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In vitro Study of the Antibiofilm Potential of Bay Leaf Extract (*Syzygium polyanthum* (Wight) Walp) against *Streptococcus sanguinis* and *Streptococcus mutans*

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Abstract

The ability to form biofilms is one of the characteristics of microbes that has the potential to cause antimicrobial resistance. The oral cavity environment, which is frequently exposed to external environments and has a specific histological structure, can lead to the accumulation of EPS, thereby increasing the risk of biofilm formation. Streptococcus is one of the bacterial groups known to have the ability to produce biofilms. Bay leaves (*Syzygium polyanthum* (Wight) Walp), a member of the Syzygium genus that has been widely used in treating infectious diseases, both traditionally and medically, due to their eugenol content, are suspected to have antibiofilm activity. This study aimed to prove the antibacterial and antibiofilm effects of ethanol extract of bay leaves against *Streptococcus sanguinis* and *Streptococcus mutans* bacteria. This study used a post-test only controlled group design, with a microtiter plate assay technique, and determination of MIC50 and MBIC50 through a probit test. The bacterial test results showed that the MIC50 values for *Streptococcus sanguinis* and *Streptococcus mutans* were 37.57% and 9.65%, respectively, while the MBIC50 values were 6.41% and 29.98%, respectively. The coefficient of determination (R2) values for both test bacteria, in both the antibacterial and antibiofilm tests, were close to 1. Thus, it can be concluded that bay leaf extract is effective as an antibacterial and antibiofilm agent against *Streptococcus sanguinis* and *Streptococcus mutans* bacteria, with a stronger antibiofilm effect than its antibacterial effect.

Keywords: bay-leaves; biofilm; Streptococcus sanguinis; Streptococcus mutans.

INTRODUCTION

Antimicrobial resistance is one of global health issues that remain unresolved until today, with reports indicating that it has increased since the Covid-19 pandemic. In addition to the increase in uncontrolled use of antimicrobials, bacterial internal factors such as the natural ability evading antimicrobials may also initiate the antimicrobial resistance, which affects the progression of disease (Pulingam et al., 2022). Mortality related to antimicrobial resistance are predicted to reach 10 million cases by 2050. (World Health Organization, 2014). The ability to producing biofilms is one of the bacterial characteristics that potential causing antimicrobial resistance. Biofilms themselves are a group of bacteria that adhere to a surface and are surrounded by an impermeable extracellular polymeric substance (EPS). (Chamat-Hedemand et al., 2020; Rather et al., 2021). Biofilm can be described as a highly dynamic and structured form of bacterial life, where they attach to a surface and produce an EPS matrix, forming a structured community with physiological properties that are no longer the same as those of free-living (planktonic) bacteria (Mendhe et al., 2023). The environment in the oral cavity, which is frequently exposed to external conditions, along with the histological structure of the organs within the oral cavity, can lead to EPS accumulation, thereby increasing the risk of biofilm formation (Le et al., 2020).

Biofilm formation occurs in several stages. The first stage is the attachment of bacteria to the location where they will settle. Attachment here includes the processes of adhesion and cohesion. The adhesion process facilitates attachment between bacterial cells and the attachment site, while the cohesion process facilitates attachment between bacterial cells. Once the attachment process is complete and stable, the bacteria will produce EPS, which then initiates the multiplication process, and forming new colonies. The next stage is the production of chemical molecules called auto-inducers by the colonies. These auto-inducers are used to facilitate the quorum sensing process, and when they reach a certain concentration, they activate receptors that produce

specific genes. The next stage is release from the colony. At this stage, the bacteria will replicate very rapidly until they fill the biofilm, and then produce saccharolytic enzymes to release themselves from their initial attachment. (Jamal et al., 2018; Mađar et al., 2020; Rather et al., 2021)

Oral microorganisms are a group of microorganisms that tend to produce EPS and form dental biofilms, such as *Streptococcus sanguinis* and *Streptococcus mutans*. Both are cariogenic because they are not only capable of producing EPS, but also capable of producing acid and are resistant to acid (Chen et al., 2020; Nomura et al., 2020). Caries lesions are formed as a result of the demineralization of tooth enamel by acids produced by cariogenic bacteria, and these bacteria can survive in a specific location of the tooth because of the EPS they produce (Mallya & Mallya, 2020; Nomura et al., 2020). Severe dental caries can reach the pulp, an area inside the tooth that is rich in nerves and capillaries, allowing oral microorganisms to enter through the bloodstream. Dental caries can also trigger periodontal tissue infections and even bleeding, allowing oral microorganisms to enter the bloodstream. In the next stage, oral microorganisms are carried by the bloodstream and can reach the heart valves, posing a risk of infective endocarditis, an infectious disease of the heart valves that has a low incidence but a high mortality rate (Bumm & Folwaczny, 2021; Nomura et al., 2020). People with prosthetic devices, especially prosthetic heart valves, are at greater risk of developing infective endocarditis. This is because prosthetic devices tend to have non-organic surfaces and do not have a direct blood supply, so there is insufficient blood flow to carry immune cells to the area to fight infection. Microbes from other parts of the body carried in the bloodstream, when they reach the heart, are more likely to attach to the surface of the prosthetic device due to the lack of immune response and the natural protective endothelial layer, thereby facilitating the process of microbial adhesion (Marques et al., 2024).

Efforts in discovering new antimicrobials are ongoing to address the increasing number of antimicrobial resistance cases. The World Health Organization (WHO) recommends medicinal plants, as part of traditional medicine, to be further researched and, if proven safe and effective, can be used in the national healthcare system (Dewi & Nafi'ah, 2022; Sharma, 2025; Stan et al., 2021). Plants from the Syzygium genus are among those expected to be able to respond to this challenge. Bay leaves (*Syzygium polyanthum* (Wight) Walp) are an example of a plant from the Syzygium genus that has been extensively researched for its antimicrobial effects (Dewi & Arlita, 2021; Harismah & Chusniatun, 2016; Iftikhonsa et al., 2021; Winarta et al., 2021). This plant is known containing eugenol, an aromatic phenol that is easily soluble in water, has low chemical stability, and is sensitive to oxidation or other chemical interactions (Teles et al., 2021; Ulanowska & Olas, 2021). Moreover, WHO has recognized eugenol as a non-mutagenic molecule and classified it as safe. The antimicrobial properties of eugenol are known from its ability to fight a number of enzymes produced by Gram-positive bacteria, such as histidine, ATPase, and protease (Hyderi et al., 2025a). Eugenol is also known to damage bacterial cell membranes, causing protein and lipid leakage from the cytoplasm (Ulanowska & Olas, 2021). The high level of eugenol utilization in the treatment of oral cavity infections is thought to be due not only to its antimicrobial activity but also to its antibiofilm activity (Zhang et al., 2017).

MATERIALS AND METHODS

This study was a laboratory experimental study using a post-test only controlled group design. The extract preparation procedure was carried out at the Pharmacology Laboratory, Faculty of Medicine, while the antibacterial and antibiofilm tests were carried out at the Pharmaceutical Biology Laboratory, Faculty of Pharmacy, Universitas Muhammadiyah Surakarta. The research subjects were colonies of Streptococcus sanguis and Streptococcus mutans bacteria obtained from the collection of the Pharmaceutical Biology Laboratory, Faculty of Pharmacy, Universitas Muhammadiyah Surakarta. The bacterial colonies that had been grown on agar media were replanted on Brain-heart Infusion Broth (BHI) media and adjusted to the McFarland 0.5 standard. The material tested was 96% ethanol extract from bay leaves (Syzygium polyanthum (Wight) Walp) provided in 5 concentration variations, which were 3.125%, 6.25%, 12.5%, 25%, and 50%. Gentamicin was used as the positive control, while Dimethyl Sulfoxide (DMSO) was used as the negative control.

Antibacterial testing was performed using the microdilution method, while biofilm testing was performed using the microtiter plate biofilm assay method (Crystal violet assay). Data were collected by tabulating the measurement results of the absorbance level (optical density) of each concentration used. The absorbance level was measured using a microplate reader at a wavelength of 590 nm (Hutomo et al., 2023). In addition, this study also conducted a quantitative analysis, the determination of MIC_{50} (Minimum Inhibitory Concentration 50) and $MBIC_{50}$ (Minimum Biofilm Inhibitory Concentration 50) values to evaluate the concentration of extracts that can inhibit planktonic growth and biofilm formation by 50%. The first statistical analysis was a normality test using the Shapiro-Wilk test and a homogeneity test using the Levene test. The next statistical analysis was a hypothesis test using the One Way Annova test, followed by a Mann Whitney post-hoc test. This study was conducted after obtaining ethical approval from the Health Research Ethics Committee (KEPK) of the

Faculty of Medicine, Muhammadiyah University Surakarta, with number No. 5353A/C.2/KEPK-FKUMS/XI/2024.

RESULTS AND DISCUSSION

Result

Antibacterial activity

The first observation, the antibacterial test, assessed the effectiveness of 96% ethanol extract of bay leaves as an antibacterial agent against both test bacteria by observing the level of media turbidity after incubation, which was expressed as the percentage of bacterial growth inhibition. The results of the antibacterial test are presented in Figure 1.

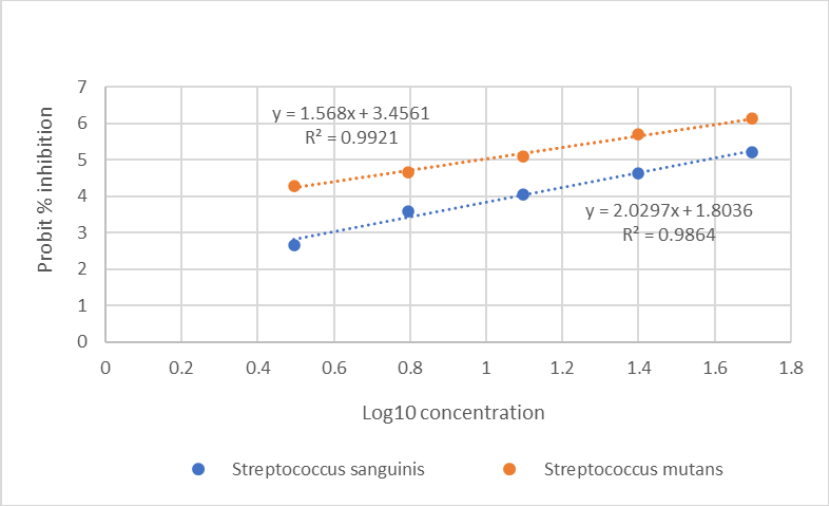
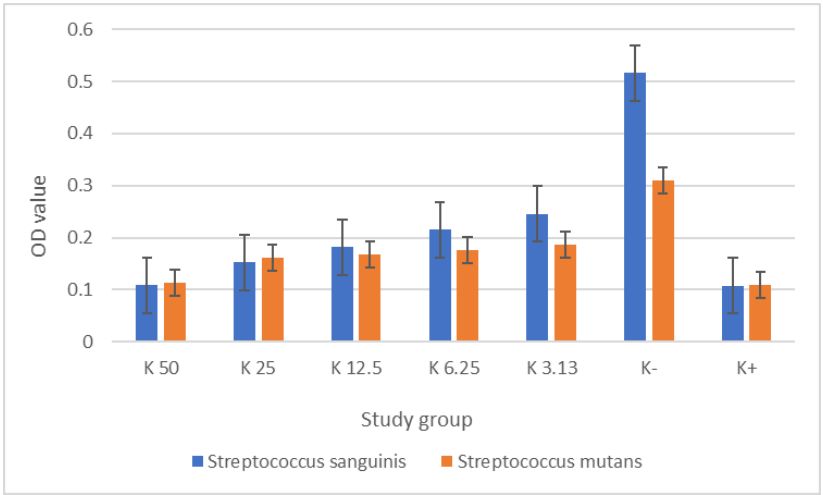


Figure 1. Graphic of probit percentage of bacterial growth inhibition.

Based on the figure above, the antibacterial effect resulted is in line to the concentration level of the bay leaf extract administered. The higher the extract concentration, the greater the antibacterial effect, with MIC_{50} for *Streptococcus sanguinis* and *Streptococcus mutans* bacteria being 37.57% (log10 extract concentration = 1.57) and 9.65% (log10 extract concentration = 0.98), respectively.

Antibiofilm activity

The optical density measurement results of the antibiofilm test of bay leaf extract at various concentrations against *Streptococcus sanguinis* and *Streptococcus mutans* bacteria are shown in Figure 2.



Note:
K 50 : Treatment group with 50% concentration of bay leaf extract
K 25 : Treatment group with 25% concentration of bay leaf extract
K 12.5 : Treatment group with 12.5% concentration of bay leaf extract
K 6.25 : Treatment group with 6.25% concentration of bay leaf extract
K3.13: Treatment group with 3.13% concentration of bay leaf extract
K - : Negative control group (DMSO)
K+ : Positive control group (Gentamicin)

Figure 2. Average optical density (OD) values in the antibiofilm test.

The OD value in the antibiofilm test shows the level of biofilm formation produced by bacteria. As shown in Figure 2, there is an inverse correlation between the concentration of bay leaf extract and the OD value. The highest concentration of bay leaf extract used resulted in the lowest OD value, which means that biofilm formation was minimal. In other words, the higher the concentration of bay leaf extract used, the greater the inhibition of biofilm formation.

The results of normality test using Shapiro-Wilk test and homogeneity test using Levene test, both on *Streptococcus sanguinis* and *Streptococcus mutans* bacteria, obtained a p-value <0.05, which means that the data obtained was not normal and not homogeneous. Thus, the next statistical test used was the Kruskal Wallis non-parametric test, and a p-value of <0.05 was obtained. This means that at least one group had a significant difference. Therefore, the statistical analysis was continued with the Mann Whitney post-hoc test to find which groups were significantly different. The hypothesis test yielded a p-value of 0.000. A p-value <0.05 indicates that there is a significant difference in the overall data. From the statistical analysis performed, it can be concluded that there is a significant effect in increasing the concentration of bay leaf extract. The higher the extract concentration, the greater the inhibitory effect on biofilm formation.

Further analysis of the bay leaf extract's ability to inhibit biofilm formation by Streptococcus sanguinis and Streptococcus mutans bacteria is presented in Figure 3.

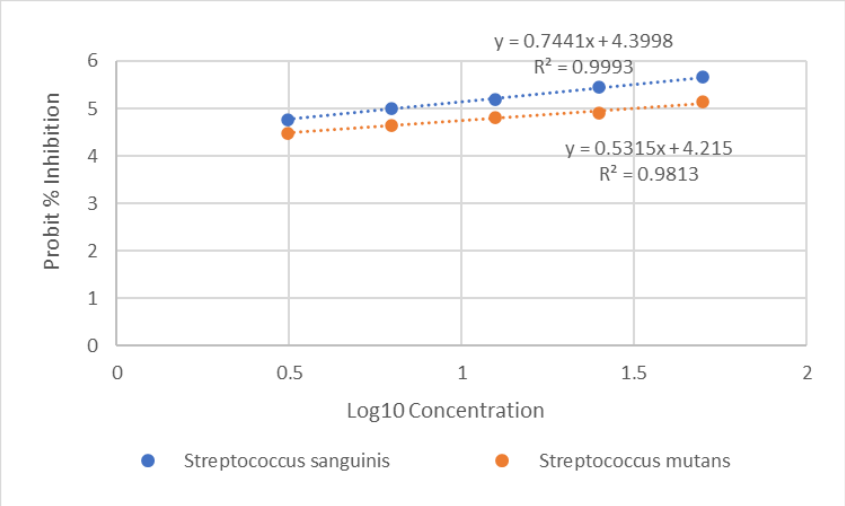


Figure 3. Graphic of probit percentage of biofilm formation inhibition.

The relationship between the antibiofilm effect and the concentration of bay leaf extract administered is consistent with the antibacterial effect. The higher the concentration of bay leaf extract, the greater the antibiofilm effect, but this antibiofilm effect appears to be stronger than its antibacterial effect. This is evident from the $MBIC_{50}$ for *Streptococcus sanguinis* and *Streptococcus mutans* bacteria, which are 6.41% (log10 extract concentration = 0.81) and 29.98% (log10 extract concentration = 1.48), respectively.

Discussion

The first observation performed in this study was the antibacterial effect of bay leaf extract on *Streptococcus sanguinis* and *Streptococcus mutans* bacteria, as shown in Figure 1. Based on this graph, it shows that bay leaf extract has a dose-dependent inhibitory effect on the growth of both bacteria. In general, an increase in extract concentration was followed by an increase in the percentage of growth inhibition. However, *Streptococcus mutans* appear to be much more sensitive, as indicated by higher inhibition values at each concentration. Antibacterial activity is said to be dose-dependent because the inhibitory effect on bacteria increases with the increase in the concentration of antibacterial compounds in the extract. When the extract concentration is still low, only a few active molecules are able to reach and damage important targets in bacterial cells, so the resulting inhibition percentage is also relatively small. However, when the dose is increased, the number of active molecules interacting with the membrane, enzymes, and bacterial cell structures increases, causing greater damage. This condition prevents the bacterial cell's defense and repair mechanisms from compensating for the damage, resulting in a gradual increase in the antibacterial effect. Pharmacodynamically, the relationship between the concentration of the active compound and its biological effect is generally proportional up to a saturation point, so that the higher the concentration of the extract, the greater the inhibitory effect on bacterial growth (Iftikhonsa et al., 2021; Ismail & Ahmad, 2019; Putri et al., 2023; Shen et al., 2023; Winarta et al., 2021).

Probit analysis was used by converting the dose-response data obtained to a probit scale to determine the effective dose. The results showed a positive and very strong relationship between the extract concentration (on a log10 scale) and the percentage of bacterial growth inhibition (on a probit scale). Both regression lines, for Streptococcus sanguinis and *Streptococcus mutans*, had very high coefficients of determination (R2), which were 0.9864 and 0.9921, respectively. R2 values close to 1.0 indicate that the linear model used was very accurate in predicting the inhibition response to changes in extract concentration (Roustaei, 2024). Using the regression equation from the graph, the MIC_{50} value, which is the lowest concentration of extract needed to inhibit 50% of bacterial growth (Probit 5.0), can be determined. For *Streptococcus sanguinis*, the MIC_{50} value is 37.57%, indicating that a relatively high concentration is needed to achieve a half-maximal effect. Meanwhile, for *Streptococcus mutans*, the MIC_{50} value was calculated to be much lower, at 9.65%, indicating that this bacteria is more susceptible and requires only a smaller concentration of extract to be inhibited. Overall, statistical probit analysis confirmed that bay leaf extract has much stronger and more effective antibacterial activity against Streptococcus mutans than against Streptococcus sanguinis. This is mainly due to the nature of Streptococcus mutans bacteria, which are very effective at producing glucan (extracellular polysaccharide) from sucrose using the enzyme glucosyltransferase (GTF). This glucan forms a very thick sticky matrix (Ahmed et al., 2023; Ma et al., 2021; Mizuta & Suzuki, 2024). The active compounds in bay leaf extract, especially eugenol, are strongly suspected to work by inhibiting GTF enzyme activity in Streptococcus mutans. Without GTF, *Streptococcus mutans* cannot form a thick glucan matrix, making it unable to adhere strongly and easier to break down (Athar, 2024; Palomares-Navarro et al., 2025).

A comprehensive analysis of the antibiofilm activity test data of bay leaf extract shows that the secondary metabolites contained in bay leaf extract are effective in inhibiting biofilm formation in both bacteria with concentration-dependent effectiveness, as well as shown di figure 2. The OD values presented in the bar chart visually show a clear relationship, where the higher the extract concentration, the lower the OD value, which means that the level of inhibition of biofilm formation increases. The OD value in the bay leaf extract group with the highest concentration, namely 50% (K50), was close to the OD value in the positive control group (K+), which was the inhibition standard, indicating that bay leaf extract has very strong antibiofilm potential. The OD value pattern in this antibiofilm test also consistently shows that Streptococcus mutans is more sensitive to bay leaf extract than *Streptococcus sanguinis*, especially at lower concentrations.

In this antibiofilm study, the smallest OD value was obtained in the positive control group with gentamicin. Thus, the selection of gentamicin as a positive control proved to be appropriate. Although gentamicin is generally classified as an antibacterial agent, this drug is also thought to have an antibiofilm effect. Gentamicin is an aminoglycoside antibiotic that works by binding to the 30S ribosomal subunit, thereby interfering bacterial protein synthesis. This binding inhibits the formation of the initiation complex and causes mRNA misreading, so that various important proteins needed by bacteria to perform initial adhesion and continue the biofilm maturation process cannot be formed properly. As a result, planktonic bacterial cells become unable to effectively attach to surfaces or other cells, thereby inhibiting the formation of the initial biofilm layer from the earliest stage (Sekar et al., 2024; Tang et al., 2014).

The relationship between concentration of bay leaf extract and probit percentage of biofilm formation inhibition in Figure 3 shows a positive linear pattern, which means that an increase in extract concentration is consistently followed by an increase in probit value, or in other words, an increase in the percentage of biofilm formation inhibition (Dewi & Arlita, 2021; Iftikhonsa et al., 2021; Shen et al., 2023; Winarta et al., 2021). This linearity pattern is reflected in the upward regression line for both bacteria. One of the most important parameters in this relationship is the slope value, because the slope describes the sensitivity of bacteria to increases in extract concentration. In Figure 3, it can be seen that the graph for Streptococcus sanguinis bacteria has a slope value of 0.7441, slightly greater than the slope value for Streptococcus mutans bacteria, which is 0.5315. The greater the slope value, the sharper the increase in the inhibitory effect when the extract concentration is increased (Roustaei, 2024). This means that *Streptococcus sanguinis* shows a stronger response to changes in dosage; a small increase in extract concentration is sufficient to produce a greater increase in the probit percentage of biofilm inhibition compared to *Streptococcus mutans*. Furthermore, the graph in Figure 3 also shows an R^2 value of 0.9993 for Streptococcus sanguinis bacteria and 0.9813 for Streptococcus mutans bacteria. As with the antibacterial test results, these two R^2 values are also close to 1, indicating that the linear regression model is very good at explaining the relationship between log10 extract concentration and probit percentage of biofilm inhibition. In short, changes in the antibiofilm effect are highly predictable from changes in extract dosage, so the dose-response relationship is considered stable and consistent (Roustaei, 2024; Shen et al., 2023)

The next observation that can be seen in Figure 3 is the antibiofilm effect of bay leaf extract, which appears to be stronger against Streptococcus sanguinis bacteria than *Streptococcus mutans* bacteria. This is contrary to the results of the antibiotic test, where *Streptococcus mutans* bacteria were found to be more sensitive than Streptococcus sanguinis. Streptococcus mutans bacteria in the planktonic phase tend to be more sensitive to

antimicrobial compounds, including eugenol and various phenolic components in bay leaf extract, because in this phase the bacteria are still individual cells that do not have the protection of Extracellular Polymeric Substance (EPS) matrix. In the planktonic state, the cell wall and membrane of the bacteria are more exposed, allowing antimicrobial compounds to interact directly with cellular targets such as membranes, enzymes, and DNA without physical barriers. In addition, the metabolism of *Streptococcus mutans* in the planktonic phase is also more active, so that disruption of protein or membrane function can more quickly cause bactericidal or bacteriostatic effects. However, when entering the biofilm phase, Streptococcus mutans is able to produce a very thick and protective glucan EPS matrix, which makes it more difficult for external active compounds to penetrate the layer and reach the bacterial cells. As a result, the effectiveness of biofilm inhibition is lower than in the planktonic phase (Athar, 2024). Furthermore, in the biofilm formation process, colonization time and the order of bacterial appearance greatly affect the thickness and level of resistance to antibiofilm agents. Streptococcus sanguinis generally acts as an early colonizer, which is the first bacterium to attach and form an initial biofilm layer that is still thin, less complex, and relatively easy to disrupt. At this stage, the biofilm is still more sensitive to chemical and physical disturbances, making antibiofilm agents more effective at penetrating and destroying its structure. In contrast, Streptococcus mutans acts as a late colonizer that arrives in the next phase and becomes the main builder of cariogenic biofilm. Biofilm at this advanced stage is mature, thicker, more organized, and reinforced by an abundant EPS matrix, making it much stronger and more resistant to antibiofilm agents. As a result, mature biofilms tend to be more difficult to destroy compared to early biofilms formed by primary colonizers such as Streptococcus sanguinis (Baty et al., 2022; Bloch et al., 2024).

The final observation is a comparison between the graphs in Figures 1 and 3, where the antibiofilm effect of bay leaf extract appears to be stronger than its antibacterial effect, as indicated by the $MBIC_{50}$ values for both bacteria being smaller than their MIC_{50} values. This finding can be understood through the differences in the mechanisms of action, biological targets, and effective concentration requirements between bacterial growth inhibition (MIC) and biofilm formation inhibition (MBIC). In antibacterial tests, the MIC value indicates the minimum concentration required to inhibit planktonic cell growth, which means that the compound must be able to penetrate the bacterial cell wall, disrupt protein or membrane synthesis, or inactivate essential enzymes. This process requires relatively high concentrations because planktonic cells have strong structural integrity and metabolic adaptation mechanisms that make them more resistant to chemical attacks, especially in Gram-positive bacteria known for their thick cell wall structure, such as *Streptococcus sanguinis* and *Streptococcus mutans*. In contrast, in the antibiofilm test, the MBIC value indicates the concentration required to prevent the initial stages of biofilm formation, which include surface adhesion, intercellular communication (quorum sensing), and EPS production. These initial processes are much more susceptible to chemical interference, so phenolic compounds such as eugenol can inhibit them at concentrations much lower than the MBIC. Therefore, even though bay leaf extract may show moderate or even low antibacterial activity at certain concentrations, its antibiofilm activity can be very high because the antibiofilm target is more easily affected. Additionally, early-stage biofilms have a structure that is still thin, loose, and not yet protected by a thick EPS matrix, so even minor disruptions to the adhesion mechanism or EPS formation are sufficient to produce a large percentage of inhibition (Elbestawy et al., 2023; Hyderi et al., 2025b; Ribeiro et al., 2024).

CONCLUSIONS

Bay leaf extract, in addition to effectively inhibiting the growth of *Streptococcus sanguinis* and *Streptococcus mutans* bacteria, has also been proven to effectively inhibit the formation of biofilms in both bacteria. This antibiofilm effect appears to be even stronger than its antibacterial effect, as indicated by a lower $MBIC_{50}$ value compared to the MIC_{50} value. The antibacterial effect of bay leaf extract was found to be stronger against *Streptococcus mutans* bacteria, while its antibiofilm effect was found to be stronger against *Streptococcus sanguinis* bacteria.

Authors' Contributions: Listiana Masyita Dewi designed the study. Azka Tarisa Atmaja and Muhammad Marcelliano Rifky Aditomo Putra carried out the laboratory work. Devi Usdiana Rosyidah, Nurhayani, and Rima Munawaroh carried out the data analysis. All authors read and approved the final version of the manuscript.

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