Egypt. J. Chem. **Vol. 66,** No. 8 pp. 185 - 190 (2023)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Exploring The Inhibitory Potential of Massoia Oil on Biofilm *Dual-Species* Culture of *Staphylococcus aureus* and *Pseudomonas aeruginosa*

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Abstract

Massoia aromatica Becc. (*Lauraceae*) is a native plant of the eastern part of Indonesia. The bark contain essential oil which has been reported to be a potential antiinfective agent against single species microbes. This research aimed to explore the potency of the massoia oil against biofilm of dual-species culture, consisted of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Pulverized massoia bark was steam-hydrodistilled to gain the essential oil (massoia oil). The oil was subjected to microdilution assay on dual-species cultures of *P. Aeruginosa* and *S. aureus* to observe the effects on the biofilm formation and degradation. Crystal violet 1% was used to stain the biofilm of which the optical density was observed by microplate reader at 540 nm. *Scanning Electron Microscope* (SEM) was used for ultrastructural observation of the cultures following sample application. Results showed that the essential oil caused the inhibition of the biofilm formation and degraded the preformed biofilm cultures tested in a concentration dependant manner . SEM revealed that the massoia oil caused cell ruptured of the *P. aeruginosa*. No observed changes in the S. aureus cells but less population density occurred. The massoia oil showed effective inhibition towards dual-species biofilm.

Keywords: Biofilm, *Massoia aromatica* Becc., *P. aeruginosa*, *S. aureus* Scanning Electron Microscope

1. Introduction

Microorganisms have a tendency to adhere to solid surfaces and form biofilms. Some cells will be bound to each other and attached to a substrate and wrapped in a matrix of extracellular polymeric substance (EPS) to form a complex structure [1,2,3,4]. Biofilms are often found in chronic infected wounds and in implanted medical devices (e.g. catheters, endotracheal tubes), the most common bacteria found are *Pseudomonas aeruginosa*, *Escherichia coli* , *Klebsiella pneumonia*, *Proteus mirabilis* [1,4,5,6]. Biofilms derived from a multispecies community of microorganisms involved in several human diseases include Gram positive bacteria (*Staphylococcus epidermidis* and *Staphylococcus aureus*), Gram negative bacteria (*P. aeruginosa* and *E. coli*) and some of the genus *Candida* sp, especially *Candida albicans* [7,8,9]. Several literatures explain that bacteria can synergistically form biofilms with other bacterial species, both physically and physiologically the biofilm structure is thicker and stronger [1,10,11].The most common method used to combat biofilms is to increase the dose of antibiotics. However, overuse of antibiotics results in the development of drug-resistant bacterial strains [1,4].

Pseudomonas aeruginosa is a gram negative bacteria that can cause serious infections in immunocompromised patients. These bacteria produce various virulence factors, which are regulated by the quorum sensing phenomenon responsible for cell

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Received date 17 October 2022; revised date 19 November 2022; accepted date 30 November.

DOI: 10.21608/ejchem.2022.169290.7100

density that regulates the expression of specific genes. The quorum sensing signal molecules, also known as autoinducers, are essential for biofilm differentiation. The quorum sensing system in *P. aeruginosa* is regulated by two signal molecules, *N-acylhomoserine lactones* (AHL) and 4-quinolones [1,12,13]. An increase in the cell population causes an increase in the concentration of autoinducers [14]. *P. aeruginosa* forms a matrix consisting of secreted extracellular polymeric substances, namely polysaccharides, proteins, lipids and extracellular DNA that surround cells in the biofilm and can also produce *4-hydroxy-2-heptylquinoline-N-oxide* (HQNO) which can help survival in extreme environments so that it is suspected that it can cause higher resistance to antimicrobial agents when compared to *S. aureus* [11,15,16].

Staphylococcus aureus is a gram-positive bacterium, can infect and cause disease usually characterized by inflammation, necrosis, and abscess formation. In general, *S. aureus* is called pyogenic because the disease it causes will form pus [9,17]. Antibiotic resistance to *S. aureus* is caused by the formation of biofilms that cause the formation of cells that are resistant to antibiotics and persistent cells that are able to adapt to antibiotics independently of the part of the cell that is the target of the antibiotic [18]. In 1960, antibiotic resistance of *S. aureus* to penicillin had reached 80% and the incidence of resistance to methicillin was first identified in the UK in 1961. *Methicillin-resistant Staphylococcus aureus* (MRSA) infection is caused by increased antimicrobial resistance to *S. aureus* due to infection control poor and widespread use of antibiotics [1,18,19,20]. This situation is exacerbated by the slow development of new antimicrobial drugs. Some plants that contain secondary metabolites of essential oils in low concentrations can inhibit the growth of Gram-positive and Gram-negative pathogenic bacteria, and can interfere with or inhibit the formation of biofilms [10,21].

Massoia aromatica Becc. (Lauraceae) can be found notably in Maluku and Papua, in the eastern part of Indonesia. This plant has a long history of usage as a traditional medicine . Massoia plant contains essential oils that can be obtained from the stem, bark, and fruit of the Massoia plant [22]. Several studies have show that essential oils have higher antibacterial activity [23]. One of the compounds contained in *Massoia aromatica* Becc. is C-10 massoia lactone which can inhibit biofilm formation and degrade mono-species and polymicrobial biofilms that have been formed [9]. Pratiwi et al. (2015) reported that the essential oil derived from the bark of *M. aromatica* can inhibit biofilm formation of *S. aureus* and *P. aeruginosa* at a concentration of 0.03% v/v, whereas at a higher concentration (0.12% v / v) can degraded the preformed biofilm. In order to explore for the essential oil potency as antiinfective, we studied the effect on the inhibitory activity against dual species biofilm. So far, the inhibitory power of essential oils from plant extracts of Massoia (*Massoia aromatica* Becc.) against dual species biofilm *S. aureus* and *P. aeruginosa* have never been studied. So, this study was made to determine the effectiveness of Massoia Oil (*Massoia aromatica* Becc.) against dual species biofilm *S. aureus* and *P. aeroginosa.*

2. Experimental

2.1 Plant material

M. aromatica barks from Sorong (Papua) were purchased in the herbal medicine traditional market in Yogyakarta, Indonesia. Identification was performed in the Pharmacognosy Laboratory and registered under Nr: BF/3507 Ident/I/2016.The pulverized barks were steam-hydrodistilled to obtain the essential oil.

2.2 Phytochemistry Analyses Of The Massoia Oil

TLC Densitometry was used to quantify the content of C-10 Massoia Lactone based on methode modified [24]. Ten milligrams of massoia oil were carefully weighed and dissolved in 1 ml of toluene. (Merck, Germany). Three micrograms of sample each sample was spotted on a TLC plate. The plate was eluted using a mobile phase consisted of toluene: ethyl acetate (93: 7)v/v in a 8 cm elution. The plate was dried at room temperature for 5 minutes to remove the solvent. Quantitative analyses was performed by using a CAMAG TLC scanner at a wavelength of 211 nm. Gap dimensions used are 8.00 x 0.40 mm and the scanning speed was 80 mm/s. Identification of compound profile contained in the oil-was done by using the annisaldehyde H2SO4 as spraying reagent. GC-MS analysis was performed to identify the chemical constituent of the massoia oil based on the method of Wu et al. (2008) using GC-MS-QP2010S (Shimadzu, Japan). Type columns used was Agilent HP 1 MS (length 30 m, diameter 0.25 mm, film thickness 0.25 um) and using helium carrier gas. MS is operated using a model of electron impact (EI) at 70eV ionization energy. The identification of compounds made with full scanning the range 28-600 m/z. The resulting chromatogram is compared against a database library NIST (National Institute of Standards and Technology) 12 and WILEY229. GC-MS analysis conducted at the Laboratory of Organic Chemistry Faculty UGM.

2.3 Biofilm Formation Inhibition Test (modified from Pierce et al., 2008)

Polystyrene 96-well flat bottom microtiter plates were used for biofilm assay. The 100 μL suspensions, each containing approximately107 CFU/mL of *dual-species S. aureus and P. aeruginosa* were included in each wells. A total of 100μL media containing massoia oil with concentrations 1% v/v; 0.5% v/v; 0.25% v/v; and 0.125% v/v, was added into each wells. Methanol was used as a solvent control, while negative control is a microbial suspension in the media. Nystatin was used as positive control. The plates were then incubated at 37 ° C for 24 h for intermediate stages of biofilm formation and 48 hours for biofilm maturation process. After rinsing using distilled water three times, the plates were dried at room temperature for 5 min to remove excess water. Afterwards, 125 mL solution of crystal violet (0.1% in distilled water) was added, followed by incubation at room temperature for 15 min. The wells were rinsed gently with running water three times and added ethyl alcohol was added into each of the wells. Optical Densities (OD) were read out at 595 nm. Assays were done in triplicate [25].

OD values were used to calculate % inhibition by the following formula:

[(OD negative control - OD test sample)/OD of the negative control] x 100% ..... (1)

2.4 Observation on utrastructural changes of multispecies biofilms

Observation on ultrastructural changes of biofilm following sample application was conducted by Scanning Electron Microscope (SEM) [26][27]. Cover slips were put into the wells of a round-bottom polystyrene microtiter plate wells containing test suspension that has been treated as for biofilm assay. After incubated at 37 ° C for 24-48 h, cover slips were carefully washed three times with sterile distilled water, then fixed with 2.5% (v / v) glutaraldehyde in buffer cacodylate for ± 24 h. Further dehydration using methanol for 30 min was done to reduce the moisture content Afterward, the samples were dried and given a layer (coating) of gold-palladium in a sputter ion. Observation has done under Scanning electron microscopy with voltage of 10 kV of which the resulted images were processed using Photoshop software.

3. Results and Discussion

3.1 Phytochemistry Analyses Of The Massoia Oil

The yield of Massoia oil obtained from steam hydrodistillation process was 0.75 % v/w. Phytochemistry analyses by using TLC to evaluate the chemical profile of the oil was as described in Fig. 1 of which the C-10 massoia lactone was observed with Rf value as 0.32 and another spot at 0.14 which both were quenched the 254 uv beam and the spot in visible ray was turn to brown color after sprayed with the annisealdehyde H_2S0_4 followed by heated at 105^0C (Figure I).

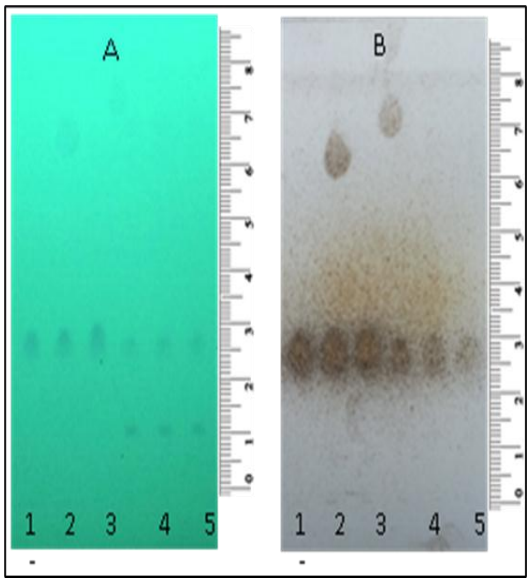

Figure I. Phytochemistry analyses by using TLC to evaluate the chemical profile of the C-10 massoialactone (3,4) and Massoia Oil (1,2) using under 254 uv (A) and after sprayed with anisaldehid sulfuric acid and heating 105 °C for 10 minutes (B).

Table 1. The C-10 massoia lactone (ML) content in the massoia oil

Sampel	% w/v ML on sampel			Mean	SD
Massoia oil	88.75	88.20	85.19	87.38	1.92

The C-10 massoia lactone content in the oil was detected as 87.38% (Std dev.:1%) by the TLC densitometry which was in accordance with the result from the GC-MS data (Table. 2) as 87.7%. The GC-MS profile was as described in table 1 confirming the dominancy of the C-10 massoialactone in the oil.

Rali et al (2007) reported that the chemical constituents of the massoia essential oil extracted from the massoia bark were the C-10 massoia lactone as the major constituent, followed by C-12 massoialactone, benzyl benzoate, linalool, borneol and beta-bisabolene. Our previous experiment also supported the results [9,28].

Table II. Analysis Of Chemical Constituents Of The Massoia Essential Oil From The Massoia Bark						
Peak	Retention Time	SI	Formula	Mol Weight	Compound Name & Abundance	Library
1	22.3	90	C10H16O 2	168	C-10 massoia lakton (87.7%)	WILEY229
2	27.5	90	C10H16O 2	168	C-10 massoia lakton (6.5%)	WILEY229
3	29.2	93	C14H12O 2	212	Benzil Benzoat (5.8%)	NIST12
*SI = similarity index						

3.2. Dual-species of Pseudomonas aeruginosa and Staphylococcus aureus

Massoia oil showed inhibitory and degradation of biofilm consisted of *P. aeruginosa* and *S. aureus* in a concentration dependant pattern. The $MBIC_{50}$ value of the massoia oil inhibition was (figure II.) 0.09% v/v and the degradation was 0.01% v/v.

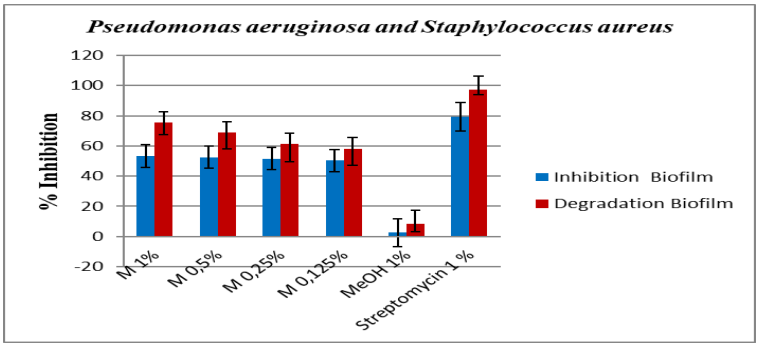

Figure II. Inhibitory Effect of The Massoia Oil towards Dual Species Culture Biofilm of *P.aeruginosa- S.aureus*.

3.3. Observation on Utrastructural changes of biofilm following massoia oil application

SEM was used to observe the differences in morphological structure of the biofilm following massoia oil treatment **[**29**].** Based on preliminary data, observation was performed on massoia oil in concentration 0.25% v/v. Untreated biofilm was used as negative control.

Treatment of the *S. aureus* and *P. aeruginosa* dual species biofilm with the massoia oil 0.25% v/v caused a decrease in the number of adhesions and cell density, as well as the inhibition of biofilm formation occurs as indicated by the rupture of the cell membrane and shrinks, causing cell death and decreased the number of cells are formed (figure III).

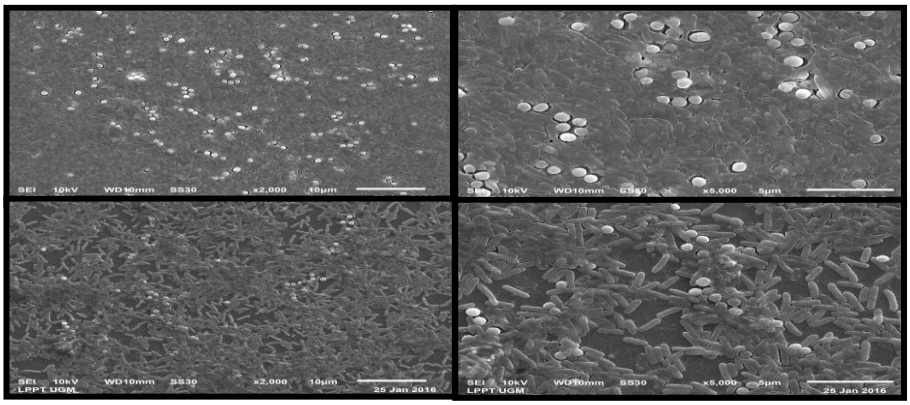

Figure III. Scanning electron microscopy micrographs of the 28 h Dual-species of *S. aureus- P. aeruginosa* biofilms on microtiter plates. **(a)** Biofilm formed in the absence of massoia oil, **(b)** Inhibition of established biofilm treated with 0.25% v/v of massoia oil (after 24 h) is illustrated.

Untreated dual-species biofilms of *P. aeruginosa* and *S. aureus* exhibited smooth cell walls and cell densities were solid. The growth of S. aureus was observed to be less dominant than *P. aeruginosa* which may be caused by phenazine derivative produced by *P. aeruginosa*. This compound affected the integrity of the cell wall as well as cause mutations in *S. aureus*. In dual species culture of *P. aeruginosa* and *S. aureus*, there is a peptidoglycan derivative, *N*-acetylglucosamine, which induces *P. aeruginosa* to produce pyocyanin which exerts antimicrobial and toxin effects, thereby reducing the viability of *S. aureus* in biofilms. it is also due to the production of diguanylate cyclase, SiaD, which is activated by Psl [30,31].

Several studies have also stated that C-10 massoialactone can inhibit the formation of monospecies and polymicrobial biofilms from several microorganisms such as *C. albicans, E. coli, P. aeruginosa, S. aureus* and can degrade the formed biofilm [32,9].

4. Conclusions

The massoia essential oil caused the inhibition of the biofilm formation and degraded the preformed biofilm dual cultures tested. SEM revealed that the massoia oil caused cell ruptured of the *P. aeruginosa* and no observed changes in the S. aureus cells despite less density of cell was observed.

5. Conflicts of Interest

The authors declare no competing interests

6. Funding

This research is funded by the Republic of Indonesia Ministry of Research-Technology and Higher Education (Nr. 619/UN1-P.III/LT/DIT-LIT/2016).

7. Acknowledgments

This manuscript is a part of graduation thesis of Mentarry Bafadal with the supervisor Triana Hertiani and Sylvia Utami Tunjung Pratiwi. The authors gratefully acknowledge the Pharmaceutical Biology Department, the Faculty of Pharmacy, Gadjah Mada University, for facilitating the research activities.

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