistent or chronic inflammation can result in tissue damage and disease progression.[1,2,3,4,5,6]

To address inflammation, anti-inflammatory drugs are widely used and are generally classified into two groups: steroidal anti-inflammatory drugs (SAIDs) and non-steroidal anti-inflammatory drugs (NSAIDs). SAIDs, such as corticosteroids, block the release of prostaglandins, reducing inflammation at its source. NSAIDs, including ibuprofen, aspirin, and naproxen, inhibit cyclooxygenase (COX), an enzyme responsible for prostaglandin synthesis. While effective, both types of drugs carry risks: SAIDs may cause immunosuppression, osteoporosis, and diabetes, while NSAIDs are associated with gastrointestinal ulcers, anemia, and renal complications.[7,8,9] These limitations necessitate the exploration of alternative, safer anti-inflammatory agents.

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*Morinda citrifolia* L., commonly known as noni, is a traditional medicinal plant widely recognized for its potential therapeutic properties, including anti-inflammatory effects. Noni fruit contains bioactive compounds such as flavonoids, iridoids, and damnacanthal, which inhibit pro-inflammatory enzymes like COX-2 and cytokines such as TNF-α and IL-6. Previous studies have demonstrated that noni extract can suppress the NF-κB pathway, reduce prostaglandin and nitric oxide production, and mitigate inflammation, making it a promising alternative for managing inflammatory conditions such as arthritis.[10,11]

Despite these promising findings, significant gaps remain in understanding the specific mechanisms and optimal dosing of noni fruit extract for anti-inflammatory purposes. For instance, while earlier studies have shown the efficacy of noni in reducing cytokine expression and NF-κB activity, its direct impact on histopathological changes and its comparative effectiveness against standard treatments like corticosteroids remain underexplored.[12,13] Additionally, while noni's therapeutic potential has been supported by biochemical and cellular studies, there is limited evidence on its effectiveness in preclinical animal models.

Freund's Complete Adjuvant (CFA), commonly used to induce inflammation in experimental settings, provides a robust model for studying anti-inflammatory agents. CFA induces localized inflammation, allowing researchers to assess both macroscopic changes, such as edema volume, and microscopic alterations, such as inflammatory cell infiltration in joint tissues.[14] Male *Rattus norvegicus* are often employed in such studies due to their stable hormonal profile and suitability for surgical and sampling procedures, further ensuring reliable results.[15,16] In this context, this study aims to address the research gap by evaluating the effectiveness of ethanol extract of *Morinda citrifolia* L. in reducing inflammation and improving histopathological outcomes in CFA-induced male rats. By comparing the effects of noni extract to those of standard treatments, this study seeks to provide critical insights into its potential as a safer, alternative anti-inflammatory agent.

## MATERIAL AND METHOD

This study employed an experimental design to assess the impact and correlation between the independent and dependent variables. The dependent variables were the reduction in inflammation, edema volume, and histopathological changes in joint tissues, while the independent variable was the ethanol extract of *Morinda citrifolia* L. The study was conducted between November 1, 2023, and February 12, 2024, at the Pharmacology Laboratory of the Faculty of Pharmacy, University of North Sumatra, and the Anatomical Pathology Laboratory at Royal Prima Hospital Medan. Ethical approval for the use of experimental animals was obtained from the Prima Indonesia University Health Research Ethics Committee (No. 061/KEPK/UNPRI/IX/2023).

### Tools and Materials

The study utilized a variety of laboratory tools and reagents essential for conducting the experimental procedures. Ethanol extracts of *Morinda citrifolia* L. were prepared through maceration followed by concentration using ethanol and silica gel chromatography for compound separation. Thirty male *Rattus norvegicus* (aged 2–3 months and weighing 150–200 g) were used as test animals, with Complete Freund's Adjuvant (CFA) employed to induce inflammation. During the treatment phase, paw edema volume was measured using a plethysmometer to evaluate the anti-inflammatory activity. Histopathological analysis of the joint tissues was performed following paraformaldehyde fixation, tissue dehydration, paraffin embedding, and Hematoxylin-Eosin (HE) staining for microscopic evaluation. Standard reagents such as PBS-azide solution, nitric acid, and hematoxylin-eosin dyes were used throughout the experimental procedures.

### Preparation of Ethanol Extract of Noni Fruit

Fresh noni fruits were washed, seeds and pulp removed, and the remaining material oven-dried. The dried material was ground into powder. Maceration was performed using 96% ethanol for 24 hours, followed by filtration and concentration with a rotary evaporator to obtain a thick ethanol extract.[17,18]

**CFA-Induced Inflammation in Test Animals**

Male rats were subplantarly injected with 0.1 mL CFA into the left hind paw to induce inflammation. After 16 days, the paw edema volume was measured using a plethysmometer. Edema measurements were performed on day 0 (before CFA injection) and on days 17, 20, 23, 26, 29, and 31 post-injection.[19]

**Study Groups and Treatments**

The 30 test animals were acclimatized for two weeks and randomly divided into six groups (n=5 per group):

1. **Normal control:** No treatment.
2. **Negative control:** CFA injection only.
3. **Positive control:** CFA injection followed by triamcinolone (0.72 mg intramuscularly) on day 17.
4. **Dose I:** CFA injection followed by 150 mg/200 g BW noni fruit extract daily from day 17 to day 31.
5. **Dose II:** CFA injection followed by 300 mg/200 g BW noni fruit extract daily from day 17 to day 31.
6. **Dose III:** CFA injection followed by 600 mg/200 g BW noni fruit extract daily from day 17 to day 31.

**Anti-inflammatory Activity Test**

The anti-inflammatory effect was evaluated by measuring the reduction in paw edema volume using a plethysmometer. The decrease in paw edema was interpreted as an indicator of anti-inflammatory activity.[20,21]

**Histopathological Analysis**

On day 32, after 15 days of treatment, joint tissues were collected from the left hind paws of the rats. The procedure involved:

1. **Dissection:** Rats were euthanized by cervical dislocation, and the left hind paw joint was excised.
2. **Tissue Processing:** The joint tissues were washed with cold 0.9% NaCl and immersed in PBS-azide solution (pH 7.4) and paraformaldehyde (PFA).
3. **Staining:** Tissue samples were processed using the Hematoxylin-Eosin (HE) staining method, involving fixation, decalcification, dehydration, infiltration, embedding in paraffin, sectioning, and staining. The slides were analyzed microscopically to evaluate inflammatory cell infiltration and synovial hyperplasia.[22,23]

**Data Analysis**

Data were analyzed using Shapiro-Wilk test was applied to assess data normality. The paired sample *t*-test was used to evaluate the effectiveness of different doses of noni fruit extract. A post-hoc LSD test determined the most effective dose for reducing edema.[24,25]

## RESULTS AND DISCUSSION

**Phytochemical Test Results**

Based on the results of phytochemical tests, the content in the ethanol extract of noni fruit (Morinda citrifolia L) contains steroids and triterpenoids, saponins, flavonoids and tannins.

**Table 1. Phytochemical screening of ethanol extract of noni fruit (*Morinda citrifolia L*)**

| Secondary Metabolite Compounds | Reagents | Result |
|---|---|---|
| Alkaloid | Bouchardart | - |
| | Maeyer | - |
| | Dragendroff | - |
| | Wagner | - |
| Terpenoids/steroids | Salkowsky | - |
| | Lieberman-Burchad | + |
| Saponins | Aquadest + 96% Alcohol | + |
| Flavonoids | $Mg_{(s)}$+$HCl_{(p)}$ | - |
| | $FeCL_3$ 5% | + |
| | NaoH 10% | - |
| | H2SO4 | - |
| Tannin | $FeCL_3$ 1% | + |
| Glycosides | Mollish | - |

Based on the results of phytochemical tests, the content in the ethanol extract of noni fruit (Morinda citrifolia L) contains steroids and triterpenoids, saponins, flavonoids and tannins that function as anti-inflammatory. The anti-inflammatory properties of flavonoids are due to their ability to inhibit cyclooxygenase and lipoxygenase, as well as the concentration of local leukocytes in line with the research of Zaky et al.[26] Found in noni fruit and has anti-inflammatory and anti-allergic effects, noni can also dilate blood vessels that are vasocontracted and improve blood circulation. Scopololetin is a molecule with medical potential, scientists think it can bind to serotonin, an important compound in humans. The scopoletin content in noni fruit can reduce prostaglandin levels by inhibiting cyclooxygenase (COX) and 5-lipoxygenase activity against arachidonic acid. Terpenoids are isometric hydrocarbon molecules found in various lipids or essential oils. These lipids play an important role in organic synthesis and cell repair in the body.[27]

The phytochemical screening of ethanol extract of *Morinda citrifolia* L. revealed the presence of terpenoids, steroids, saponins, flavonoids, and tannins, which are well-documented for their anti-inflammatory properties. Terpenoids and steroids, identified through the Lieberman-Burchard test, inhibit key inflammatory pathways, including cyclooxygenase (COX) and lipoxygenase (LOX), thereby reducing the synthesis of pro-inflammatory mediators such as prostaglandins and leukotrienes. Saponins, detected using Aquadest and 96% alcohol, contribute to anti-inflammatory activity by stabilizing cell membranes and reducing vascular permeability, which minimizes edema formation. Flavonoids, confirmed through the $FeCl_3$ 5% test, exert both anti-inflammatory and antioxidant effects by inhibiting COX and LOX enzymes while protecting tissues from oxidative damage caused by inflammation. Similarly, tannins, identified using the $FeCl_3$ 1% test, exhibit astringent properties that reduce tissue swelling and inflammatory exudates while downregulating cytokine production, including tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6). The absence of alkaloids and glycosides suggests that the anti-inflammatory effects of the extract are primarily mediated by the detected compounds. Collectively, these bioactive constituents explain the extract's efficacy in reducing paw edema and histopathological markers of inflammation, particularly in the high-dose treatment group. This phytochemical composition highlights *Morinda citrifolia* L. as a promising natural anti-inflammatory agent with potential therapeutic applications for managing inflammatory conditions.

**Thin Layer Chromatography Test (KLT)**

Based on the results of Thin Layer Chromatography (KLT) test, the content in the ethanol extract of noni fruit (*Morinda citrifolia L*) is scopoletin with an Rf value of 0.51. The Thin Layer Chromatography (TLC) analysis of the ethanol extract of *Morinda citrifolia* L. confirmed the presence of scopoletin, a coumarin derivative,

with an Rf value of 0.51, indicating its significant contribution to the extract's pharmacological properties. Scopoletin is a well-documented bioactive compound with potent anti-inflammatory, antioxidant, and immunomodulatory effects. It exerts anti-inflammatory activity by inhibiting pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), and suppressing the activation of nuclear factor kappa B (NF-κB), a transcription factor that regulates inflammatory gene expression. Furthermore, scopoletin inhibits the cyclooxygenase-2 (COX-2) enzyme, thereby reducing prostaglandin synthesis, which is responsible for pain, swelling, and redness during inflammation. Its antioxidant properties complement these effects by scavenging reactive oxygen species (ROS) and reducing oxidative stress, which helps protect tissues from damage and preserves cellular integrity. Additionally, scopoletin's immunomodulatory activity balances immune cell responses, such as macrophages and lymphocytes, preventing excessive inflammatory reactions that could lead to tissue damage. The identification of scopoletin in the extract underscores its potential as a key contributor to the anti-inflammatory effects observed in this study, particularly in reducing paw edema and improving histopathological parameters. This finding supports the therapeutic value of *Morinda citrifolia* L. as a natural source of anti-inflammatory agents and highlights scopoletin's synergistic interaction with other bioactive compounds present in the extract.

**Effect of Mouse Leg Volume with Pletismometer**

Measurement of rat paw volume with a pletismometer was carried out on day 0, namely before CFA induction and then after CFA induction on day 17, 20, 23, 26, 29 and day 31, with the results in the table 2.

**Table 2. Measurement of Mouse Paw Volume Using a Plethysmometer (µL)**

| Group | Day-0 | Day-17 | Day-20 | Day-23 | Day-26 | Day-29 | Day-31 |
|---|---|---|---|---|---|---|---|
| Normal Control | 4.42 ± 0.26 | 4.40 ± 0.21 | 4.38 ± 0.24 | 4.26 ± 0.19 | 4.29 ± 0.20 | 4.28 ± 0.22 | 4.30 ± 0.21 |
| Negative Control | 4.22 ± 0.58 | 9.03 ± 1.12 | 8.94 ± 1.12 | 8.78 ± 1.10 | 8.70 ± 1.09 | 8.56 ± 1.07 | 8.40 ± 1.09 |
| Positive Control (Triamcinolone) | 3.65 ± 0.25 | 7.34 ± 0.82 | 6.93 ± 0.61 | 6.21 ± 0.44 | 5.46 ± 0.55 | 4.98 ± 0.53 | 4.39 ± 0.46 |
| Dose I (150 mg/200 g BW) | 3.71 ± 0.19 | 7.73 ± 0.79 | 7.58 ± 0.74 | 7.30 ± 0.71 | 7.01 ± 0.66 | 6.76 ± 0.61 | 6.28 ± 0.58 |
| Dose II (300 mg/200 g BW) | 4.06 ± 0.40 | 7.30 ± 0.78 | 6.93 ± 0.73 | 6.54 ± 0.72 | 6.22 ± 0.67 | 5.64 ± 0.61 | 5.09 ± 0.57 |
| Dose III (600 mg/200 g BW) | 3.35 ± 0.23 | 8.11 ± 1.07 | 7.56 ± 1.01 | 6.86 ± 0.92 | 6.42 ± 0.86 | 5.59 ± 0.76 | 4.95 ± 0.69 |

The results presented in Table 2 demonstrate the progressive reduction in paw volume across different treatment groups, reflecting the anti-inflammatory effects of *Morinda citrifolia* L. extract. In the normal control group, the paw volume remained consistent throughout the study, indicating the absence of inflammation and serving as a baseline. Conversely, the negative control group, which was induced with CFA but received no treatment, exhibited a significant increase in paw volume from Day-17 to Day-31, confirming persistent inflammation caused by the CFA induction. The positive control group treated with triamcinolone showed the most substantial reduction in paw volume, with values decreasing from 7.34 µL on Day-17 to 4.39 µL on Day-31, demonstrating the potent anti-inflammatory and immunosuppressive effects of triamcinolone.

The lowest average decrease in the volume of rat's feet after CFA induction from day-17 to day-31 was the negative control group, from 9.03 µL to 8.40 µL, while the highest average decrease in the volume of rat's feet after CFA induction

from day-17 to day-31 was the positive control group, from 7.34 µL to 4.39 µL. The results of the average decrease in the volume of rat feet after CFA induction from day-17 to day-31 given dose therapy I, II and III, were most effective in dose group III, namely from 8.11 µL to 4.95 µL. This shows that the administration of triamcinolone is more effective in reducing the volume of udema than the administration of ethanol extract of noni fruit. Blocking the phospholipase A2 enzyme in the phospholipid layer of cell membranes, triamcinolone has anti-inflammatory and immunosuppressant effects. This action prevents the formation of arachidonic acid by blocking the breakdown of leukocyte lysosomal membranes. Reduces the expression of cyclooxygenase (COX) and lipoxygenase (LOX), which in turn inhibits the production of prostaglandins and leukotrienes. Corticosteroids manifest anti-inflammatory effects through inhibiting the migration of macrophages and leukocytes to the affected site by restoring dilation and permeability of blood vessels. This action leads to reduced edema, erythema and pruritus. An important anti-inflammatory mechanism is mediated by the inhibition of nuclear factor kappa-B (NF-kappa-B), which leads to decreased expression of interleukin-6 (IL-6), interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), and COX-2.[28]

These findings highlight the anti-inflammatory potential of *Morinda citrifolia* L. extract, particularly at higher doses, in mitigating CFA-induced inflammation. However, the extract's efficacy remains lower than that of triamcinolone, indicating that while the extract provides substantial anti-inflammatory effects, it may serve better as a complementary or adjunctive therapy rather than a standalone treatment. The dose-dependent reduction in paw volume underscores the importance of optimizing dosing strategies for maximizing therapeutic outcomes.

**Effectiveness of Ethanol Extract of Noni Fruit (*Morinda citrifoilia L*)**

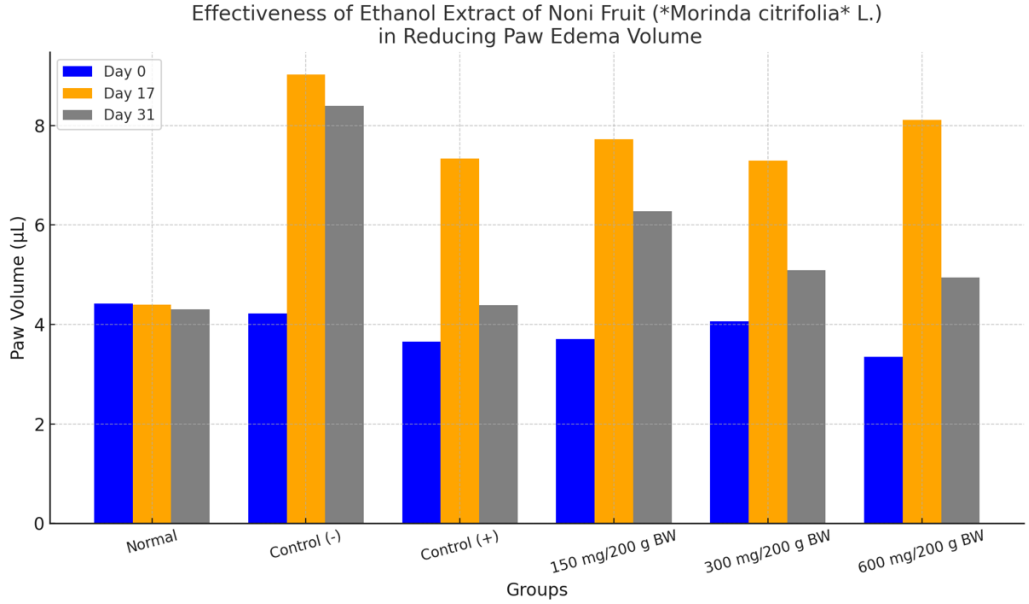


**Figure 1. Effectiveness of Ethanol Extract of Noni Fruit (Morinda citrifoilia L) in Reducing the Volume of Udem in Mouse Legs**

The graph demonstrates the effectiveness of ethanol extract of *Morinda citrifolia* L. and triamcinolone in reducing paw edema in CFA-induced mice, measured at different time points (Day 0, Day 17, and Day 31). The normal control group maintained consistent paw volumes throughout the study, indicating no inflammatory response. In contrast, the negative control group showed a marked increase in paw volume following CFA induction, with minimal reduction by Day 31, reflecting persistent inflammation in the absence of treatment. The positive control group, treated with triamcinolone, exhibited a significant reduction in paw volume

from Day 17 to Day 31, demonstrating its potent anti-inflammatory effects. Among the groups treated with noni fruit extract, a dose-dependent reduction in paw volume was observed. The group receiving the highest dose (600 mg/200 g BW) showed the greatest efficacy, with paw volumes approaching those of the positive control group by Day 31. Lower doses (150 mg/200 g BW and 300 mg/200 g BW) were less effective but still reduced paw edema compared to the negative control. These findings highlight the potential anti-inflammatory effects of *Morinda citrifolia* L. extract, particularly at higher doses, while emphasizing that its efficacy is lower than that of triamcinolone.

The effects of the treatments highlight the ability of ethanol extract of *Morinda citrifolia* L. and triamcinolone to modulate the inflammatory response and reduce tissue swelling. The high-dose noni extract (600 mg/200 g BW) showed a notable reduction in edema, suggesting its effectiveness in decreasing vascular permeability and inflammatory mediator production, which are key processes in the progression of inflammation. The bioactive compounds, such as scopoletin, flavonoids, and tannins, likely contribute by inhibiting cyclooxygenase (COX) and lipoxygenase (LOX) pathways, thus reducing prostaglandin and leukotriene synthesis. The improvement in edema reduction over time in the treated groups also indicates a potential cumulative effect of the extract with prolonged administration. However, while the noni extract reduced inflammation significantly, its effects were not as rapid or pronounced as triamcinolone, which acts through more direct mechanisms, such as suppressing cytokine expression and stabilizing lysosomal membranes. This positions the noni extract as a potential complementary therapy in managing inflammation, particularly in cases where corticosteroid use may be contraindicated.

**Table 3. Post-Hoc Test Results Using LSD (Least Significant Difference)**

| Day | Control (-) | Control (+) | Dose 1 (150 mg/200 g BW) | Dose 2 (300 mg/200 g BW) | Dose 3 (600 mg/200 g BW) |
|---|---|---|---|---|---|
| Day 0 | 0.667 | 0.667 | 0.829 | 0.667 | 0.829 |
| Day 17 | 0.003 | 0.003 | 0.796 | 0.796 | 0.606 |
| Day 20 | 0.002 | 0.002 | 0.314 | 0.137 | 0.779 |
| Day 23 | 0.001 | 0.001 | 0.000 | 0.002 | 0.000 |
| Day 26 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| Day 29 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| Day 31 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |

The effectiveness of ethanol extract of noni fruit (Morinda citrifoilia L) which is most effective in reducing the volume of udema in the legs of male rats induced by CFA can be seen using the Post-Hoc test with LSD. Based on the doses used, namely 150 mg/200 gBB, 300 mg/200 gBB and 600 mg/200 gBB of the three doses of extracts have effectiveness as anti-inflammatory. However, the dose of 150 mg/200 gBB has shown effectiveness on day 23 and the dose of 600 mg/200 gBB is the most effective dose in reducing the volume of edema in the legs of male rats induced by CFA. The most effective dose of noni fruit ethanol extract in reducing the volume of udema is in dose group III of 600 mg/200 gBB. Scoploletin, flavonoids, triterpenoids, and steroid chemicals are included in the ethanol extract of noni fruit. Scopolonetin contains anti-inflammatory and anti-allergic effects in addition to dilating narrowed blood vessels and improving blood circulation. The analgesic effect of flavonoid concentrations of noni fruit is due to its ability to block the enzyme cyclooxygenase and its substrates from binding oxygen, thereby causing prostaglandin synthesis.[29]

## Mouse Foot Histopathology

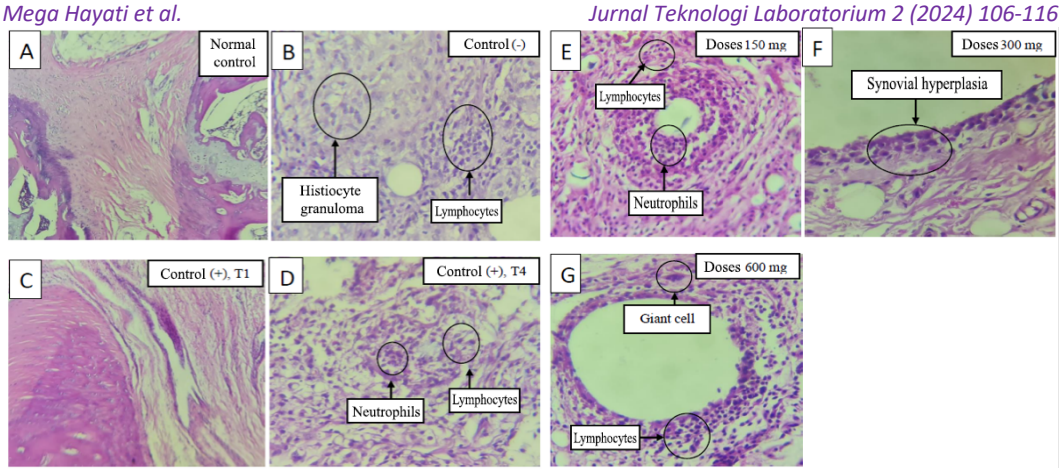


**Figure 2. Rat paw histopathology**

The results of the histopathological examination of the negative control group can be seen in Figure 2 (B) that there is an infiltration of inflammatory cells in the tissue of the rat's foot joints in the form of inflammatory cell infiltrates of lymphocytes, plasma cells, PMN neutrophils and the distribution and group of histiocytes forming a granuloma structure. The histopathology results of the positive control group (triamcinolone injection) can be seen in Figure 2 (C) and (D) that almost all rats (rats 1,2,3 and 5) show a cleaner picture of the foot joint tissue and are close to a normal picture. The histopathology results of dose groups 1, 2 and 3 (ethanol extract of noni fruit) can be seen in Figure 2 (E), (F) and (G) that there is an infiltration of inflammatory cells in the rat foot joint tissue in the form of infiltrates of lymphocyte inflammatory cells, plasma cells and scattered, histiocyte groups forming a granuloma structure accompanied by the form of many-nucleated datia cells and there is also synovial hyperplasia. The histopathological picture of inflammatory cell infiltrates and thickened synovials in rats given mengkudu fruit extract doses of 150 mg / 200 gBB, 300 mg / 200 gBB and 600 mg / 200 Gbb, this indicates that the ethanol extract of mengkudu fruit (Morinda citrifolia L) cannot improve the histopathology of joint tissue in CFA-induced rat feet.

Inflammation that occurs in rat feet after being induced by CFA, interpret the parameters of histopathological changes in rat foot joint tissue after the administration of ethanol extract of noni fruit as an inflammatory therapy. The results showed that the tissues in the rat's feet experienced inflammation in all groups of rats except the normal control group and the positive control group (triamcinolone injection), it can be seen in Figure 2 (C) and 2 (D) that almost all rats (rats 1, 2, 3 and 5) showed a cleaner picture of the foot joint tissue and approached a normal picture. Only in rat 4 which still shows inflammatory cell infiltration. This is in line with research according to Katzung et al., 2023 triamcinolone inhibits the postpolyphase enzyme, which results in the inhibition of the release of arachidonic acid, which is needed to activate the next enzyme.[30]

## CONCLUSION

The ethanol extract of *Morinda citrifolia* L. demonstrates significant anti-inflammatory activity, as evidenced by its ability to reduce paw edema and inflammatory cell infiltration in CFA-induced rats. Among the tested doses, the 600 mg/200 g BW dose exhibited the highest efficacy, with reductions in paw volume and improvements in histopathological parameters approaching those of the positive control (triamcinolone). However, the extract did not achieve complete tissue recovery as observed with triamcinolone, suggesting its anti-inflammatory effects are less potent. The presence of bioactive compounds such as scopoletin, flavonoids, and tannins contributes to the anti-inflammatory effects through mechanisms like COX and LOX inhibition, reduction of cytokine production, and

modulation of immune responses. These findings imply that while *Morinda citrifolia* L. extract shows potential as a natural anti-inflammatory agent, it is best suited as a complementary therapy or for conditions where corticosteroid use is contraindicated. Future research should focus on refining extraction methods, exploring higher doses, and conducting clinical trials to validate its therapeutic application in managing chronic inflammatory conditions.

## AUTHORS' CONTRIBUTIONS

Mega Hayati prepared the samples, Adek Amansyah designed the protocol, implemented the protocol, and Fioni wrote the manuscript. Ali Napiah Nasution and Ermi Girsang reviewed and edited the manuscript. All authors have read and approved the final manuscript.

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## DATA AVAILABILITY STATEMENT

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

## DISCLOSURE STATEMENT

There is no conflict of interest.

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## Original Research

# *Effect of Sechium edule extract on blood sugar levels and pancreatic histopathology in male Rats with type II diabetes mellitus*

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**Abstract:** Diabetes mellitus (DM) is a metabolic disorder characterized by elevated blood glucose levels (hyperglycemia) and the presence of glucose in the urine (glucosuria). The etiology of diabetes varies depending on its type, including Type 1 diabetes, which is caused by autoimmune destruction of pancreatic beta cells responsible for insulin production; Type 2 diabetes, resulting from insulin resistance; and gestational diabetes, triggered by hormonal changes during pregnancy affecting insulin sensitivity. Insulin plays a crucial role in glucose metabolism by converting glucose into glycogen for storage. This study aimed to evaluate the effect of Siamese pumpkin extract (Sechium edule) on blood glucose levels in male Wistar rats (Rattus norvegicus) with type II diabetes. A pre-test and post-test control group experimental design was employed, using 25 rats divided into five groups: a negative control group, a positive control group, and treatment groups receiving chayote extract at doses of 14 mg/kgBW, 28 mg/kgBW, and 42 mg/kgBW, respectively. Streptozotocin (STZ) was administered to induce diabetes in all groups except the negative control. Data analysis included normality tests, homogeneity tests, ANOVA, and post-hoc multiple comparison tests. The results demonstrated that Siamese pumpkin extract contains active compounds such as alkaloids, saponins, flavonoids, steroids, triterpenoids, tannins, and glycosides. Among the treatment groups, the administration of chayote extract at a dose of 42 mg/kgBW resulted in the most significant reduction in blood glucose levels compared to the lower doses. Histopathological analysis of pancreatic tissue revealed no abnormalities, necrosis, or degeneration across all treatment groups. The superior efficacy of the 42 mg/kgBW dose is attributed to the higher concentration of active compounds, enhanced glucose-lowering mechanisms, and improved systemic distribution.

**Keywords:** Type II Diabetes Mellitus; Blood Sugar Level; Pancreatic Histopathology; Siamese Pumpkin Extract.

## INTRODUCTION

Diabetes mellitus (DM) is a disorder of carbohydrate metabolism characterized by elevated blood glucose levels (hyperglycemia) and the presence of glucose in urine (glucosuria). Hyperglycemia negatively impacts overall health by promoting the formation of free radicals or reactive oxygen species through oxidative-reduction mechanisms, which increase electron donors in the mitochondrial electron transport chain[1]. This metabolic imbalance can impair pancreatic beta-cell function, resulting in inadequate insulin production or insulin resistance, both of which contribute to hyperglycemia[2]. In such cases, glucose accumulates in the bloodstream without being utilized by cells due to resistance at insulin receptor sites[3]. DM is broadly classified into type 1, characterized by autoimmune destruction of pancreatic beta cells, and type 2, which is associated

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with insulin resistance, peripheral tissue dysfunction, and beta-cell insufficiency[4]. I

n Indonesia, diabetes is a significant health challenge marked by chronic hyperglycemia and metabolic disturbances in carbohydrates, fats, and proteins[5]. As of 2023, the World Health Organization (WHO) estimates that more than 422 million people worldwide suffer from diabetes, which caused 1.5 million deaths in 2019[6]. Globally, diabetes prevalence is approximately 28.3% in Pakistan, 10% in the United States, and 8.5% among Indonesians aged ≥15 years[7]. In Medan, Indonesia, the Basic Health Research (RISKESDA) report of 2018 noted a prevalence of 58.73%[8]. Type 2 diabetes mellitus poses a serious threat to developing nations like Indonesia, where 80% of cases occur in low- and middle-income regions[9].

Type 2 diabetes mellitus poses a serious threat to developing nations like Indonesia, where 80% of cases occur in low- and middle-income regions[10]. Such complications are influenced by genetic, environmental, and lifestyle factors, as well as delays in diagnosis and treatment[11]. The underlying mechanism involves vascular dysfunction due to oxidative stress, which damages endothelial cells and other tissues unable to regulate glucose transport[12].

Modifiable risk factors for diabetes include poor dietary habits, smoking, obesity, hypertension, stress, lack of physical activity, and alcohol consumption[13]. While diabetes prevalence is similar between genders, women may be at slightly higher risk due to physiological factors such as pregnancy-related weight gain, higher life expectancy, and greater prevalence of obesity and hypertension[14].

Patient non-adherence to pharmacological treatments often exacerbates diabetes, prompting interest in alternative therapies using phytopharmaceuticals. These plant-based treatments are considered cost-effective, widely available, and associated with fewer side effects[15]. One such plant, chayote (Sechium edule), is traditionally used for medicinal purposes. Belonging to the Cucurbitaceae family, chayote is a nutrient-rich fruit commonly consumed as a vegetable or in herbal preparations[16].

Chayote exhibits a range of biological activities, including antioxidant, antimicrobial, diuretic, antihypertensive, and hypocholesterolemic effects[17]. Its active compounds include flavonoids, alkaloids, and saponins, which are known to support cardiovascular health[18]. Additionally, its potassium content may enhance its hypoglycemic potential[19]. This study investigates the anti-diabetic efficacy of chayote extract in male Wistar rats (Rattus norvegicus) induced with streptozotocin (STZ), focusing on its glucose-lowering effects and histopathological impact on pancreatic tissue. Unlike prior studies, this research aims to elucidate the optimal dosage of chayote extract for therapeutic use, thereby providing a approach to diabetes management.

## MATERIAL AND METHOD

This study employed a pre-test and post-test only control group experimental design[20]. The research was conducted at the Pharmacology Laboratory of the Faculty of Pharmacy, University of North Sumatra, and the Anatomical Pathology Laboratory of the Royal Prima Medan Hospital from January to April 2024. Ethical approval was obtained from the Prima Indonesia University Health Research Ethics Committee (034/KEPK/UNPRI/XII/2023).

### Experimental Design

The study utilized 25 male Wistar rats (Rattus norvegicus), randomly divided into five groups (five rats per group) to evaluate the effect of chayote extract on blood glucose levels. The groups were as follows:

1. **Negative Control (K-)**: No streptozotocin (STZ) induction or chayote extract treatment.
2. **Positive Control (K+)**: Induced with STZ (45 mg/kg body weight) but no chayote extract treatment.

3. **Treatment 1 (P1)**: Induced with STZ (45 mg/kg body weight) and administered chayote extract (14 mg/kg body weight).
4. **Treatment 2 (P2)**: Induced with STZ and administered chayote extract (28 mg/kg body weight).
5. **Treatment 3 (P3)**: Induced with STZ and administered chayote extract (42 mg/kg body weight).

The rats were acclimatized for one week before the study. On day 0, fasting blood glucose levels were measured. Rats in the treatment and positive control groups were fasted for 8 hours before receiving STZ induction via intraperitoneal injection (45 mg/kg body weight dissolved in 0.9% NaCl). Hyperglycemia was confirmed 3 days post-induction (fasting blood glucose levels between 200–349 mg/dL)[21]. Chayote extract was administered orally every morning for 4 weeks. The negative control and treatment groups of rats (rattus wistar strain) were given swimming exercises for 20 minutes, the treatment was given every 3 times a week and lasted for 4 weeks[22].

**Preparation of Chayote Extract**

Chayote (Sechium edule) was extracted using the maceration method. Fresh chayote was processed with 95% ethanol for 72 hours, followed by an additional 48-hour maceration. The filtrate was concentrated using a rotary evaporator to obtain a thick ethanol extract[23].

**FTIR (Fourier Transform Infrared) screening**

FTIR can be used quantitatively because it can determine a compound in a sample. through at a particular wavelength is directly proportional to the amount of kinetic energy associated[24].

**Phytochemical Screening**

Qualitative tests were performed to identify secondary metabolites, including alkaloids, flavonoids, saponins, and tannins in the ethanol extract of chayote[16].

**Induction of Diabetes**

Streptozotocin (STZ) was prepared at a dose of 45 mg/kg body weight, dissolved in 0.9% NaCl. Following STZ induction, rats were provided 1% glucose solution to prevent hypoglycemia[25]. Hyperglycemia was confirmed on the third day using a glucometer.

**Histopathological Preparation**

Pancreatic tissue was collected and fixed in 10% formalin[26]. The tissue was processed through stages of dehydration, clearing, embedding in paraffin, and sectioning into 2 µm slices[27]. Hematoxylin and eosin (H&E) staining was performed, and slides were observed under a light microscope for histopathological changes such as inflammation, necrosis, or degeneration[26,27].

**Statistical Analysis**

Data were analyzed using one-way ANOVA ($p \leq 0.05$) after confirming normality (Shapiro-Wilk test, $p > 0.05$) and homogeneity (Levene's test, $p > 0.05$). Post hoc Mann-Whitney tests were applied to determine differences between groups[28,29].

## RESULTS AND DISCUSSION

**Phytochemical Screening**

The results of the phytochemical screening test on the 96% ethanol extract of chayote were observed and determined based on the reactions formed with specific reagents.

**Table 1. Phytochemical Screening Results of 96% Ethanol Extract of Chayote (Sechium edule)**

| Compound Group | Test Reagents | Result |
|---|---|---|
| Alkaloids | Bouchardat | Positive |
| | Mayer | Positive |
| | Dragendorff | Positive |
| Terpenoids/Steroids | Salkowski | Positive |
| | Liebermann-Burchard | Positive |

| | | |
|---|---|---|
| Saponins | Distilled water + 96% ethanol | Positive |
| Flavonoids | Magnesium (Mg) + HCl | Positive |
| Tannins | 1% Ferric chloride ($FeCl_3$) | Positive |
| Glycosides | Molisch | Positive |

Table 1 shows the results of the phytochemical test of the chemical components of chayote fruit show that chayote extract contains alkaloids, terponoids/steroids, saponins, flavonoids, tannins, glycosides. The table provides an overview of the secondary metabolite compounds detected in the study, along with the reagents used for qualitative analysis and their respective results. The presence of alkaloids was confirmed through tests with Bouchardat, Mayer, and Dragendorff reagents, all yielding positive results, which indicates a significant presence of these compounds. Terpenoids and steroids were also detected using the Salkowski and Liebermann-Burchard reagents, both showing positive outcomes that confirm their presence.

Saponins were identified using a mixture of distilled water (aquadest) and 96% alcohol, with the test producing a positive result, highlighting the presence of these bioactive compounds. Similarly, flavonoids were confirmed using a combination of magnesium (Mg) and hydrochloric acid (HCl), as evidenced by a positive reaction. The presence of tannins was verified through a test with 1% ferric chloride ($FeCl_3$), which yielded a positive outcome. Lastly, glycosides were detected using Molisch's test, as shown by the positive result.

Overall, the results indicate that the tested extract contains a diverse range of secondary metabolite compounds, including alkaloids, terpenoids/steroids, saponins, flavonoids, tannins, and glycosides. These findings underscore the potential bioactivity and therapeutic properties of the extract.

**FTIR Test Results of 96% Ethanol Extract of Siamese Pumpkin**

The results of the FTIR test of 96% chayote ethanol extract to determine the functional groups contained in the sample can be observed in the figure below:

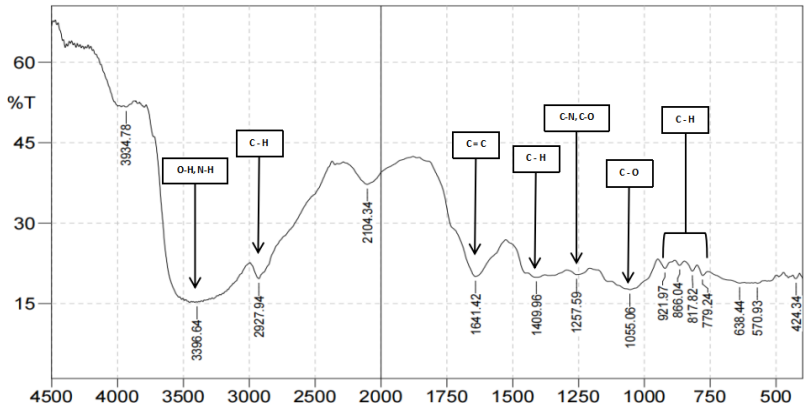


**Figure 1. FTIR Test Results of Siamese Pumpkin Extract**

The FTIR spectrum of the sample, presumably chayote (Sechium edule) extract, reveals the presence of several functional groups that are characteristic of bioactive compounds. A broad absorption peak observed at 3369.78 $cm^{-1}$ corresponds to O-H stretching vibrations, indicating the presence of hydroxyl groups commonly found in alcohols and phenolic compounds. Additionally, this peak also reflects N-H stretching, which suggests the presence of amines or amides. A distinct peak at 2927.94 $cm^{-1}$ is attributed to C-H stretching, characteristic of aliphatic hydrocarbons. A minor absorption at 2104.34 $cm^{-1}$ indicates the potential presence of triple bonds, such as C≡C or C≡N, although this feature is less pronounced. The peak at 1641.42 $cm^{-1}$ corresponds to C=C stretching, indicative of alkenes or aromatic compounds, while the absorption at 1409.95 $cm^{-1}$ reflects C-H bending vibrations, likely associated with methyl or

methylene groups. Another significant feature is the band at 1257.56 $cm^{-1}$, attributed to C-N stretching, which suggests the presence of amines or amide functional groups. The strong absorption at 1055.06 $cm^{-1}$ corresponds to C-O stretching, indicative of alcohols, esters, or ethers. In the fingerprint region (below 1000 $cm^{-1}$), peaks such as 783.22 $cm^{-1}$ and 424.34 $cm^{-1}$ represent C-H bending in aromatic or substituted compounds, providing structural specificity to the sample.

Figure 1 shows at wave numbers 779.24 cm1, 817.82 cm1, 866.04 cm1 and 921.97 cm1 indicate the presence of strong alkene and aromatic C-H functional groups. Strong C-H functional groups of alkane compounds are also present at wave numbers 1409.96 cm1, and 2927.94 cm1. At wave number 1257.59 cm1 shows the presence of C-N functional groups of amine compounds, amides and C=O functional groups of strong alcohol, ether, carboxylic acid and ester compounds. At wave number 1641.42 cm1, it shows the presence of C=C functional group of alkenes compound which changes. O-H functional groups of phenol compounds, nomoner alcohols and hydrogen bond alcohols are changing, sometimes widening and also the presence of N-H functional groups of amine and amide compounds which are present at wave number 3396.64 cm1. Overall, the FTIR spectrum confirms the presence of diverse functional groups, including hydroxyl, aliphatic hydrocarbons, amines, esters, and aromatic compounds. These findings align with the phytochemical composition of the extract, supporting the presence of secondary metabolites such as flavonoids, alkaloids, tannins, and saponins, which contribute to its bioactive properties.

**Blood Sugar Level Measurement Results**

Table 2 shows that in group K + treatment with STZ the average blood sugar level value is 0.26mg/dl. In group K- with treatment not given STZ and chayote extract, the average blood sugar level value is 0.00mg/dl. In group F1 with treatment given STZ and chayote extract, the average blood sugar level value is 0.47mg/dl. In group F2 with treatment given STZ and chayote extract, the average value of blood dula level is 0.54mg/dl. In group F3 with treatment given STZ and chayote extract, the average value of blood dula level is 0.61mg/dl.

The table presents data on the changes in blood glucose levels of rats (Rattus norvegicus) over time across five groups: Positive Control (K+), Negative Control (K-), and three treatment groups (F1, F2, F3). Each group contains three test subjects, and glucose levels were recorded at various time points (H1, H3, H6, H9, H12, H15, H18, H21, H24, H28), representing hours or days of observation.

The K+ group shows a minimal average reduction in glucose levels (0.26%), while the K- group exhibits no significant reduction (0.00%), indicating the absence of treatment effects in these groups. In contrast, the treatment groups (F1, F2, and F3) demonstrate progressively greater percentages of blood glucose reduction. F1 shows an average reduction of 0.47%, F2 achieves 0.54%, and F3 exhibits the highest reduction of 0.61%. This trend suggests a dose-dependent effect of the administered chayote extract, with F3 being the most effective in lowering blood glucose levels. The data underline the potential of chayote extract as a hypoglycemic agent, with its effectiveness increasing at higher doses. The controlled experimental design, including positive and negative controls, ensures the reliability of the results and highlights the therapeutic potential of chayote extract for managing diabetes.

**Table 2. Blood sugar level measurement results**

| Group | K+ | | | K- | | | F1 | | | F2 | | | F3 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Rat** | **1** | **2** | **3** | **1** | **2** | **3** | **1** | **2** | **3** | **1** | **2** | **3** | **1** | **2** | **3** |
| **H1** | 465 | 496 | 547 | 82 | 80 | 90 | 283 | 307 | 585 | 342 | 561 | 578 | 478 | 549 | 563 |
| **H3** | 469 | 490 | 532 | 80 | 82 | 88 | 274 | 303 | 554 | 318 | 532 | 563 | 444 | 511 | 533 |
| **H6** | 460 | 485 | 530 | 81 | 84 | 91 | 261 | 295 | 534 | 307 | 510 | 531 | 418 | 467 | 510 |
| **H9** | 453 | 472 | 526 | 83 | 81 | 90 | 238 | 277 | 496 | 278 | 463 | 493 | 374 | 443 | 457 |
| **H12** | 433 | 453 | 505 | 78 | 84 | 93 | 221 | 258 | 458 | 244 | 448 | 451 | 344 | 407 | 411 |
| **H15** | 426 | 438 | 493 | 80 | 80 | 92 | 203 | 237 | 415 | 218 | 429 | 406 | 312 | 373 | 393 |
| **H18** | 407 | 419 | 475 | 79 | 78 | 90 | 190 | 226 | 375 | 196 | 388 | 378 | 276 | 321 | 354 |
| **H21** | 386 | 405 | 441 | 78 | 82 | 88 | 179 | 217 | 335 | 179 | 334 | 347 | 235 | 278 | 322 |
| **H24** | 367 | 382 | 413 | 79 | 80 | 89 | 166 | 201 | 285 | 162 | 285 | 321 | 207 | 245 | 296 |
| **H28** | 349 | 367 | 405 | 80 | 81 | 90 | 151 | 189 | 254 | 149 | 248 | 297 | 163 | 211 | 253 |
| **Percent Decrease** | **0.25** | **0.26** | **0.26** | **0.02** | **0.01** | **0.00** | **0.47** | **0.38** | **0.57** | **0.56** | **0.56** | **0.49** | **0.66** | **0.62** | **0.55** |
| **Average** | **0.26** | | | **0.00** | | | **0.47** | | | **0.54** | | | **0.61** | | |

**Table 3. Mean blood glucose levels and statistical comparisons among experimental groups**

| Group | Mean (mg/dL) | Standard Deviation (mg/dL) | Significance (P-Value) Compared to K+ |
|---|---|---|---|
| Positive Control (K+) | 458.07 | 40.61 | - |
| Negative Control (K-) | 112.76 | 91.67 | 0.000 |
| 14 mg/kgBW (F1) | 287.46 | 92.28 | 0.001 |
| 28 mg/kgBW (F2) | 361.86 | 97.33 | 0.234 |
| 42 mg/kgBW (F3) | 373.83 | 104.75 | 0.456 |

The table 3 summarizes the mean blood glucose levels, standard deviations, and statistical comparisons among experimental groups to evaluate the effect of chayote extract on diabetic rats. The positive control group (K+) exhibited the highest mean blood glucose level (458.07 ± 40.61 mg/dL), while the negative control group (K-) had the lowest (112.76 ± 91.67 mg/dL). The treatment groups, receiving chayote extract at doses of 14 mg/kgBW (F1), 28 mg/kgBW (F2), and 42 mg/kgBW (F3), demonstrated intermediate reductions in glucose levels, with means of 287.46 ± 92.28, 361.86 ± 97.33, and 373.83 ± 104.75 mg/dL, respectively.

Statistical analysis using post hoc tests revealed significant differences ($P < 0.05$) between the positive control group (K+) and the treatment groups (F1 and F2), as well as between the negative control group (K-) and all other groups. However, no significant differences were observed between the treatment groups (F1 vs. F2, F2 vs. F3, or F1 vs. F3), indicating comparable hypoglycemic effects across the three doses of chayote extract. The lack of significant differences among treatment groups suggests a threshold effect where increasing the dose beyond 14 mg/kgBW does not result in proportionately greater glucose reduction.

Overall, the findings highlight the efficacy of chayote extract in lowering blood glucose levels, with significant improvements compared to the positive control group. These results suggest that chayote extract has potential as a natural therapeutic agent for diabetes management, with comparable effectiveness across the tested doses.

**Histopathology of Pancreatic Wistar Male Rats**

The use of chayote extract at a dose concentration of 42 mg/KgBB produced a length of Langerhans islets of 851.70 µm, wider than other doses.

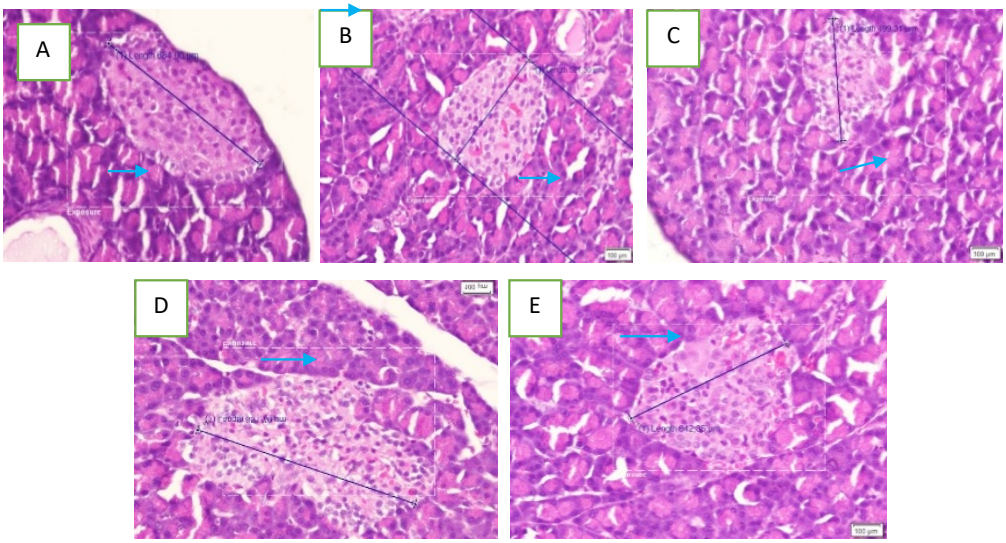


**Figure 2. Histopathology of Pancreatic Wistar Male Rats**

Figure 2 show the histopathology of the pancreas in male Wistar rats across different experimental groups. In the positive control group, the length of the islets of Langerhans was observed to be 684.00 µm. In the negative control group, the length of the islets of Langerhans measured 521.30 µm. The treatment group receiving a 14 mg/kgBW dose of chayote extract showed an islet length of 499.31 µm, while the group treated with a 28 mg/kgBW dose displayed the longest islet length at 851.70 µm. The 42 mg/kgBW dose group showed an islet length of 642.35 µm. Across all groups, no morphological abnormalities, necrosis, or degeneration were observed. Additionally, the exocrine glands of the pancreas remained intact, as indicated by the blue arrows.

Phytochemical analysis of chayote extract confirmed the presence of various active compounds, including alkaloids, flavonoids, steroidal saponins, triterpenoids, tannins, and glycosides. These compounds exhibit antioxidant and anti-inflammatory properties that inhibit carbohydrate absorption, enhance insulin sensitivity, and protect pancreatic beta cells, thereby accelerating diabetes recovery[30]. This activity contributes to lowering blood glucose levels by reducing the activity of key enzymes such as alpha-amylase and alpha-glucosidase, which are responsible for breaking carbohydrates into monosaccharides that can be absorbed by the intestine. Consequently, blood sugar levels remain stable[18].

The FTIR analysis further identified functional groups, such as ethers (C-O), associated with tannins and flavonoids in the chayote extract. Flavonoids, known for their antioxidant properties, can protect pancreatic beta cells from oxidative damage caused by reactive oxygen species[31]. Flavonoids also inhibit enzymes involved in carbohydrate metabolism and improve insulin sensitivity. Additionally, the potassium content in chayote stimulates insulin secretion, while niacin, a component of nicotinamide adenine dinucleotide (NAD), facilitates glycogenesis, further contributing to glucose reduction[7].

Chayote is a low-calorie food with a low glycemic index, rich in fiber, potassium, and bioactive compounds, making it beneficial for individuals with diabetes. Its high potassium-to-sodium ratio (62:1 per 100 grams) promotes heart and vascular health. Moreover, its high soluble fiber content slows carbohydrate digestion and absorption, preventing postprandial blood sugar spikes. Steamed chayote, with its filling starch content, serves as an alternative to excessive staple food consumption[7].

The one-way ANOVA test indicated that the hypoglycemic effect of chayote extract was most significant at a 42 mg/kgBW dose compared to 14 mg/kgBW and 28 mg/kgBW doses. This can be attributed to the higher concentrations of active compounds, such as flavonoids and alkaloids, at the highest dose. These compounds improve insulin sensitivity, reduce insulin resistance, and protect pancreatic beta cells. Secondary metabolites, particularly flavonoids, act as potent antioxidants that mitigate oxidative stress, prevent degenerative diseases such as diabetes, and restore insulin receptor sensitivity[32]. These findings underscore the potential of chayote extract as a natural therapy for diabetes management.

## CONCLUSION

The study provides compelling evidence that chayote extract significantly reduces blood glucose levels, protects pancreatic tissue, and exhibits antioxidant properties that mitigate diabetes-induced oxidative stress. The findings underscore the potential of chayote as a natural adjunct therapy for diabetes management, with its multifaceted mechanisms of action contributing to glucose regulation and beta-cell protection. However, further clinical research is necessary to confirm its efficacy and safety in humans.

## AUTHORS' CONTRIBUTIONS

Marti silfia prepared the samples, designed the protocols, executed the protocols, and wrote the manuscript. Gusbakti Rusip, Linda Chiuman reviewed and supervised the manuscript. All authors have read and approved the final manuscript.

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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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# *Evaluation of the anti-acne effectiveness of Andaliman (Zanthoxylum acanthopodium DC) nanoemulgel in propionibacterium acnes-induced male wistar rats*

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**Abstract:** Acne vulgaris is an inflammatory disorder of the pilosebaceous unit, characterized by lesions such as comedones, papules, pustules, nodules, and cysts. This study aimed to assess the effectiveness of Andaliman Extract Nanoemulgel (Zanthoxylum Acanthopodium DC) in treating acne lesions in male Wistar rats (Rattus Novergicus) induced with Propionibacterium acnes. This experiment involved 30 male Wistar rats aged 6-8 weeks, weighing 150-200 grams, divided into 6 groups: 1) normal, 2) negative control (P. acnes induction and base gel), 3) positive control (P. acnes induction and Mediklin TR gel), 4) Treatment 1 (P. acnes induction and 2% Nanoemulgel), 5) Treatment 2 (P. acnes induction and 3.5% Nanoemulgel), and 6) Treatment 3 (P. acnes induction and 5% Nanoemulgel). The Nanoemulsion was applied for 10 days to evaluate the healing of acne lesions on the skin of Wistar rats. The results indicate that Andaliman Extract Nanoemulgel significantly promoted the healing of lesions caused by Propionibacterium acnes. In conclusion, Nanoemulgel with Andaliman fruit extract at concentrations of 2% and 5% demonstrated the highest effectiveness in anti-acne treatment.

**Keywords:** Acne Vulgaris; Nanoemulgel Andaliman Extract; *Propionibacterium Acnes*.

## INTRODUCTION

Acne vulgaris (AV) is a chronic inflammatory condition of the pilosebaceous unit, primarily triggered by the accumulation of excess sebum and the proliferation of *Cutibacterium acnes* (formerly known as *Propionibacterium acnes*).[1] The condition commonly affects predilection sites such as the face, neck, shoulders, chest, back, and upper arms.[2] According to the Global Burden of Disease (GBD) report, the incidence of AV is approximately 85% among individuals aged 12–25 years, with the highest prevalence observed in females aged 14–17 years (83–85%) and males aged 16–19 years (95–100%).[3] A survey in Southeast Asia reveals that AV cases account for 40–80% of dermatological issues.[3] Based on the European S3 Acne Guideline, AV can be classified into comedonal acne, papulopustular acne (mild to moderate severity), severe papulopustular acne, moderate nodular acne, severe nodular acne, and conglomerate acne.[4]

The pathogenesis of AV is influenced by several factors, including increased sebum production, abnormal keratinocyte proliferation, bacterial colonization, and inflammation. These factors can be exacerbated by lifestyle choices, such as diet, inadequate skin hygiene, cosmetic usage, and stress.[5] Although various topical and systemic therapies are available for AV, many have

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limitations, including potential side effects, resistance, and high recurrence rates. These challenges highlight the need for alternative, effective treatments that leverage natural resources.

Indonesia, renowned for its biodiversity, has abundant natural resources that have been traditionally used for medicinal purposes due to their safety, availability, and minimal side effects.[6] One such resource is Andaliman (*Zanthoxylum acanthopodium DC*), a plant endemic to the North Tapanuli and Toba Samosir regions in North Sumatra.[7] Andaliman thrives at elevations of 900–2000 meters above sea level with an annual rainfall of 170–180 days.[8] Traditionally used as a spice in Batak cuisine and for medicinal purposes, Andaliman is rich in bioactive compounds such as flavonoids, alkaloids, saponins, and terpenoids.[9] These compounds exhibit a wide range of pharmacological activities, including antibacterial, anti-acne, anti-aging, antiviral, anticancer, antihyperglycemic, hepatoprotective, antifungal, and antipreeclampsia properties. Additionally, Andaliman is rich in essential oils with potent antibacterial and antioxidant activities.[10]

Topical formulations, such as lotions, ointments, and creams, are commonly used for AV treatment. However, they often face limitations such as poor drug penetration, low drug-loading capacity, and stability issues.[11] Transparent gels, though offering improved stability, may not effectively deliver lipophilic drugs. To address these limitations, nanoemulgel formulations have been developed. Nanoemulgels are emulsions with droplet sizes ranging from 1 to 100 nm, suspended in a hydrogel base. This formulation enhances drug delivery by temporarily disrupting the lipid bilayer structure, allowing for better penetration of active substances.[12,13] The inclusion of oil, surfactant, and cosurfactant components further increases therapeutic efficacy.[14]

While previous studies have demonstrated the anti-acne potential of Andaliman fruit extract, there is limited research on its incorporation into advanced nanoemulgel formulations. Additionally, the effectiveness of nanoemulgel formulations of Andaliman extract in managing acne induced by *Propionibacterium acnes* in animal models remains unexplored. This study aims to evaluate the anti-acne efficacy of Andaliman nanoemulgel in white male Wistar rats induced with *Propionibacterium acnes*, addressing the gap in utilizing this potent natural resource in innovative drug delivery systems.

## MATERIAL AND METHOD

### 2.1. Materials and Tools

Wistar rats 6-8 weeks, oven, evaporator, blender, 40 mesh sieve, UV-Vis spectrophotometer, digital balance, Buchner funnel, filter paper, Particle Size Analyzer, magnetic stirrer, ultrasonicator, centrifuge, waterbatch, stirrer, vortex mixer, dissolution tester, laboratory glassware, analytical balance, stirrer, gloves, and documentation tools, Metil Paraben, Kitosan, Propyl Paraben, Methanol, Aquadest, Tween 80, PEG 400, Carbopol 940. Acetate Acid 1% and Ethanol 96%.

### 2.2. Methods

Two kilograms of Andaliman fruit were dried in an oven with a blower at 55°C for 5 hours. The dried fruit was then ground, yielding 700 grams of powder, and extracted via maceration. Methanol was used as the solvent with a ratio of 1:3 (w/v) Methanol serves as a polar and versatile solvent, capable of extracting both polar and non-polar components. The extraction process utilized various concentrations of methanol to optimize the yield of antioxidants [15]. According to Ghanimi R., *et al*. the methanol extract proven to have a greater concentration of flavonoids than the other extracts (such as ethanol, ethyl acetate and water extracts) [16]**.** The maceration process with the solvent was carried out for 24 hours.

The maceration filtrate was concentrated using an evaporator at 55°C, yielding a thick extract [17].

Nanoemulsions were invented using spontaneous emulsification method. Emulsion system contain of an oil phase and water phase. Spontaneous emulsification technique was done by adding the oil phase to water phase dropwise[18]. During the water phase, Methyl Paraben and Propyl Paraben were dissolved in heated distilled water, which was then cooled, followed by the addition of Tween 80 in distilled water. The mixture was stirred using a magnetic stirrer for 30 minutes at 5000 rpm (Mass 1). PEG was then added to the andaliman extract and stirred with a magnetic stirrer for 20 minutes at 5000 rpm (Mass 2). Mass 1 and Mass 2 were gradually combined dropwise using a pipette. The mixture was then stirred with a magnetic stirrer for 8 hours at 5000 rpm and subsequently sonicated [17].

The Nanoemulgel was prepared by first dispersing 10mL of carbopol 940 in warm distilled water for 24 hours and dissolving chitosan in 10 mL of 1% acetic acid. The dissolved chitosan was neutralized with 10 mL of 0.1 N NaOH until reaching pH 5. A portion of the swollen Carbopol 940 was transferred to a mortar and mixed with methylparaben dissolved in 96% ethanol until homogenous. Triethanolamine was added and mixed until homogenous. The remaining nanoemulsion and carbopol were slowly added while stirring until a homogeneous gel mass formed. Chitosan was then slowly added while stirring until a homogeneous gel mass formed [14,19]. Carbopol 940 was widely use as gelling agent in cosmetic, known for its excellent compatibility, stability, non-toxic nature, and soft application on the skin. It serves several functions, including maintaining emulsion stability, suspending solid particles in liquids and regulating the consistency of cosmetic formulations [20]. Meanwhile Chitosan enhance the mechanical structures of Nanoemulsion through electrostatic interactions and increasing the stability of the emulsified system [21].

Subsequently, 30 male Wistar strain rats were acclimatized for 1 week.

Wistar rats were randomly divided into six groups where each group consisted of 5 individuals:

1. Group 1 = Normal, no stressed.
2. Group 2 = Negative control, induced *P.acnes* 0.2 mg/kgBB within intradermal, gel base.
3. Group 3 = Positive control, induced *P.acnes* 0.2 mg/kgBB within intradermal, Mediklin TR gel applied on skin once a day for 10 days.
4. F1 = Treated 1, induced P.acnes 0.2 mg/kgBB within intradermal, Nanoemulgel 2%.
5. F2 = Treated 2, induced P.acnes 0.2 mg/kgBB within intradermal, Nanoemulgel 3,5%.
6. F3 = Treated 3, induced P.acnes 0.2 mg/kgBB within intradermal, Nanoemulgel 5%.

Each control and treatment group was induced with Propionibacterium acnes. On the 2nd day post-induction, acne lesions were measured. In the treatment group, Nanoemulgel was applied topically once a day to the rats' skin and observed for up to 10 days.

The data analysis using IBM SPSS 25 involved acne severity, which was initially analyzed using descriptive statistics. Subsequently, the normality of the data distribution was assessed using the Shapiro-Wilk test. This test was selected due to small sample size and it appropriateness for detecting deviations from normality. If the data followed a normal distribution, the analysis continued with One Way ANOVA and Post Hoc tests. However, if data transformation did not achieve normality, non-parametric analysis such as the Kruskal-Wallis test was used instead.

## RESULTS AND DISCUSSION

Andaliman fruit, scientifically known as *Zanthoxylum Acanthopodium*, was extracted using the maceration method, resulting in a methanol extract with the following characteristics:

**Table 1. Characteristic of *Zanthoxylum Acanthopodium* fruit Extract**

| Characteristic | Amount |
|---|---|
| Fesh Herbal Weight (gr) | 2 kg |
| Weight Herbal Powder (gr) | 700 g |
| Solvent Volume (ml) | 5000 ml |
| Extract Weight (gr) | 75.30 g |
| Yield (%) | 10.76 % |

Table 1 shows that 2 kilograms of fresh andaliman fruit, yielded 75.3 grams of extract. Therefore, the yield obtained from the methanol extract of andaliman is 10.76%. Andaliman fruit extract underwent a phytochemical analysis consisting of phytochemical screening followed by flavonoid, alkaloid and total tanin measurements. Screening of phytochemical results are described in Table 2.

**Table 2. Phytochemical screening of Methanol *Zanthoxylum Acanthopodium* fruit Extract**

| Phytochemical | Reagents | Results |
|---|---|---|
| Flavonoid | $FeCl_3$ 5% | + |
| | Mg(s) + HCL(p) | - |
| | NaOH 10% | - |
| | $H_2SO_4$(p) | - |
| Alkaloid | Bouchardart | + |
| | Mayer | + |
| | Wagner | + |
| | Dragendorff | + |
| Terpenoid dan Steroid | Lieberman-Burchard | - |
| | Salkowsky | - |
| Tanin | $FeCl_3$ 1% | - |
| Saponin | Aquadest+Alkohol 96% | + |
| Glycoside | Mollish | + |
| Antosianin | HCL 2M | - |

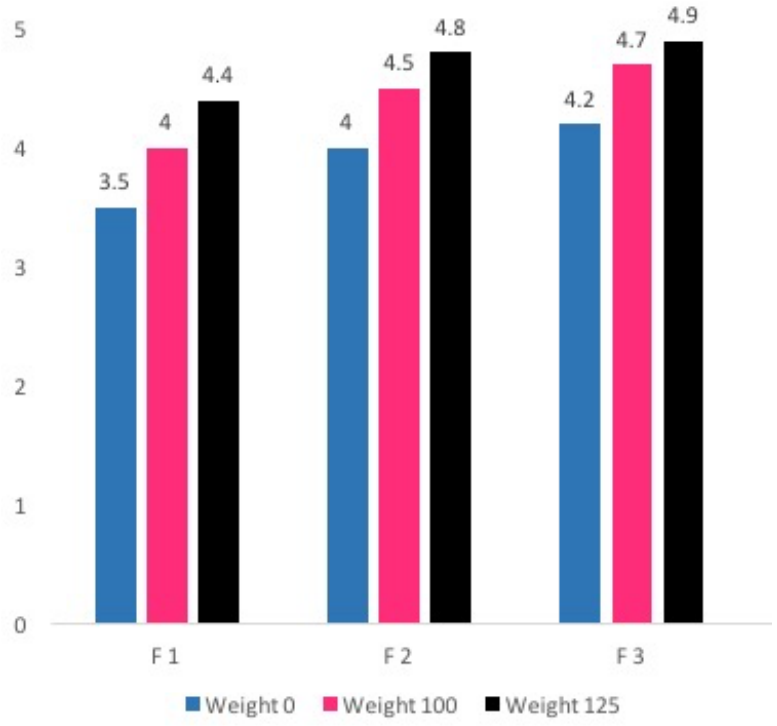
Based on Table 2, phytochemical screening showed that the *Zanthoxylum Acanthopodium* fruit extract had phytochemicals, including flavonoid, alkaloid, saponin dan glycoside.

**Table 3 Evaluation of Organoleptic physics characteristic of *Zanthoxylum Acanthopodium* fruit extract**

| Organoleptic Evaluation | F1 | F2 | F3 |
|---|---|---|---|
| Odor | distinctive smell | distinctive smell | distinctive smell |
| Color | brown | brown | brown |
| Appearance | nanoemulgel | nanoemulgel | nanoemulgel |
| Homogenity | homogen | homogen | homogen |
| pH | 6.42 | 6.38 | 6.36 |

Based on Table 3 The physics characteristic of Nanoemulgel *Zanthoxylum Acanthopodium* fruit extract has the same odor, color, appearance, and homogenity, with a pH range of 6.36-6.42. Spreadability shown on Table 4

**Table 4 Spreadability of physics characteristic of *Zanthoxylum Acanthopodium* fruit extract**




| Formulation | F1 | | | F2 | | | F3 | | |
|---|---|---|---|---|---|---|---|---|---|
| Weight (gr) | 0 | 100 | 125 | 0 | 100 | 125 | 0 | 100 | 125 |
| Spreadability (cm) | 3.5 | 4.0 | 4.4 | 4.0 | 4.5 | 4.8 | 4.2 | 4.7 | 4.9 |

**Table 5 Comparison of Acne Severity Measurement**

| Groups | Mean Average | Mean Score |
|---|---|---|
| Group 2 | 13.33 ± 3.00 | 12.58 |
| Group 3 | 13.17 ± 3.17 | 11.26 |
| F3 | 13.83 ± 5.00 | 13.63 |
| F2 | 11.83 ± 3.00 | 9.59 |
| F1 | 12.50 ± 5.00 | 13.22 |

Table 5 presents the results of Kruskal-Wallis test, which showed a significant difference between treatment groups indicates the effectiveness of Andaliman Nanomulgel in reducing acne lesions. Analysis of mean ranks indicated that group F3 had a higher average rank (Mean Rank = 13.63) compared to group 2 (Mean Rank = 11.26), group 3 (Mean Rank = 12.58), F2 (Mean Rank = 9.59), and F1 (Mean Rank = 13.22). The significant Kruskal-Wallis test values (Asymp. Sig. < 0.05) for all treatment groups suggest a significant difference in treatment effects on different days of experimentation (H1, H3, H6, H8, H10).

Mean rank analysis showed treatment F3 had the highest average rank, followed by F1, group 3, group 2, and F2. This indicates that Andaliman

Nanoemulgel, especially in treatment F3, which contains the highest bioactive compounds in 5% Nanoemulgel.

*Propionibacterium acnes* is a bacterium that causes acne vulgaris [22]. *Propionibacterium acnes* produces proteins that degrade skin tissue, leading to inflammation. Phytochemical screening data of methanol extract from Andaliman revealed the presence of several phytochemical compounds such as flavonoids, alkaloids, saponins, and terpenoids. According to research of (Fajryana et al., 2022), these compounds have been found to inhibit the growth *of Propionibacterium acnes* [23].

According to (Ira Syaputri et al., 2022), flavonoids are effective antimicrobial agents against various microorganisms because they have the ability to form complexes with bacterial cell proteins through hydrogen bonding. This interaction destabilizes the bacterial cell wall and cytoplasmic membrane, rendering the proteins biologically inactive, thereby rendering the bacterial cell proteins inactive biologically. Alkaloids possess antibacterial properties by intercalating with DNA, disrupting the peptidoglycan components of bacterial cells. Saponins absorbed onto the cell surface cause damage by increasing membrane permeability, leading to cell death. Terpenoid compounds inhibit bacterial growth by disrupting the formation process of cell membranes and walls [24]. Research of Nafyad *et.al*., Methanol crude extracts showed better antimicrobial activity in medicinal plants [25]. Study conducted by Chiuman *et.al*., proves Methanol and Nanoemulsion Andaliman showed antibacterial activities against E.Coli [26]. Methanol extract are limited solubility in water, it penetrate the outer membrane of bacteria and disturbed cellular function, metabolism, and loss of cellular constituents, leading their death [27].

Nanoemulgel is a type of emulsion with droplet sizes ranging from 1-100 nm. It is suspended in a hydrogel making it ideal for topical delivery. According to (Imanto et al., 2019) and (Indalifiany et al., 2021), nanoemulgel is one of the most promising topical delivery systems because it offers dual release capabilities: gel and nanoemulsion [14,28]. The addition of drug solutions can enhance stability and drug release. In this study, the administration of 5% and 2% Andaliman Nanoemulgel was effective as an antimicrobial and anti-inflammatory agent.The Kruskal-Wallis test revealed that treatment F3 demonstrated the most significant reduction in acne severity, as indicated by a mean rank of 13,63. Based on the study of Amelia *et al*., proves that higher concentration of Andaliman shown to be more effective in inhibiting bacteria growth [29].

These findings are consistent with research conducted by (Hanum Ismanelly and Laila, 2018), where ethanol extract of Andaliman significantly healed acne within 4 weeks. Physical observations also showed a reduction in inflammation levels due to the properties of Andaliman that are absorbed by the skin, influenced by metabolism, moisture, and skin thickness. Flavonoids present in Andaliman are effective antibacterial and anti-inflammatory agents for acne-prone skin [30].

## CONCLUSION

Based on the research findings, the following conclusions can be drawn from this study: Nanoemulgel Andaliman fruit extract at doses of 5% and 2% are the most effective doses in reducing the number of acne lesions and decreasing inflammation. The phytochemical screening of methanol extract from Andaliman identified several compounds including flavonoids, alkaloids, saponins, and terpenoids. These phytochemicals effectively act as antimicrobial agents that inhibit acne in rats. The results suggest that Andaliman Nanoemulge, particularly at 5%concentration, could be developed as an alternative topical treatment for acne vulgaris, offering potential benefits over conventional therapies due to its natural origin and effectiveness.

## 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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