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Original Research Article

Antibacterial and Antibiofilm Activities of Ternate Blue Pea (*Clitoria ternatea*) Flower Extract against *Staphylococcus aureus*

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ABSTRACT

The Ternate Blue Pea, renowned for its antibacterial properties and native to the northern Maluku islands, has been a subject of scientific investigation. Despite previous studies on this plant, its potential antibiofilm activity against *Staphylococcus aureus* (SA) has remained unexplored. This research aimed to assess the efficacy of Ternate Blue Pea flower extract (TBPE) in inhibiting and eradicating SA biofilm activity. Antibiofilm activity was evaluated using the microtiter broth method, with the minimum biofilm inhibitory concentration ($MBIC_{50}$) and minimum biofilm eradication concentration ($MBEC_{50}$) values serving as determinants. The antibiofilm mechanism was elucidated through scanning electron microscopy (SEM). Statistical analysis was conducted using ANOVA ($p<0.05$). Results revealed that TBPE inhibited SA growth by 85.20% ± 0.01, slightly lower than the control activity of Vancomycin at 88.00% ± 0.01. In the antibiofilm test, TBPE exhibited significant efficacy in inhibiting biofilm during the mid-phase (81.40% ± 0.01) and preventing biofilm formation in the maturation phase (78.14% ± 0.01) of biofilm, whereas Vancomycin demonstrated slightly higher inhibitory activity in both the mid-phase (85.40% ± 0.01) and maturation phase (82.00% ± 0.01) of biofilm. TBPE disrupted the SA biofilm formation by 74.44% ± 0.01, while Vancomycin was marginally more effective with the disruption value of 77.00% ± 0.01. SEM analysis confirmed that TBPE could inhibit and breakdown SA biofilm by damaged the extracellular polymer (EPS) matrix in biofilms. In conclusion, TBPE exhibits promising antibiofilm activity against SA.

***Keywords*:** Antibacterial, Antibiofilm, Telang Ternate, *Staphylococcus aureus*, Periodontal Disease

Introduction

Periodontal disease (PD) is a prevalent and persistent inflammatory condition impacting the periodontium, comprising the gingiva, periodontal ligament, and alveolar bone.[1,2] PD, a non-communicable disease, poses a substantial global health challenge, with severe manifestations affecting approximately 10% of the global adult population.[3] If left untreated, PD can result in irreversible damage to the periodontal attachment, alveolar bone loss, tooth mobility, and eventual tooth loss.[4] The etiology of periodontitis involves the overgrowth of oral bacteria, with around 600-700 identified species contributing to tissue damage, inflammation, and potential tooth loss if not promptly addressed.[5-7]

Among the myriad oral bacteria, *Staphylococcus aureus* (SA) is naturally present in the oral microbiota.[8,9] Research indicates that this particular species is often found in the biological flora of teeth, forming biofilm formations, particularly in dysbiotic flora associated with PD and peri-implantitis.[10]

Moreover, various studies highlight the common isolation of SA from conditions such as angular cheilitis, jaw osteomyelitis, parotitis, endodontic infections, and mucositis.[11] These findings underscore the potential role of the oral disease-associated microbiota as a reservoir for diverse clinically significant pathogens, including multidrug-resistant *Staphylococcus*.[12]

Eliminating biofilms poses a considerable challenge due to the protective polysaccharide matrix that enhances antimicrobial resistance, impeding the penetration of drugs. This difficulty is particularly pronounced in combating dental plaque biofilm, a persistent issue linked to potential tooth loss.[13,14] The formidable nature of biofilms necessitates antibiotic doses up to 1000 times higher than those effective against suspended bacteria.[15] In the United States, biofilms are a predominant cause of microbial infections, contributing to 80% of cases. Within the oral environment, biofilms serve as a conducive habitat for bacteria, such as *Staphylococcus aureus* (SA), fostering their growth and providing a shield against external threats. Moreover, biofilms facilitate bacterial attachment and invasion of host tissues, resulting in various deleterious effects. Addressing infections stemming from biofilms emerges as a substantial challenge.[16,17] The resilience of oral biofilms, encapsulated within a polysaccharide matrix, further complicates their elimination. The exploration of novel drugs is imperative in this context.[18] This study specifically investigates the

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efficacy of telang ternate extract against SA biofilm, a pivotal factor in the development of Periodontal disease.

Indonesia, celebrated for its extraordinary plant diversity, second only to Brazil, boasts a documented array of 20,000 plant species. Despite this wealth, a substantial fraction of Indonesia's plant resources remains undiscovered, and a mere 300 species find application in traditional medicine. One standout among these is *Clitoria ternatea*, a member of the *Fabaceae* family, recognized as a highly esteemed medicinal plant in Ternate island in North Maluku Indonesia. This annual *herbaceous* plant is distinguished by its captivating deep blue flowers, with both the flowers and leaves serving as commonly utilized medicinal components.[19] *Clitoria ternatea* (Ternate Blue Pea) plant demonstrate efficacy in addressing various conditions, including red eyes, fatigue, throat and skin ailments, urinary tract disorders, and toxins. Pounded leaves of the telang flower are employed in treating festering wounds, while when boiled and combined with other plants, it proves beneficial in addressing vaginal discharge, as documented by Febrianti in 2022.[20] Febrianti et al. (2022) observed that ternate Blue Pea plant extract (TBPE) is rich in secondary metabolites such as flavonoids, phenols, saponins, alkaloids, and tannins. These compounds confer antibacterial properties and offer additional benefits, including antifungal, antioxidant, anticancer, analgesic, and wound healing effects.[20] The antibacterial potential of the Ternate Blue Pea plant prompts further exploration, particularly in the context of the native Ternate island in North Maluku plants, specifically as an antibacterial and antibiofilm agent. While research on the Ternate Blue Pea bioactivity is extensive, there remains a significant gap, particularly in its role as an antibacterial and antibiofilm agent against *Staphylococcus aureus* (SA), one of a causative agent of periodontal disease (PD). Recognizing the vast potential of the Ternate Blue Pea flower, this study aims to investigate its antibacterial and antibiofilm activity against SA biofilm during the mid-phase, maturation, and degradation stages of biofilm formation.

Materials and Methods

Materials

Laminar Air Flow, Ternate Blue Pea flower, Standard biofilm-forming *Staphylococcus aureus* ATCC 25923, Vancomycin, DMSO 1%, NaCl, McFarland 0.5 standard, sterile aquadest, Brain-heart Infusion (BHI) (Merck, Germany), Mueller-Hinton Broth liquid medium (Merck, Germany), PBS (Phosphate Buffer Saline) solution, crystal violet 1% (Merck, Germany), incubator (IF-2B) (Sakura, Japan), micropipette pipetman (Gilson, France), microplate flat-bottom polystyrene 96 well (Iwaki, Japan), microtiter plate reader (Optic Ivymen System 2100-C, Spain), autoclave (Sakura, Japan), Scanning Electron Microscopy, and analytic scales (AB204 -5, Switzerland), multichannel micropipette (Socorex, Swiss).

Plant Collection and Preparation

The plant materials were obtained from local areas in Dufa-Dufa, Ternate, Indonesia. *Clitoria ternatea* were collected in June 2023. Plant determination was carried out by Prof. Dr. Ir Paulus Matius, at the Mulawarman Herbarium, Laboratory of Tropical Forest Ecology and Biodiversity Conservation, Faculty of Forestry, Mulawarman University with voucher number: 127/UN17.4.08/LL/2023. The Ternate Blue Pea plants were washed and drained. The kernel parts were separated from the shell, cut, dried in an oven (40^{o}C for 6-8 hours), and blended.

Extraction of Plant material

The powdered plant material (750 g) was macerated in 5.000 mL n-hexane, ethyl-acetate, ethanol, and water for 24 hours each, filtered, and to dryness. This process was repeated with the dregs and the next solvent in sequence.[21]

Preparation of Bacteria Test

The SA bacteria was cultured in a BHI medium for a day at 37°C. The bacterial density was verified using a spectrophotometer and adjusted to the McFarland 0.1 standard. If the density did not meet the standard range of 0.5-1.5×10^{8} CFU/mL, the media was diluted accordingly. After completing the standard, the bacteria were ready for testing.[22]

Antibacterial Activity Screening Test Of Telang Ternate Extract

Test bacteria were grown on BHI Medium, incubated (37^{o}C for 24 hours), pre-cultured in Mueller-Hinton Broth liquid medium, and re-incubated at 37^{o}C for 24 hours. Turbidity density was adjusted to Mc-Farland standard. The positive control used was vancomycin.[22]

Inhibition Test of Biofilm Formation Mid-Phase (24 H) And Maturation Phase (48 H) Using The Microbroth Dilution Method

To examine the biofilm inhibition activity of the extract tested, the microtiter plates were washed with distilled water to eliminate non-adherent cells, followed by air drying at room temperature for 5 minutes. Subsequently, a 125 µL of 1% crystal violet solution was added into each well to stain the living and dead cells, and any other biofilm constituents. After incubation at room temperature for 15 minutes, the plate then rinsed with running water to remove the purple colour. As much as 200 µL of 96% ethanol were added to each wells and the Optical Density (OD) was measured using a microplate reader at a wavelength of 595 nm.

The OD results are then used to calculate the percentage inhibition in the following equation:

$$\frac{(Average\ OD\ of\ negative\ control - Average\ OD\ of\ sample)}{Average\ OD\ of\ negative\ control} \times 100\%$$

The concentration that can inhibit at least 50% of biofilm formation is considered Minimum biofilm inhibitory concentration ($MBIC_{50}$).[23]

Biofilm Eradication Test

The method for eliminating biofilm is comparable to that of preventing the formation of biofilm, but it takes more time. Biofilm degradation takes five days, whereas biofilm inhibition usually takes one to two days, depending on the desired level of inhibition. In this procedure, a microtiter plate was loaded with the biofilm, followed by an incubation period of 48 hours at 37°C. To remove non-adherent cells, the plates undergo a thorough cleansing three times with sterile distilled water. Subsequently, each well then filled with a medium containing various concentrations of TBPE, and the mixture undergoes another 48-hour incubation at 37°C. As a positive control, 1% w/v vancomycin was employed. Post-incubation, adhering cells were eliminated through washes with sterile PBS. The biofilm eradication rate was quantified by adding 125 µL of 1% crystal violet solution to each well, allowing it to stand at room temperature for 15 minutes. Following a PBS wash of the microplate, the formed biofilm in each well was dissolved by adding 200 µL of 96% ethanol. Optical density (OD) readings were then acquired at a wavelength of 595 nm using a microplate reader.[24]

Scanning Electron Microscopy (SEM) Observation

The TBPE with bacterial test suspension was placed in a microtiter plate with coverslips and incubated at 37°C for 24-48 hours to perform biofilm. The coverslip was washed with sterile distilled water and fixed with glutaraldehyde. Methanol was used to reduce water and the test was observed using SEM with a voltage of 10 kV.[25]

Data Analysis

Data from the study was subjected to statistical analysis using ANOVA normality test, which utilized the Shapiro-Wilk method. The normality level for the test was $p < 0.05$, and the data were evaluated with the Statistical Package for The Social Sciences (SPSS) (version 20).

Results and Discussion

Antibacterial Effect Of Telang Ternate of TBPE Extract Against Staphylococcus aureus

This study found that telang ternate extract at a concentration of 1% w/v effectively inhibited SA growth, with %inhibition of 85.20% ± 0.01 which was not significantly different ($p>0.05$) from drug control with %inhibition of 88.00% ± 0.01, indicating that the antibacterial activity of telang ternate extract against SA growth was able to match the antibacterial activity of drug control. Figure 1 shows that the effectiveness of the extract depends on its concentration, with higher concentrations resulting in greater inhibition of SA growth.

This study revealed that TBPE, at a concentration of 1% w/v, effectively impeded the growth of *Staphylococcus aureus* (SA), demonstrating a %

inhibition of 85.20% ± 0.01. Importantly, this inhibition was not significantly different ($p>0.05$) from the drug control at a concentration of 1% w/v, which exhibited a % inhibition of 88.00% ± 0.01. This suggests that the antibacterial efficacy of TBPE against SA growth is comparable to that of the drug control. Figure 1 illustrates a concentration-dependent effectiveness of the extract, indicating that higher concentrations lead to greater inhibition of SA growth.

The antibacterial activity of TBPE against SA is intricately linked to the presence of secondary metabolite compounds, including alkaloids, flavonoids, saponins, terpenoids, tannins, steroids, and anthocyanins. Alkaloids, as demonstrated,[26] predominantly exhibit antibacterial activity through cell wall depolarization, interaction with bacterial DNA, and inhibition of mRNA transcription.[26] Similarly, the activity of flavonoids, as described by Shamsudin et al. (2022), involves the suppression of nucleic acid synthesis, cytoplasmic membrane function, and energy metabolism, resulting in the inhibition of bacterial growth.[27] The mechanism of antibacterial action of saponins, as explained by Tagousop et al. (2021), is rooted in the disruption of the cell membrane, leading to the formation of pores that cause leakage of cell proteins and enzymes.[28] This is further supported by Ashraf *et al.*[29], who stated that phytochemical compounds can either directly eliminate bacteria or collaborate with conventional antibiotics to restrain factors that contribute to bacterial resistance. Alternatively, these compounds inhibit the molecular targets crucial for bacterial growth and division.[29]

Inhibitory Activity of TBPE Against Mid-Phase (24 hours) Staphylococcus aureus Biofilms

The extract derived from the Ternate Blue Pea flower, at a concentration of 1% w/v, exhibited robust antibiofilm activity against *Staphylococcus aureus* (SA) during the mid-phase biofilm, showcasing a % inhibition of 81.40% ± 0.01. Notably, this inhibition was not significantly different ($p>0.05$) from the drug control at a concentration of 1% w/v, which demonstrated a % inhibition of 85.40% ± 0.01, as depicted in Figure 2. Additionally, the telang ternate extract, even at the lowest concentration of 0.125%, demonstrated a substantial inhibition of 65.30% ± 0.01.

These findings suggest that inhibiting bacteria in biofilm formation is more challenging, as the % inhibition decreases when compared to bacteria in planktonic form. TBPE exhibited a robust inhibition of bacteria in the planktonic form by 85.20% ± 0.01, whereas the inhibition was slightly lower at 81.40% ± 0.01 for bacteria that had formed biofilms during the mid-phase.

This observation aligns with the assertion of Sharma et al. (2019), which highlights that microbial cells in biofilm form tend to develop antibiotic resistance compared to their planktonic counterparts.[30] The resistance is attributed to the presence of extracellular polymeric substances (EPS), which enhance protection against antimicrobial agents, thus increasing drug tolerance. EPS acts as both a physical barrier and a diffusion hindrance to various antimicrobial agents, thereby restricting drug access to the deeper layers of the biofilm.[31]

Inhibitory Activity of TBPE Against Maturation Phase (48 hours) Staphylococcus aureus biofilms

During this phase, there was a notable decline in the inhibitory efficacy of TBPE against Staphylococcus aureus (SA) biofilms compared to the planktonic form and mid-phase biofilm. This decline can be attributed to the complete formation of SA biofilms during this stage, resulting in heightened protection. The increased production of extracellular polymeric substances (EPS) in this biofilm stage acted as a formidable shield and nutrient source for the sustained survival of SA biofilms. Consequently, the antibiofilm activity diminished in comparison to the mid-stage biofilm and the planktonic form.[32]

At a concentration of 1% w/v, TBPE showed a 78.14% ± 0.01 inhibitory activity during the maturation phase biofilm. Importantly, this outcome did not exhibit a significant difference ($p>0.05$) from the inhibitory activity of the drug control, which displayed a superior inhibitory activity of 82.00% ± 0.01.

These findings substantiate the notion that as biofilm growth time prolongs, an increase in matrix arrays occurs, resulting in a stronger and more intricate biofilm structure. This structural complexity, in turn, diminishes the effectiveness of both the test compound and drug control in inhibiting biofilms. Hamzah (2018) corroborates these observations by asserting that antimicrobial agents face greater challenges in penetrating biofilm defenses during the maturation phase.[33]

Staphylococcus aureus Biofilm Eradication Activity from TBPE

The efficacy of TBPE in eradicating *Staphylococcus aureus* (SA) biofilm was assessed, revealing that at concentration of 1% w/v TBPE could inhibited biofilm formation by 74.44% ± 0.01.

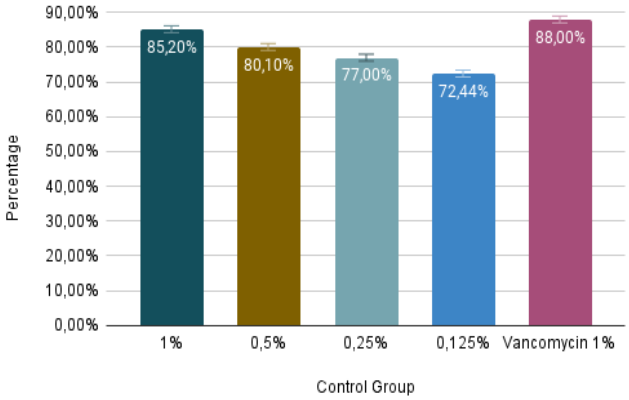

Figure 1: Antibacterial Activity Of Telang Ternate Extract Against *Staphylococcus aureus*

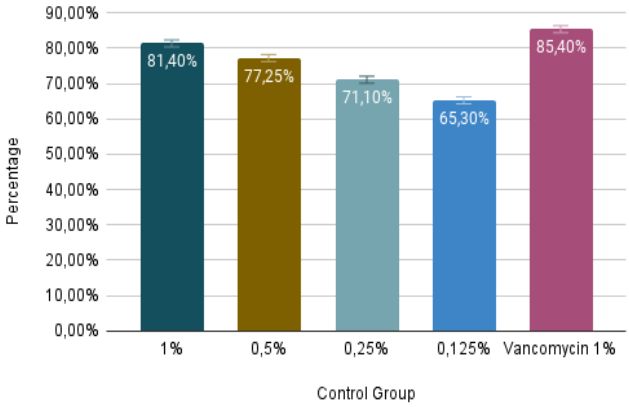

Figure 2: Percentage of Inhibitory Activity Of Telang Ternate Extract Against Mid-Phase (24 hours) *Staphylococcus aureus* Biofilms

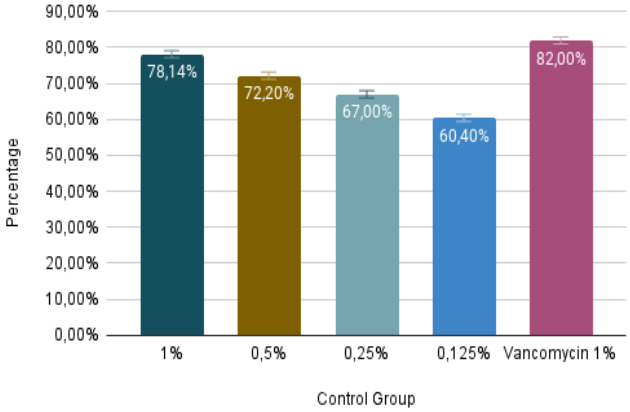

Figure 3: Percentage Inhibitory Activity of Telang Ternate Extract Against Maturation Phase (48 Hours) *Staphylococcus aureus* Biofilms

As depicted in Figure 4, the drug control exhibited a slightly higher effectiveness than the TBPE at the tested concentration, achieving a reduction of SA biofilm by 77.00% ± 0.01.

During this biofilm stage, there was a noticeable decrease in the inhibitory activity compared to the mid-stage of biofilm (81.40 ± 0.01) and the maturation stage (78.14% ± 0.03). The prolonged development of the biofilm during this stage led to the formation of more intricate and stable structures in the *Staphylococcus aureus* (SA) biofilm. This increased complexity prompted a higher production of extracellular polymeric substances (EPS) designed to shield SA, thereby playing a role in the noted reduction of inhibitory activity during the degradation phase. Biofilm microbes in the degradation phase established structured cell communication and synergized with one another to form highly complete and thick compositions of EPS and nutrients. The dense EPS matrix observed in the microbial biofilm slime within the 96-well microplate during the degradation phase further indicates the formidable defense against antimicrobial compounds. Consequently, destroying biofilms in this phase becomes considerably challenging compared to other phases. This outcome aligns with Pratiwi's statement (2020) that the biofilm degradation phase, being the longest stage of biofilm formation, is distinguished by the formation of a more extensive and complex EPS structure, fortifying SA's defense against antimicrobial agents and antibiofilms.[34]

Scanning Electron Microscopy (SEM) Observation

SEM analysis, as depicted in Figure 5, demonstrated that TBPE effectively hinders and disintegrates the *Staphylococcus aureus* (SA) biofilm (Figure 5b). Additionally, the SEM observation indicated that untreated SA exhibited a densely populated cellular structure within the extracellular polymeric substances (EPS) matrix, providing a protective environment for the organism (Figure 5a).

The SEM analysis unveiled that untreated biofilm possesses a densely packed structure with high cell density and the capacity to produce EPS. Biofilms are formed through the cooperative efforts of various bacterial species, resulting in the development of robust physical and physiological structures. Biofilms treated with telang ternate extract at a concentration of 1% w/v exhibited cell lysis, damage to the EPS matrix, and contraction. This phenomenon may be associated with the presence of secondary metabolites that enhance cell membrane permeability and disrupt mature biofilms. Increased membrane permeability enables antibacterial components to penetrate cells, leading to cellular damage.[35,36]

Conclusion

In conclusion, this study evaluated the inhibitory activity of Ternate Blue Pea extract against the formation of *Staphylococcus aureus* biofilm. The extract demonstrated robust inhibitory effects during the mid-phase of *Staphylococcus aureus* biofilm formation. However, a decline in inhibitory activity was observed during the maturation and degradation phases. Subsequent investigations should focus on elucidating the extract's mechanism of action, exploring its effectiveness across various biofilm phases, and conducting dose-response studies to optimize its potential as an antibiofilm agent.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

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

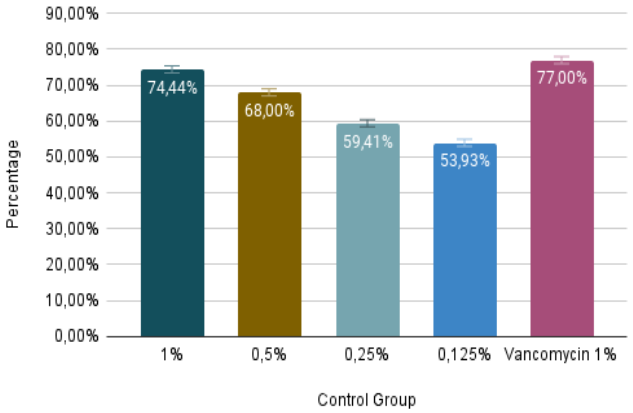

Figure 4: Percentage of *Staphylococcus aureus* Biofilm eradication activity from telang ternate extract

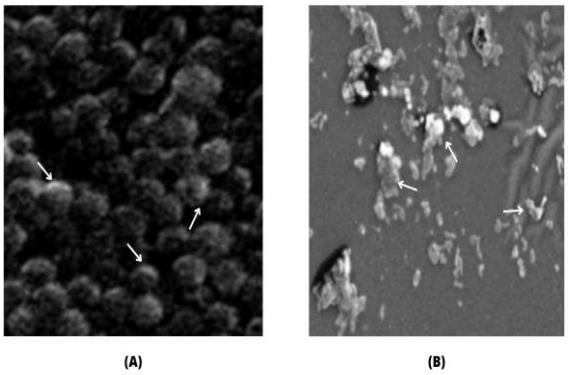

Figure 5: Figure 5a Result of Scanning Electron Microscopy Biofilm with No Treatment, Figure 5b Result of Scanning Electron Microscopy Biofilm with administration of telang ternate extract 1% b/v

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