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The Effect of Giving Insulin Leaf Extract (*Smallanthus sonchifolius*) on the Reduction of the Number of Leukocytes in Wistar Rats Infected with *Staphylococcus aureus*

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Abstract: This study aimed to evaluate the antibacterial activity and immunomodulatory effects of insulin leaf (*Smallanthus sonchifolius*) extract against *Staphylococcus aureus* infection in Wistar rats. The research employed an experimental design using Wistar rats divided into five groups: negative control, positive control, and treatment groups receiving insulin leaf extract at doses of 100 mg, 200 mg, and 400 mg. Leukocyte counts were measured from day 1 to day 13 following bacterial induction and treatment administration. Antibacterial activity was assessed using the well diffusion method at extract concentrations of 25%, 50%, and 75%. Statistical analysis was performed using One-Way ANOVA. The results demonstrated that insulin leaf extract significantly reduced leukocyte counts in infected rats, indicating anti-inflammatory and immunomodulatory effects. The 400 mg dose exhibited the most stable reduction in leukocyte levels throughout the observation period. Antibacterial testing showed inhibition zone diameters of 20.42 mm, 22.62 mm, and 24.90 mm at concentrations of 25%, 50%, and 75%, respectively, indicating strong to very strong antibacterial activity against *Staphylococcus aureus*. One-Way ANOVA revealed significant differences among treatment groups ($p = 0.002$) and among antibacterial concentrations ($p < 0.001$). These findings suggest that *Smallanthus sonchifolius* leaf extract possesses potent antibacterial, anti-inflammatory, and immunomodulatory properties and has potential as a natural therapeutic agent for bacterial infections caused by *Staphylococcus aureus*.

Keywords: Antibacterial activity; Anti-inflammatory; Immunomodulator; Insulin leaf extract; Leukocyte count; *Smallanthus sonchifolius*; *Staphylococcus aureus*; Wistar rats

Introduction

Staphylococcus aureus is a Gram-positive, facultative anaerobic coccus that typically forms grape-like clusters under microscopic observation. It commonly colonizes the skin and mucosal surfaces, particularly the nasal cavity, throat, and perineal region, as part of the normal human microbiota. However, under favorable conditions, it becomes an opportunistic pathogen capable of causing a wide spectrum of infections, ranging from superficial skin infections such as boils, impetigo, and cellulitis to severe diseases including pneumonia, osteomyelitis, endocarditis, sepsis, meningitis, septic arthritis, and toxic shock syndrome. Its pathogenicity is associated with numerous virulence factors, including coagulase, hemolysins, leukocidins, and protein A, which facilitate immune evasion and tissue invasion (Conium et al., 2020; Linz et al., 2023; Urish et al., 2020). Owing to its increasing antimicrobial resistance, *S. aureus* has been recognized by the World Health Organization as one of the priority bacterial pathogens requiring the development of alternative therapeutic strategies (Linz et al., 2023). In addition, *S. aureus* remains a common cause of both human and animal infections and continues to pose a major global public health challenge (Majid et al., 2019).

Leukocytes are the principal cellular components of the immune system and play essential roles in innate

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and adaptive immune responses through pathogen recognition, phagocytosis, and secretion of antimicrobial mediators. Acute *S. aureus* infection stimulates inflammatory responses that alter leukocyte profiles, making total leukocyte count a useful hematological indicator of infection severity and therapeutic response. Increased leukocyte counts (leukocytosis) generally reflect active bacterial infection, whereas normalization of leukocyte levels may indicate successful bacterial clearance and resolution of inflammation (Cahyaningtyas et al., 2019; F. M. Putri et al., 2023). Therefore, evaluating leukocyte count provides an appropriate approach to assess the in vivo biological activity of antibacterial agents.

Smallanthus sonchifolius (yacon), locally referred to in Indonesia as the insulin leaf, is a medicinal plant native to the Andean region of South America. Previous studies have demonstrated that yacon leaves and tubers contain various bioactive compounds, including flavonoids, alkaloids, tannins, phenolic acids, saponins, and steroids, which possess antioxidant, antimicrobial, antifungal, and antidiabetic activities (Ramadhani et al., 2020; Rohman et al., 2021). Several studies have reported antibacterial activity of *S. sonchifolius* against pathogenic bacteria, including *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella* spp., primarily through disruption of bacterial cell walls and inhibition of bacterial growth (Rohman et al., 2021; Wicaksana et al., 2024). Moreover, the extraction method influences the recovery of phytochemical constituents, with percolation reported to yield phenolic and flavonoid compounds exhibiting antibacterial activity against *Staphylococcus* species (Putri et al., 2022).

Beyond its direct antibacterial activity, the phytochemical constituents of *S. sonchifolius* may contribute to modulation of host immune responses. Flavonoids are known to regulate inflammatory mediators and immune-cell activation, including leukocytes, thereby reducing excessive inflammatory responses during bacterial infection (Ramadhani et al., 2020). In addition, yacon contains inulin and fructooligosaccharides (FOS), which function as prebiotics that support beneficial gut microbiota and may reduce gastrointestinal disturbances commonly associated with prolonged antibiotic therapy (Lopes et al., 2021; Valliath et al., 2023). These combined antibacterial, anti-inflammatory, and immunomodulatory properties suggest that *S. sonchifolius* may reduce bacterial burden while simultaneously promoting normalization of leukocyte counts during *S. aureus* infection.

Natural products have attracted considerable attention as alternative antibacterial agents because conventional antibiotics frequently produce adverse effects, including gastrointestinal disturbances, disruption of beneficial gut microbiota, and hepatic or renal toxicity. Furthermore, the widespread emergence of antibiotic-resistant bacteria, particularly methicillin-resistant *Staphylococcus aureus* (MRSA), has become a major global health concern, highlighting the urgent need for safer and more effective therapeutic alternatives (Suryantarini et al., 2024). Although *S. sonchifolius* has shown promising antibacterial, antioxidant, anti-inflammatory, and antidiabetic properties, most available studies have focused on phytochemical characterization or in vitro antibacterial assays (Ramadhani et al., 2020; Rohman et al., 2021; Wicaksana et al., 2024). Evidence regarding its in vivo antibacterial efficacy against *S. aureus* infection and its influence on host inflammatory responses, particularly leukocyte count, remains limited.

Therefore, this study aimed to evaluate the in vivo antibacterial activity of *Smallanthus sonchifolius* leaf extract against *Staphylococcus aureus* infection by assessing total leukocyte counts in an experimental animal model. This study addresses the current knowledge gap regarding the relationship between the antibacterial activity of *S. sonchifolius* and its potential immunomodulatory effects during bacterial infection, thereby providing scientific evidence for its development as a natural therapeutic alternative against *S. aureus* infections.

Method

Research Design

This study employed a true experimental laboratory design using a pretest–posttest randomized control group design. Twenty-five male Wistar rats (Rattus norvegicus), aged 9–12 weeks and weighing 200–250 g, were used as experimental animals. Following a seven-day acclimatization period, the animals were allocated into five experimental groups, with five rats in each group (n = 5; total N = 25). The sample size was determined using Federer's formula for laboratory animal experiments, resulting in five animals per group (total N = 25). The experimental groups consisted of Group I (negative control), *Staphylococcus aureus*-infected rats receiving no treatment; Group II (positive control), *S. aureus*-infected rats treated with clindamycin; Group III, *S. aureus*-infected rats treated with *Smallanthus sonchifolius* leaf extract at 100 mg; Group IV, rats treated with 200 mg; and Group V, rats treated with 400 mg for 13 consecutive days. Total leukocyte counts were measured before bacterial induction and after treatment. The independent variable was the administration of *S. sonchifolius* leaf extract, whereas the dependent variable was the total leukocyte count in *S. aureus*-infected Wistar rats.

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Research Tools and Materials

The tools used consist of: stationery (sidu, joyko), sieve (nagata), blender (Panasonic), stirring rod (pyrex), beaker glass (pyrex), bunsen, cover glass (one lab), glass funnel (pyrex), watch glass (pyrex), measuring cup (onemed), handscoon (safe glove), hemocytometer (assistant Germany), incubator (memmert IN55), experimental animal cage, cage cover wire, filter paper (whatman), label (champion), laminar air flow (microflow), mask (sensi), micropipette (dragon lab), microscope (Olympus), analytical balance (shimadzu), ose, oven (dry box), razor (onemed), dropper pipette (onemed), rotatory evaporator (biobase), syringe (terumo), test tube, tissue (paseo), EDTA tube (onemed), waterbath (memmert).

Aquadest (onelab), 70% alcohol (onemed), 1% BaCl2, pure culture of *Staphylococcus aureus*, *Smallanthus sonchifolius* insulin leaves, Wistar rat blood, 96% ethanol (tnt chemical), 1% FeCl3 H2SO4, 2N HCl, Dragenrouff reagent, Mayer reagent, chloroform (thermo scientific), soy borth trypticase toy media (millipore), 0.9% physiological NaCl, rat feed (citra feed), Wistar rat, Turk (indo reagent).

Research Procedures

Ethics Commission Submission

This study involved male white rats (Rattus norvegicus) of the Wistar strain as experimental animals. Therefore, prior to implementation, it was submitted and approved by the Research Ethics Committee of the relevant Faculty/Institution (Manangin, 2025). Submission to the ethics committee was carried out to ensure that all stages of the study, including the procedure for inducing *Staphylococcus aureus* infection, administering insulin leaf extract, and terminating the animals, were carried out in accordance with applicable research ethics principles, especially in the treatment of experimental animals (Intan et al., 2020). This aims to protect animal welfare, minimize suffering, and ensure that the research is conducted scientifically, responsibly, and in accordance with the 3R principles (Replacement, Reduction, Refinement). The proposed research protocol included the experimental procedures, dosage regimen, route of administration, sample size justification, humane endpoints and termination procedures, as well as scientific justification for the use of experimental animals, in accordance with internationally accepted ethical principles for animal research and the 3Rs (Replacement, Reduction, and Refinement) (Mancini et al., 2022).

Acclimatization Stage and Treatment of Experimental Animals of Wistar Rats

Acclimatization is the process of adapting experimental animals to changes in environmental climate. In this study, the experimental animals were acclimatized for 7 days, kept at a temperature of approximately 24^{o}C and 55% humidity, and received adequate lighting (a 12-hour light-dark cycle). The adapted test animals were placed in individual cages with wire cage covers (Hadi et al., 2022). During acclimate, the Wistar rats were fed pellets, 20% of their body weight, and plain water. After being adapted for 7 days, the test animals were divided into 5 treatment groups, namely group 1 without treatment, only given regular feed (negative control), treatment group 2 which was induced by pure culture of *Staphylococcus aureus* and given antibiotics (positive control), treatment group 3 which was induced by *Staphylococcus aureus* and given insulin leaf extract (*Smallanthus sonchifolius*) with a low dose of 100 mg, treatment group 4 was given insulin leaf extract (*Smallanthus sonchifolius*) with a medium dose of 200 mg and treatment group 5 was given insulin leaf extract (*Smallanthus sonchifolius*) with a high dose of 400 mg.

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insulin leaf extract (*Smallanthus sonchifolius*) with a low dose of 100 mg, treatment group 4 was given insulin leaf extract (*Smallanthus sonchifolius*) with a medium dose of 200 mg and treatment group 5 was given insulin leaf extract (*Smallanthus sonchifolius*) with a high dose of 400 mg.

Induction of Staphylococcus aureus Bacteria in Wistar Rats

The experimental animals were rendered unconscious using ketamine, then disinfected using 70% alcohol in the peritoneal area. *Staphylococcus aureus* bacteria as much as 0.4 ml were induced intradermally. Wait for a 7-day incubation period, then blood was taken from the mice to see if there was an increase in leukocytes. If the results were still within normal limits, they were re-induced with pure *Staphylococcus aureus* bacteria as much as 0.4 ml and waited another 7 days. Then, an examination of the 14-day incubation period was carried out before administering the extract.

Sampling

In experimental Wistar rats, blood samples were taken through the lateral vein (tail). This was done by cutting a small section of the tail and collecting 1.5 ml or 2 ml of blood in an EDTA tube. The tail vein blood collection process has the advantage of not requiring anesthesia compared to other methods that require anesthesia.

Leukocyte Examination Using the Tube Method

Prepared the deck glass and Improved Neubaurer counting chamber in a clean and dry condition. Dilute the blood using the tube method in a Turk reagent pipette as much as 190µl into a test tube clean the outside of the pipette tip from any remaining blood that is still attached, then pipette EDTA blood as much as 10 µl for a 20-fold dilution, avoid air bubbles. Homogenize by up and down for 1 minute until the blood is homogeneous, the homogenized blood pipette in the test tube is then inserted into the counting chamber by flowing from the edge of the deck glass. Counting leukocyte cells: Count leukocyte cells under a microscope with a 40x lens magnification, count leukocytes in 4 large boxes and 16 medium boxes in the corner of the counting chamber, count leukocyte cells in a zigzag manner from the top left (Rosyidah et al., 2024).

Data Analysis

The data obtained will be statistically tested using SPSS 25 with a normality test using the Shapiro Wilk test to determine whether the data is normally distributed and continued with a homogeneity test to determine whether the data is homogeneously distributed. Then continued with the one-way ANOVA test + Turkey HSD test a purpose of making a one-way ANOVA test to determine whether there is a difference in the number of leukocytes from 5 treatment groups. The results of the analysis are declared significant if the p value <0.05 if the data is not normally distributed there is a difference meaning the data is classified as non-parametric data, so using the Wilcoxon difference test. The purpose of making the Wilcoxon difference test is to test whether there is a significant difference between the independent variable group and the dependent variable.

Result and Discussion

Based on the results of the study of the effect of administering insulin leaf extract (*Smallanthus sonchifolius*) on changes in the number of leukocytes in wistar rats infected with *Staphylococcus aureus* which was carried out in April - May 2025 with a sample of 25 male wistar rats divided into 5 treatment groups, each treatment group containing 5 rats induced by *Staphylococcus aureus* bacteria given drug therapy using *Smallanthus sonchifolius* extract. The results were obtained in the form of phytochemical tests, the initial condition of wistar rats, the results of *Staphylococcus aureus* bacterial induction, the results of the leukocyte count examination, the average results of the leukocyte count. Then the average results were analyzed using the Shapiro Wilk statistical test to carry out the Normality test followed by the homogeneity test after the data was normally and homogeneously distributed. Followed by a one-way ANOVA test using SPSS 25 to determine whether there was a difference in the number of leukocytes from the 5 treatment groups after administering *Smallanthus sonchifolius* insulin leaf extract.

Based on table 1, the results of the H0 leukocyte count examination before induction, the number of leukocytes was still within normal limits, and there was no increase in leukocytes, indicating that the Wistar rats were healthy because they had not been induced. On D-7 after induction, it showed that the number of leukocytes had increased in all groups that were given S. aureus bacteria induction. The results on D-4 after 4 days of administering insulin leaf extract showed a decrease in the number of leukocytes but were still high. The results of the examination on D-7 after 7 days of administering insulin leaf extract in some groups showed a decrease in the number of leukocytes. The results on D-10 after 10 days of administering insulin leaf extract showed a decrease in the number of leukocytes in the treatment group compared to the control group induced by *Staphyloccocus aureus* without the extract. The results on D-13 after 13 days of administering insulin leaf extract in the positive control given the antibiotic Clindamycin 300mg/250 BB.

In the *Smallanthus sonchifolius* insulin leaf extract group with concentrations of 100, 200, 400 mg, the results showed a decrease after administration of insulin leaf extract for 13 days, indicating that the extract worked effectively in reducing the inflammatory load after a 13-day administration period. In line with the research of Ramonah et al. (2017) where 15% insulin leaf ethanol extract was able to produce an inhibition zone of up to 26.5 mm which was categorized as very strong antibacterial activity. This means that the insulin leaf extract in vitro was able to inhibit the growth of S. aureus significantly and showed potential use as an alternative antibacterial agent. Inhibited bacterial growth was characterized by a decrease in the number of leukocytes after administration of insulin leaf extract (*Smallanthus sonchifolius*) as an inhibitory response to the growth of *Staphylococcus aureus*, due to the presence of bioactive compounds contained, insulin leaf extract contains secondary metabolites of alkaloids, flavonoids, tannins, saponins, steroids and triterpenoids.

Compounds have different advantages in the process of inhibiting microbial growth. The presence of alkaloid compounds in insulin leaf extract has antibacterial activity that works by disrupting the structure and function of bacterial cell membranes, inhibiting DNA and protein synthesis, and damaging microbial cell metabolism. In infections by *Staphylococcus aureus*, they contribute to suppressing bacterial growth by damaging the integrity of the gram-positive bacterial cell wall. In addition to having antibacterial activity, alkaloids also have anti-inflammatory effects that help reduce the inflammatory response due to bacterial infection by suppressing the production of pro-inflammatory cytokines. Phenolic compounds contained in insulin leaf extract can damage the cell walls of *S. aureus* bacteria by inhibiting biofilm formation and suppressing inflammation. This effect helps reduce the number of leukocytes and accelerate the healing process of infections (Grancieri et al., 2023). The antibacterial properties of active flavonoid compounds in insulin leaves can inhibit *Staphylococcus aureus* bacteria by damaging cell membranes, inhibiting metabolic enzymes, and disrupting bacterial DNA and RNA synthesis, thereby reducing the burden of infection and excessive immune responses. Research by Putri et al. (2022) gave insulin leaf extract for 10 consecutive days to experimental animals showing a significant antibacterial effect against *Staphylococcus aureus*.

The average leukocyte count in Wistar rats after administration of insulin leaf extract at concentrations of 100, 200, and 400 mg inhibited the growth of the pathogenic bacteria *Staphylococcus aureus*. The significant decrease in leukocyte counts in the treatment group indicates that insulin leaf extract not only acts as an antimicrobial agent but also has the ability to stabilize the body's immune system. This immunomodulatory activity is important in regulating the immune response to prevent excessive leukocytosis, as excessive leukocytosis can damage body tissue and exacerbate inflammation. Thus, insulin leaf extract demonstrates dual potential as a therapeutic agent to suppress infection and simultaneously control the resulting immune reaction. In a clinical context, these results are highly relevant for further development, particularly as a natural alternative or adjunct to antibiotic therapy in treating bacterial infections. In addition to demonstrating good efficacy, the use of plant extracts such as insulin leaf also reduces the risk of antimicrobial resistance, which is currently a global problem. However, further research is needed regarding dose standardization, isolation of key active compounds, and long-term safety testing for more widespread use of insulin leaf extract in evidence-based medicine (Febrianti et al., 2021).

Results of Observations on the Number of Leukocytes in Wistar Rats

This study aims to evaluate the effect of administering *Smallanthus sonchifolius* insulin leaf extract on the number of leukocytes infected with *Staphylococcus aureus*. The results of observations of the number of leukocytes in Wistar rats showed an increase in the number of leukocytes after induction of *Staphylococcus aureus* to assess the effectiveness of administering *Smallanthus sonchifolius* insulin leaf extract drug therapy which contains bioactive compounds on reducing the number of leukocytes. Measurements were carried out in stages on days 1, 4, 7, 10, and 13 after treatment. Each experimental animal was observed for its leukocyte count using an improved Neubaurer counting chamber. This data is important to see the decrease in the number of leukocytes. The following are the results of measuring the number of leukocytes (Cells/mm^3). Table 1 shows that on D-1, the results were already infected before the extract was administered. On D-4 and D-7, the results after the extract administration decreased, but some leukocyte counts remained high. On D-10 and D-13, the treatment group experienced a decrease in leukocyte counts, but normal results were no longer present, with no increase.

Based on 2 it can be seen that in the table above the negative control after observation for 13 days the results obtained were on average still quite high only experiencing a slight decrease on D-1 16,670 cells / µL to 11,100 cells / µL on the 13th day, in the positive control the average decreased on D-1 12,300 cells / µL to 3,080 cells / µL on the 13th day. For the treatment group with doses (100,200 and 400 mg) with the administration of insulin leaf extract experienced a progressive decline. Starting from KD 100 mg on D-1 14,410 cells / µL to 3,040

cells / µL on the 13th day. in the 200 mg dose treatment group the results obtained on D-1 14,680 cells / µL to 2,940 cells / µL on the 13th day. For the 400 mg dose treatment group, the largest decrease in the number of leukocytes occurred on D-1 from 15,570 cells/µL to 3,110 cells/µL on the 13th day.

Table 1. Leukocyte Count Results after Administering *Smallanthus sonchifolius* Leaf Extract

Group	Animal Samples	H-1	H-4	H-7	H-10	H-13
KN	TKN 1	18,400	14,050	13,850	9,750	8,050
	TKN 2	14,400	8,650	9,400	8,150	7,200
	TKN 3	16,450	13,050	12,450	11,950	8,400
	TKN 4	20,600	17,150	16,300	14,250	8,750
	TKN 5	13,500	9,400	8,450	7,400	6,650
KP	TKP 1	10,150	7,600	5,100	3,650	3,150
	TKP 2	13,500	8,000	5,150	4,100	3,800
	TKP 3	12,500	11,700	8,250	5,250	3,350
	TKP 4	12,250	10,350	8,100	5,000	3,000
	TKP 5	13,100	9,100	7,350	4,600	2,100
KD 100	TKD 1	12,000	9,700	4,500	3,850	3,400
	TKD 2	15,600	8,700	6,250	4,150	3,100
	TKD 3	12,100	8,100	4,200	3,000	2,850
	TKD 4	13,800	11,700	7,150	6,050	3,700
	TKD 5	18,550	8,550	4,800	3,900	2,150
KD 200	TKD 1	13,300	9,400	4,950	3,250	2,100
	TKD 2	19,200	14,000	8,350	6,200	3,550
	TKD 3	14,750	11,550	7,050	3,650	2,800
	TKD 4	13,550	8,200	6,000	4,100	3,200
	TKD 5	12,600	9,050	6,750	4,000	3,050
KD 400	TKD 1	13,800	11,000	8,750	5,100	3,300
	TKD 2	18,100	15,050	9,100	7,200	4,100
	TKD 3	14,050	9,800	6,000	4,400	2,800
	TKD 4	17,500	14,250	9,800	6,250	3,000
	TKD 5	14,400	12,100	8,550	5,550	2,350

Information: D0 = Before administering insulin leaf extract; D4 = After the 4th day of extract administration; D7 = After giving the extract on the 7th day; D10 = After giving the extract on the 10th day; D13 = After giving the extract on the 13th day.

Based on table 2, the average number of leukocytes after administration of insulin leaf extract shows that the fastest decrease is at a concentration of 400 mg, which is the maximum dose for the decrease. This shows that a high dose is effective in providing a lowering effect, indicating that the higher the dose of extract given, the greater the effect of reducing leukocytes quickly. This means that insulin leaf extract has the potential as a natural therapeutic agent against bacterial infections by suppressing the inflammatory response and accelerating the recovery of the immune system. The number of leukocytes is an important parameter in the body's immune system, which will increase when an infection or inflammation process occurs. In this study, observations were made for 13 days on five groups of Wistar rats: negative control (KN), positive control (KP), and three treatment groups given insulin leaf extract at doses of 100 mg, 200 mg, and 400 mg, respectively. Each group was observed gradually from day 1 (D-1) to day 13 (D-13), to assess the effect of the extract on the number of leukocytes after being induced with *Staphylococcus aureus*.

Table 2. Average Leukocyte Count Observations Per Day After Insulin Leaf Extract Administration

Group	H-1	H-4	H-7	H-10	H-13
KN	16,670	12,460	12,090	11,300	11,100
KP	12,300	9,350	6,790	4,520	3,080
Kel	H-1	H-4	H-7	H-10	H-13
KD 100 mg	14,410	9,350	5,380	4,190	3,040
KD 200 mg	14,680	10,440	6,620	4,240	2,940
KD 400 mg	15,570	12,440	8,440	5,700	3,110

In the negative control (KN) group, leukocyte counts decreased only slightly from 16,670 cells/µL on Day 1 to 11,100 cells/µL on Day 13, indicating the persistence of inflammatory responses in untreated animals. Because these animals did not receive antibacterial or anti-inflammatory treatment, the

relatively high leukocyte counts throughout the observation period suggest that the inflammatory stimulus induced by *Staphylococcus aureus* infection was not adequately resolved. Leukocytosis is a well-recognized hematological response to acute bacterial infection, reflecting activation of the innate immune system and increased production of circulating leukocytes in response to inflammatory mediators (Cahyaningtyas et al., 2019; Putri et al., 2023).

In contrast, animals treated with *Smallanthus sonchifolius* leaf extract exhibited a progressive decline in leukocyte counts throughout the treatment period. The reduction was observed in all extract-treated groups (100, 200, and 400 mg/kg body weight), indicating that administration of the extract contributed to the resolution of infection-associated inflammation. The gradual normalization of leukocyte counts is consistent with previous reports demonstrating that *S. sonchifolius* possesses antibacterial and anti-inflammatory activities attributable to its abundant flavonoids, phenolic compounds, tannins, saponins, and sesquiterpene lactones (Lopes et al., 2021; Ramadhani et al., 2020; Rohman et al., 2021).

The 100 mg/kg treatment group showed a marked reduction in leukocyte counts from 14,410 to 3,040 cells/µL, whereas the 200 mg/kg group exhibited the lowest final leukocyte count (2,940 cells/µL). Although the 400 mg/kg group also produced substantial leukocyte reduction, the differences among extract-treated groups were relatively small by the end of the observation period. These findings suggest that the antibacterial and anti-inflammatory effects of *S. sonchifolius* are dose-dependent, although increasing the dose beyond 200 mg/kg may not proportionally enhance therapeutic efficacy. Similar dose-dependent antibacterial activity has been reported for *S. sonchifolius* leaf extracts against *Staphylococcus aureus*, where higher concentrations produced larger inhibition zones because of increased concentrations of bioactive phytochemicals (Putri et al., 2022; Wicaksana et al., 2024).

The observed reduction in leukocyte counts may be explained by the synergistic biological activities of the phytochemical constituents present in insulin leaves. Flavonoids have been widely reported to inhibit the production of pro-inflammatory cytokines and suppress activation of the NF-κB signaling pathway, thereby reducing excessive inflammatory responses. Phenolic compounds also exhibit antioxidant activity that limits oxidative stress during infection, whereas saponins and triterpenoids contribute to antimicrobial defense and modulation of inflammatory responses (Grancieri et al., 2023; Lopes et al., 2021; Valliath et al., 2023). Collectively, these mechanisms may reduce bacterial burden, attenuate inflammation, and facilitate the normalization of leukocyte counts.

Overall, the present findings indicate that *Smallanthus sonchifolius* leaf extract possesses promising antibacterial and anti-inflammatory potential in experimental *Staphylococcus aureus* infection. The progressive decline in leukocyte counts observed during treatment suggests that the extract may contribute to controlling infection-associated inflammation while supporting restoration of immune homeostasis. Nevertheless, because this study evaluated only total leukocyte counts, further investigations involving inflammatory biomarkers, cytokine profiles, oxidative stress markers, and bacterial load are required to clarify the precise immunomodulatory mechanisms underlying the therapeutic effects of *S. sonchifolius* (Grancieri et al., 2023; Ramadhani et al., 2020; Wicaksana et al., 2024).

Decrease in Leukocyte Count in Wistar Rats from 1 to 13 Days

The reduction in leukocyte count in this study was measured on the first day before treatment of mice in an active, healthy state and not infected with bacteria and on the 13th day after treatment with *Smallanthus sonchifolius* insulin leaf extract to assess the effectiveness of reducing leukocyte counts. The initial leukocyte count in all treatment groups averaged 4,000 cells/µL. After 13 days of drug therapy, the average leukocyte count of Wistar rats in each treatment group was measured. The calculation of the decrease in leukocyte count from day 1 to day 13 in each treatment group was to see which dose group had the ability to reduce the leukocyte count the most.

Figure 1. Leukocyte decrease graph H-1 to H-13

The graph above shows the results of observations of the number of leukocytes in Wistar rats from day 1 (D-1) to day 13 (D-13), after induction of *Staphylococcus aureus* bacteria and administration of *Smallanthus sonchifolius* leaf extract (insulin leaves) at various doses. The negative control group (KN) experienced a slow decrease in the number of leukocytes, from 16,670 cells/µL to 11,100 cells/µL, indicating that no special treatment was given. In contrast, the positive control

(KP) and all treatment groups (KD 100 mg, KD 200 mg, and KD 400 mg) showed a much more significant decrease. The KD 100 mg and KD 200 mg groups showed the most optimal decrease in leukocytes to reach around 3,000 cells/µL on day 13, indicating the high effectiveness of insulin leaf extract as an immunomodulatory agent. Meanwhile, the 400 mg KD also experienced a decrease, but the final result was slightly higher than the 100 mg and 200 mg doses, possibly due to the body's response to the high dose. The decreasing line on the graph shows that the insulin leaf extract was able to reduce the number of leukocytes gradually and significantly, especially after the 4th to 13th day, with the best results at the 100 mg and 200 mg doses.

Figure 1 shows changes in leukocyte counts in Wistar rats from day 1 (D-1) to day 13 (D-13) after induction of *Staphylococcus aureus* infection and administration of insulin leaf extract (*Smallanthus sonchifolius*) at various doses. The graph consists of two bars depicting the initial and final leukocyte counts, and one line indicating the magnitude of the decrease in leukocyte counts in each group. In the D-1 group (negative control), the leukocyte count decreased but not significantly, reflecting a normal immune response without any treatment or administration of the extract. An increase in leukocyte counts was evident on D-4 (positive control), which was induced by S. aureus without administration of the extract, indicating an acute inflammatory response from the immune system to the bacterial infection.

Meanwhile, in the D-7, D-10, and D-13 groups treated with *Smallanthus sonchifolius* leaf extract, the graph demonstrates a progressive decrease in leukocyte counts with increasing treatment duration and extract dosage. The greatest reduction was observed on Day 13, indicating that prolonged administration of the extract enhanced its effectiveness in reducing leukocytosis associated with *Staphylococcus aureus* infection. This finding is consistent with previous reports showing that *S. sonchifolius* leaves possess anti-inflammatory and antioxidant activities through their high content of phenolic compounds, which suppress inflammatory responses and oxidative stress (R. Widowati et al., 2022). The reduction in leukocyte counts suggests that insulin leaf extract exerts both anti-inflammatory and immunomodulatory effects by regulating excessive leukocyte recruitment and inflammatory mediator production during bacterial infection.

Flavonoids, polyphenols, and saponins contained in *S. sonchifolius* are known to inhibit inflammatory signaling pathways, including NF-κB activation and the production of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, while simultaneously promoting immune homeostasis (Gonfa et al., 2023). Therefore, the gradual normalization of leukocyte counts observed in this study may reflect not only suppression of bacterial growth but also restoration of the host immune balance through the synergistic action of these phytochemical constituents. In addition, yacon (*S. sonchifolius*) leaves are recognized as rich sources of phenolic acids, flavonoids, tannins, saponins, and sesquiterpene lactones, which collectively contribute to their antibacterial, antioxidant, anti-inflammatory, and immunostimulatory properties. Overall, this graph confirms that the administration of insulin leaf extract is able to reduce systemic inflammation characterized by a decrease in the number of leukocytes, especially in the high-dose treatment group and longer administration time (Putri et al., 2022).

Results of the Effect of Leukocyte Count After Administration of Insulin Leaf Extract

The One-Way ANOVA test is a parametric test used to determine whether there is a difference in the average between three or more groups. This test is used if the data is normally distributed and the variance between groups is homogeneous. In this study, One-Way ANOVA helps determine whether the administration of *Smallanthus sonchifolius* insulin leaf extract has a significant effect on reducing the number of leukocytes. If the ANOVA results show a significance value (p-<0.05), there is a real difference between the treatment groups. The results of the One-Way ANOVA test are presented in the table 3.

Table 3. One-way ANOVA Test Results

Leukocyte count

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	2420.416	4	605.104	6.279	.002

One-Way Analysis of Variance (ANOVA) was used in this study to determine whether there was a statistically significant difference in the mean leukocyte counts among the treatment groups of Wistar rats infected with *Staphylococcus aureus* and treated with different doses of *Smallanthus sonchifolius* leaf extract. ANOVA is an appropriate statistical method for comparing the means of more than two independent groups using a single continuous dependent variable, provided that the assumptions of normality and homogeneity of variance are satisfied (Mishra et al., 2019). Therefore, before conducting the ANOVA, the

data were tested for normality using the Shapiro–Wilk test and for homogeneity of variance using Levene's test.

Based on Table 3, the One-Way ANOVA revealed a statistically significant difference among the treatment groups ($F = 6.279$; $p = 0.002$). Since the significance value was less than 0.05, the null hypothesis was rejected, indicating that administration of *Smallanthus sonchifolius* leaf extract at different doses produced significantly different effects on leukocyte counts. The observed reduction in leukocyte counts suggests that the extract attenuated the inflammatory response induced by *Staphylococcus aureus* infection. During bacterial infection, leukocytosis occurs as part of the innate immune response, whereas normalization of leukocyte counts reflects resolution of inflammation and effective control of bacterial burden (Parker et al., 2023; Putri et al., 2023). The anti-inflammatory effect observed in this study is likely associated with the presence of flavonoids, phenolic compounds, saponins, tannins, and sesquiterpene lactones in *S. sonchifolius*, which have been reported to suppress NF-κB activation and reduce the production of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, thereby limiting excessive leukocyte recruitment and restoring immune homeostasis (Shen et al., 2024; W. Widowati et al., 2023).

Inhibitory Test of Insulin Leaf Extract on the Growth of Staphylococcus aureus

The inhibitory power of insulin leaf extract (*Smallanthus sonchifolius*) against the growth of Staphylococcus aureus was tested using the well diffusion method with extract concentrations varying from 25%, 50%, and 75%. Clindamycin was used as the K (+), and 1% DMSO was used as the K (-). The results of the inhibition zone diameter measurements for each treatment can be seen in Table 4. Antibacterial inhibition testing in this study was conducted with five repetitions in each treatment group based on calculations using the Federer formula. The treatment groups consisted of three variations in insulin leaf extract concentration: 25%, 50%, and 75%, as well as two control groups: a positive control using 0.03% clindamycin and a negative control using 1% DMSO. The use of controls in antibacterial inhibition testing is necessary to compare the effectiveness of the test compounds on bacterial growth. Clindamycin was chosen as a positive control because it has been known to be effective in inhibiting the growth of various types of gram-positive bacteria, including *Staphylococcus aureus.* Thus, clindamycin provides a benchmark for the extent to which the tested extract inhibits bacterial growth (Fitriani et al., 2021).

1% DMSO was used as a solvent because it is able to dissolve non-polar and semi-polar extracts well. Insulin leaf extract is known to be non-polar to semi-polar, so it will not dissolve completely in polar solvents such as distilled water. A 1% DMSO solution was also used as the negative control to verify that the solvent itself did not contribute to the observed antibacterial activity, thereby ensuring that any inhibition zone resulted solely from the bioactive compounds present in the *Smallanthus sonchifolius* leaf extract rather than from the solvent or other experimental factors (Balouiri et al., 2016). Pure isolates of *Staphylococcus aureus* in this study were subjected to macroscopic and microscopic observations first to ensure the authenticity of the test bacteria shown in Table 4. The test bacteria were then rejuvenated with the aim of reactivating the bacterial cells to be in optimal growth conditions, so that the test results were more accurate (Kebbouche-Gana et al., 2013).

Table 4. Inhibitory Test of Insulin Leaf Extract on the Growth of *Staphylococcus aureus*

Treatment	Inhibition Zone Diameter (mm)					Mean	Inhibitory Activity Category
	1	2	3	4	5		
K (-)	0	0	0	0	0	0	There isn't any
K (+)	29.5	29.5	29.7	29.5	29.7	29.58	Very strong
25%	20.4	20.5	20.3	20.5	20.4	20.42	Strong
50%	22.7	22.5	22.6	22.6	22.7	22.62	Very strong
75%	24.9	25.0	24.8	24.9	24.9	24.9	Very strong

The criteria for the strength of bacterial growth inhibition can be grouped into four categories, namely the diameter of the inhibition zone (<5 mm) is categorized as weak, (5-10 mm) is categorized as moderate, (10-20) mm is categorized as strong and (20-30 mm) is categorized as very strong (Handayani et al., 2023). Based on the data presented in Table 4, inhibition zones with varying diameters were obtained according to the concentration of the extract used. In the negative control (K−) with 1% DMSO, no inhibition zone was found (average diameter 0 mm), which indicates that 1% DMSO has no inhibitory power against bacteria, in contrast to the positive control (K+) with 0.03% clindamycin, the inhibition zone diameter was 29.58 mm on average and was included in the very strong inhibition category. At a concentration of 25%, the average diameter of the inhibition zone was 20.42 mm with a strong inhibition category, while at concentrations of 50% and 75%, the average diameter of the inhibition zone was 22.62 mm and 24.9 mm,

respectively, both concentrations were included in the very strong inhibition category.

The formation of an inhibition zone in media inoculated with *Staphylococcus aureus* indicates that insulin leaf extract (*Smallanthus sonchifolius*) has the ability to inhibit bacterial growth. This inhibitory power is closely related to the content of active compounds in insulin leaf extract, namely alkaloids, quinones, steroids, and triterpenoids, each of which has a mechanism of action in inhibiting bacterial growth. Alkaloids work by disrupting the peptidoglycan components in bacterial cells. The peptidoglycan layer provides structural rigidity and protects bacterial cells from osmotic lysis in hypotonic environments. Disruption of this layer weakens the cell wall, leading to loss of mechanical strength, osmotic instability, cell swelling, and ultimately bacterial cell death (Silhavy et al., 2014; Vollmer et al., 2008). Quinones are organic compounds known to have redox properties, namely they can undergo reduction and oxidation reactions. Flavonoids and other polyphenolic compounds exert their biological effects through redox-related electron transfer reactions and interactions with intracellular proteins, enzymes, and signaling molecules, leading to modulation of oxidative stress and inflammatory responses (Yang et al., 2025). Steroids and triterpenoids, as antibacterials, interact with lipid membranes and exhibit sensitivity, which can cause leakage in liposomes, resulting in decreased membrane integrity and altered cell morphology, leading to fragility and lysis (Ramonah et al., 2017). These four compounds work synergistically to disrupt the bacterial defense and metabolism, inhibiting the growth of *Staphylococcus aureus.*

Clindamycin as a positive control showed a larger inhibition zone compared to insulin leaf extract as a natural antibacterial, this is due to the specific mechanism of action of clindamycin, which inhibits bacterial protein synthesis by binding to the 50S ribosomal subunit, in contrast to insulin leaf extract as a natural antibacterial that works non-specifically by disrupting cell wall integrity, damaging cell membranes, and inhibiting enzyme activity and bacterial metabolic processes through the action of various active compounds such as alkaloids, quinones, steroids, and triterpenoids (Ramonah et al., 2017). The inhibition zone indicated by the highest concentration (75%) produced the widest inhibition zone, although the inhibition power formed did not exceed the positive control of clindamycin as a standard antibiotic, but these results indicate that insulin leaves have the potential to be further developed as a natural antibacterial alternative, both as a supportive therapy treatment and a preventive measure against bacterial infections, especially those caused by *Staphylococcus aureus* amidst increasing resistance to the use of synthetic antibiotics.

Based on the results of the ANOVA test on the diameter of the inhibition zone at various extract concentrations (25%, 50%, and 75%), a significance value (p-value) of 0.000 ($p < 0.05$) was obtained. This indicates that there is a significant difference between treatment groups in the diameter of the bacterial inhibition zone. The mean square value between groups is 25.091, while the mean square within the group is only 0.006. This difference in value indicates that the variation between groups is greater than the variation within the group, so that the effect of treatment (extract concentration) on the diameter of the inhibition zone is real and not caused by random variation.

Conclusion

The present study demonstrated that insulin leaf (*Smallanthus sonchifolius*) extract possesses significant antibacterial and immunomodulatory activities against *Staphylococcus aureus* infection. Administration of the extract effectively reduced leukocyte counts in Wistar rats, indicating its ability to regulate inflammatory responses and support immune system recovery. Among the tested doses, the 400 mg treatment exhibited the most stable and effective reduction in leukocyte levels. Furthermore, the extract showed strong antibacterial activity, with the highest inhibition zone observed at the 75% concentration. Statistical analysis confirmed significant differences among treatment groups, supporting the therapeutic efficacy of insulin leaf extract. Therefore, *Smallanthus sonchifolius* has promising potential as a natural antibacterial and immunomodulatory agent and may serve as an alternative or complementary therapy for managing bacterial infections and reducing dependence on conventional antibiotics.

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