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The biofilm eradication activity of C-10 massoialactone against Staphylococcus aureus

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ABSTRACT

C-10 massoaialactone is a major component of stem bark essential oils Massoia aromatica Becc, which has potential as antibacterial and antifungal but antibiofilm activity against S. aureus has never been reported. The discovery of anti-biofilm drug candidates is needed to overcome infections associated with biofilms. This study aims to determine the eradication activity of C-10 massoialactone against S. aureus biofilms. Antibacterial testing and eradication activity of S. aureus biofilms was determined using the microtiter broth method. The effectiveness of C-10 massoilactone against S. aureus biofilm was analyzed by calculating the value of minimum biofilm eradication concentration. The mechanism of action of C-10 massoilactone against S. aureus biofilms was tested using scanning electron microscopy (SEM). C-10 massoilactone 1% gives antibacterial activity of 86.80% ± 0.01 and can eradication S. aureus biofilm by 68.98% ± 0.01 and not much different from the eradication activity of chloramphenicol drug control. The results also provide evidence that C-10 masoaialactone can damage the extracellular polymeric substance (EPS) matrix and S. aureus biofilm morphology. Therefore, C-10 masoaialactone is very potential to be developed as a candidate for a new antibiofilm drug against S. aureus biofilm.

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INTRODUCTION

Antibiotic resistance to S. aureus is one of the current problems caused by the formation of biofilms that can cause the formation of cells that are resistant to antibiotics and form persister cells that can adapt to antibiotics in a way that does not depend on the part of cells that are targeted by antibiotics. S. aureus resistance to penicillin antibiotics has reached 80%. Methicillin and vancomycin antibiotics have caused resistance to S. aureus such as S. aureus Methicillin Resistance (MRSA) and S. aureus Vancomycin Resistance (VRSA) (Chambers and DeLeo, 2009); (Lewis, 2010).

Biofilm is a biofilm defined as a collection of microbial cells that are attached toa surface and encased in an Extracellular Polymeric Substances matrix(EPS) that they produce themselves (Abbas *et al.*, 2013); (Hamzah *et al.*, 2020) Bacterial and fungal infections caused by biofilms are very difficult to treat, to kill both bacteria and fungi in the form of biofilms requires 1000 times the dose of antimicrobial needed to achieve the same results as planktonic cells (Hetrick *et al.*, 2009); (Nuryastuti *et al.*, 2018). Biofilms can be controlled by utilizing chemical compounds obtained from natural materials (Sandasi *et al.*, 2010); (Hertiani *et al.*, 2010).

Indonesia has a very high source of plant diversity and is the second-largest in the world in terms of biodiversity. People use their knowledge to use plants to replace modern medicines in maintaining health and treating diseases (Nurwijayanto *et al.*, 2019).

In recent years there has been increasing attention on the discovery of GRAS compounds (generally regarded as safe) from natural ingredients to treat new and existing diseases. Higher plants are exotic species for this discovery because they contain many potentially active secondary metabolites (Lagendijk, 2015).

C-10 massoialactone is the main compound found in masoyi and is also found in fruit oil in small amounts (Atmadja, 2009); (Hamzah, 2017); (Sa'roni and Adjirni, 1999). Masoyi plants (Massoia aromatica Becc.) Grow in Indonesia, especially in Maluku and Papua (Hertiani *et al.*, 2016). Essential oils are obtained from the bark, stem, and fruit of the Masoyi. Massoia aromatica has a long history of traditional medicine (Rali *et al.*, 2007).

Empirically, the masoyi plant's bark is used, among others, for diarrhea, convulsions, fever, tuberculosis, muscle aches, headaches, and chronic constipation (Hertiani *et al.*, 2016); (Widowati and Pudjiastuti, 1999).

Until now, no one has reported about the eradication activity of C-10 massoilactone against S. aureus biofilms. Therefore, it is important to look for potential new S. aureus biofilm agents obtained from plant compounds that lead to the discovery of new drugs.

METHODS

Materials

Materials used were C-10 massoialactone compounds from the isolation of Massoia aromatica Becc were obtained from West Papua, Indonesia. Other materials include the following: crystal violet (Merck, Germany), ethyl acetate (Merck, Germany), Brain heart infusion (Oxoid) (Merck, Germany), RPMI 1640 (Sigma-Aldrich), ethanol 95 % (Merck, Germany), chloramphenicol (Sigma-Aldrich, Germany).

Equipment

Laminar Air Flow, incubator (IF-2B) (Sakura, Japan), micropipette pipetman (Gilson, France), multichannel micropipette (Socorex, Swiss), microplate flat-bottom polystyrene 24 well (Iwaki, Japan), microtiter plate reader (Optic Ivymen System 2100-C, Spain), spectrophotometry (Genesys 10 UV Scanning, 335903) (Thermo Scientific Spectronic, USA), autoclave (Sakura, Japan), incubator with orbital shaker S1500 (Stuart, UK), analytical scales (AB204 -5, Switzerland).

Bacterial Strains

A standard strain of Staphylococcus aureus (ATCC 25923) was cultured in tryptic soy broth (TSB) medium and incubated at 37°C for 72 h. The optical densities (OD600) of microbial cultures will be adjusted to 0.1 (equal of the 0.5 McFarland standard ~1,5 x 10^8 CFU/ml), and subsequently diluted in fresh medium to OD600 0.01.

Antibacterial effect of C-10 massoailactone against S. aureus

Antibacterial test was carried out using the microdilution method. The test was carried out on a 96 wells flat-bottom polystyrene microtiter plate with a series of test compound levels of 1%, 0.5%, 0.25%, 0.125% b / v. Positive control using 1% b / v chloramphenicol. Negative control in the form of a microbial suspension and solvent control was adjusted to the test compound's solvent. BHI media and S. aureus bacterial suspensions were inserted into each wells microplate and then incubated at 37^0C for 24 hours. Microplate absorbance readings were carried out using a microplate reader at a wavelength of 595 nm.

Eradication activity of C-10 massoialactoen against S. aureus biofilm

A total of 100 μL of S. aureus suspension was added to each well microtiter plate and then incubated at $\pm$ 37ºC for 48 hours for the formation of the biofilm maturation phase. After the incubation period, the plates are washed using 150 μL sterile aquadest three times to remove non-attached cells. A total of 100 μL of media containing pure isolates with series concentration (1% b / v, 0.5% b / v, 0.25% b / v, 0.125% b / v), were added to each washed wells. As a media control media are used without microbial growth, and microbial suspensions are used as negative controls. As a positive control used, a microbial suspension which was given antibacterial chloram-

phenicol levels of 1% b / v. Biofilms were formed after each well was incubated for 48 hours with a bacterial suspension in the RPMI media. After the biofilm is formed, the suspension in the microplate is discarded. The plate was washed using distilled water three times and dried at room temperature for 5 minutes to remove the remaining water. A total of 125 μL of 1% crystal violet solution was added to each well to color the formed biofilm, dead cells, and living cells, which are constituent components of biofilms. The plates were then incubated at room temperature for 15 minutes. After incubation at room temperature, wash with running water three times to clean the remaining crystals violet, and 200 μL 96% ethanol was added to each well to dissolve the biofilm formed. Testing is done with three replications. Reading the biofilm eradication activity results using a 595 nm Optical Density (OD) microplate reader, conducted as in the reading of the results of biofilm inhibition. The OD value is then used to calculate the percent degradation of biofilm in the following equation:

Od growth control–Od sample/Od growth control x 100

Sample levels that can degrade at least 50% of biofilm formation are considered $MBEC_{50}$ (Minimal Biofilm Eradication Concentration) (*Lagendijk*, 2015); (Hamzah *et al.*, 2018).

Scanning electron microscopy (SEM)

Cells were grown directly on the slipcover or catheter and incubated at 37^0C for 24 hours for the middle phase. Test compounds are compounds known to provide $MBIC_{50}$ / $MBEC_{50}$ activity at levels of 0.25% and 0.5% v / v. After the mid-phase biofilm is formed, the coverslip is carefully washed with PBS 1% twice, followed by washing 2% paraformaldehyde, 2% glutaraldehyde, and sodium cacodylate 0.15 M and the sample were prepared to be observed under SEM. Sputter specimens were coated with a layer of gold, and samples were observed under Scanning electron microscopy (SEM) JEOL JSM-6400. Images processed using software photoshop (Hess *et al.*, 2012); (Sofer and Denstedt, 2000).

RESULTS AND DISCUSSION

Antibacterial effect of C-10 massoialactone against s.aureus

C-10 massoialactone 1% b / v gave antibacterial activity against S. aureus at 86.80% ± 0.01 and lower than the control drug chloramphenicol 1% at 90.06% ± 0.01. The results of C-10 massoailactone

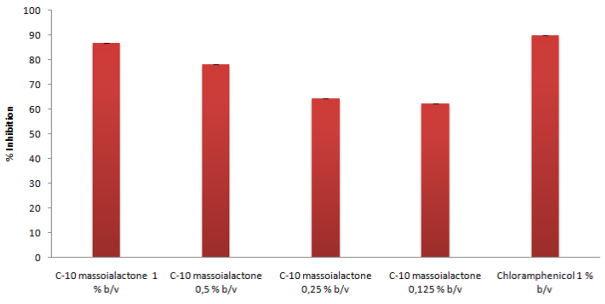

Figure 1: The antibacterial activity of C-10 massoilactone against S. aureus

as an antibacterial showed that the higher the concentration used, the greater the antibacterial activity obtained (Figure 1).

This study provides evidence that C. 10 massoialactone can provide antibacterial activity of S. aureus. This statement is stated by (Barros *et al.*, 2014), which says that massoialactone is a compound that can irritate the skin, but shows excellent antimicrobial activity. The mechanism of inhibition of C-10 massoialactone as an antibacterial due to C-10 massoialactone inhibits the biosynthesis pathway of S. aureus peptidoglycans resulting in the cell wall of S. aureus bacteria becoming fragile so that the cell undergoes lysis where S. aureus is a Gram-positive bacterium, which has 40 peptidoglycan pathways which are a cell of S. aureus 50% of the cell wall building material.

Eradication activity of C-10 massoialactone against S. aureus biofilm

C-10 massoialactone gives eradication activity to S. aureus biofilms. Eradication activity of a 1% C-10 massoialactone biofilm given was 68.98% ± 0.01, while the chloramphenicol drug control 1% was 71.10% ± 0.01 (Figure 2).

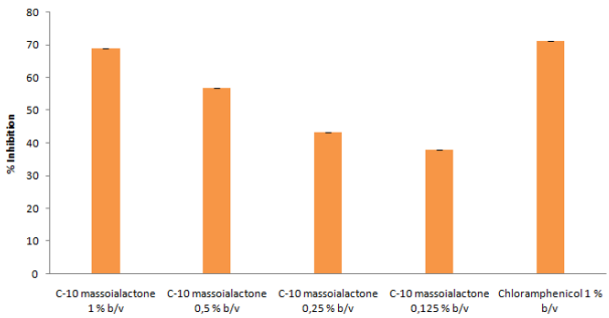

Figure 2: Eradication activity of C-10 massoilactone against S.aureus biofilms

These results indicate that there is a decrease in inhibitory activity given C-10 massoialactone on eradication compared with its action against antibacterial. This is because at the stage of erad-

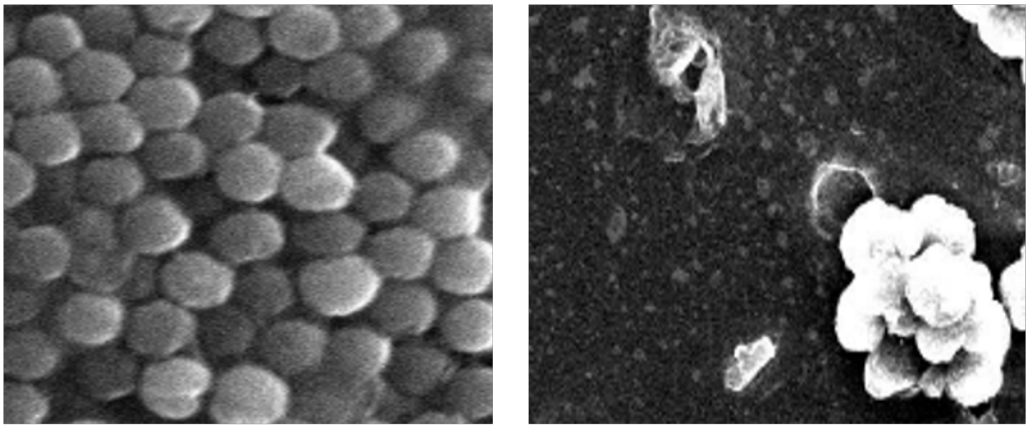

Figure 3: biofilm S. is taken using Scanning Electron Microscopy (SEM) (A) Before giving C-10 Massoialactone, (B) After administration of C-10 Massoialactone compounds.

ication makes biofilm growth faster than the inhibition provided by test compounds, and this causes the inhibitory activity given by the test compound is not as large as the activity assigned to the antibacterial due to the synergy and the length of growth of S. aureus biofilms at a stage that causes the growth of more biofilm colonies. This is according to the research stated by (Madsen *et al.*, 2016) which says that long-term coexistence of bacterial and fungal species in a place can stimulate to form biofilms that synergize with one another in an involved community. Besides that statement, (Costerton *et al.*, 1995); (Hamzah *et al.*, 2018) suggest that when antibiotics are given, then these antibiotics only eliminate or decrease planktonic bacteria, but the bacteria in biofilm persist when given antibiotics. When treatment with antibiotics is complete, biofilms will form more planktonic cells, resulting in recurring infections.

S. aureus forms biofilms through the polysaccharide intercellular antigen (PIA)-dependent, PIA-independent dan extracellular DNA (eDNA) pathway. S. aureus produces PIA in vitro from UDP-N-acetylglucosamine through the intercellular adhesion (ica) locus. The PIA-dependent pathway shows the formation of biofilms occurs when microbes produce PIA which act as bridges between cells and causes the accumulation of biofilm layers (Archer *et al.*, 2011); (Atshan *et al.*, 2012).

These results prove that C-10 massoialactone can be used as a candidate for S. aureus antibiofilm because C-10 massoialactone is capable of eradicating S. aureus biofilm. The mechanism of C-10 massoialactone eradication against S. aureus biofilms by penetrating biofilms through the polysaccharide matrix and dissolving lipids in the biofilm matrix, in addition to other mechanisms of C-10 massoialactone by degrading cell walls by causing damage to the cytoplasmic membrane and membrane proteins as well as dissolving lipids in the biofilm matrix, in addition to other mechanisms of C-10 massoialactone by degrading cell walls to cause damage to the cytoplasmic membrane and membrane proteins as well as reduction of the proton gradient to the membrane potential to damage to the ATP synthesis mechanism.

The result of Scanning Electron Microscopy (SEM) S. aureus biofilm with no treatment

The untreated S. aureus biofilm shows the density of dense, structured, and encased cells by the EPS matrix (Figure 3). From these results, it can be seen that the S. aureus bacteria form a bond that is interconnected between the bacteria with each other. This proves that the bacteria together make the EPS matrix as a self-defense system of antimicrobial compounds, thus causing antimicrobial compounds to have difficulty in penetrating their defenses.

This result is by the statement (Donlan, 2002); (Hamzah *et al.*, 2020); (Lagendijk, 2015) which suggests that microbial biofilms can function as protectors so that the microbes that form biofilms usually have resistance to normal antimicrobials and avoid the host cell's immune system. Besides the occurrence of synergistic interactions, microbes can benefit from the presence of each other. For example, microbes work together to increase resistance to antimicrobial agents or metabolic exchange products, known as cross-phenomenon,

to modify environmental conditions in biofilms (PH, oxygen concentrations) to support the growth of others (Short *et al.*, 2014).

Scanning Electron Microscopy (SEM) S. aureus biofilm with the administration of C-10 massoialactone compounds 0,5 % b/v.

Our results show that the C-10 massoilactone compound can damage the S. aureus biofilm defense. This is evidenced by the destruction of the S. aureus biofilm matrix after exposure to the C-10 massoilactone compound. Also, the administration of C-10 massoilactone reduces the level of biofilm cell damage and damage the morphological structure of S. aureus biofilms (Gambar Figure 3). Matrix damage and biofilm morphology are caused due to the hydrophobic nature of C-10 massoilactone, which helps target lipids located in bacterial and mitochondrial cell membranes, consequently increasing membrane permeability, which causes ion leakage and other cell content.

This result is supported by the statement (Nazzaro *et al.*, 2013) that essential oils can also directly influence bacterial cell morphology, causing total damage to the microbial cell structure entirely.

CONCLUSIONS

The C-10 massoilactone compound has antibacterial activity and eradication of S. aureus biofilms. Based on Scanning Electron Microscopy (SEM), C-10 massoilactone can damage the EPS matrix and the morphology of S. aureus biofilms. Therefore C-10 massoilactone is very potential to be developed as a candidate for C-10 massoilactone antibiotics against S. aureus.

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Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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