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**Anti-biofilm Activity of EtOAc Extract from Indonesian Mangrove-Derived Fungi against *Staphylococcus aureus***

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cultivated fungi and extracted the material, and screening anti-biofilm

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| *\*Corresponding author:* | **ABSTRACT**  The abundance of natural resources in coastal areas offers huge potential for the discovery of medicinal compounds. Because mangrove has the ability to grow in challenging ecosystems, it is reasonable to expect mangrove-derived fungi with diverse potential as drugs. Mangrove-derived fungi are still unexplored as a source of bioactive metabolites that are important in developing biopharmaceuticals. This study aimed to screen ethyl acetate extract from fungi associated with mud and mangrove plants as a source of anti-biofilm agents. In this study, eight isolates of fungi associated with mangroves fermented on selective media of shrimp shell waste and the EtOAc extract were tested against *S. aureus* bacteria. Compounds that have anti-biofilm activity can be observed by staining with crystal violet and measuring their optical density (OD) at 630 nm. The results showed that isolates 20AA03RF, 20BA04RF, 20BB04RF, 20BA0502RF, 20BB0501RF, 20CD01RF, 20CL0102RF, and 20CL02RF had inhibitory activity against biofilm formation at a concentration 250 µg/mL. The 20CD01RF isolate had the greatest biofilm inhibitory activity compared to other samples and was selected for further research. The results of this study are important to be used as the main information for further research in the search for anti-biofilm agent compounds.  ***Keywords****: anti-biofilm, fungi, mangrove, Staphylococcus aureus* |
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**Introduction**

In recent years, the failure of treatment using conventional antibiotics in the medical world has increased. World Health Organization reports the expansion of multi-drug resistant infections in 2,164,568 people in 66 countries [1]. This is exacerbated by the formation of biofilms by multidrug-resistant bacteria. The presence of biofilm formation increases the complexity of infection by altering antimicrobial susceptibility and rendering conventional treatment unusable [2]. The presence of bacterial biofilms surrounded by extracellular polymeric substances (EPS) [3] leads to a 10 to 1000-fold increase in resistance compared to planktonic cells [4] making it difficult to overcome. EPS consists of proteins, lipids, polysaccharides, and extracellular DNA that play a role in pathogen infection [5]. The extracellular matrix formed in the biofilm can penetrate the compound to the deepest layer of the biofilm [6].

Currently, researchers were looking for alternative anti-biofilm agents that are safe, cost-effective, and more environmentally friendly by using materials that are biodegradable. However, the development of the anti-biofilm compound is still under-investigated [7]. The difficulty of eradicating biofilm infections and the limited availability of effective drugs as anti-biofilm agents have made natural products one of the leading sources of drugs [8]. Natural products have a lower toxicity effect than synthetic products [9]. The presence of structural complexity with more stereogenic centers and fewer halogen or nitrogen atoms [10] in NPs is expected to provide attack on more difficult targets [11].

Plants have a number of sources of compounds, especially their secondary metabolites which have the potential as antibacterial, antifungal, and antibiofilm [12]. Compounds derived from plants have the 5 largest classes of activity to inhibit the formation of biofilms, namely phenolics, essential oils, terpenoids, lectins, alkaloids, polypeptides, and polyacetylenes [13]. Previous studies have reported that several alkaloids, namely marine bisindole [14] and isoquinolines [15] can inhibit the formation and destroy bacterial biofilms. Fungi originating from marine organisms showed a considerable anti-biofilm effect against Gram-positive and Gram-negative bacterial strains [16].

Mangrove is one of the plants that live in a unique ecosystem located between the coast and the sea scattered on the coast of Indonesia, especially Lampung. The place where it grows is always flooded with water with high salinity [17]. Mangroves have been shown to interfere with the growth of bacteria, planktonic fungi, and sessile [18]. Microbes originating from mangroves have adapted to soil acidity, salinity, temperature, and constant tidal changes. Characteristics of marine microbes that are different from the terrestrial environment produce complex secondary metabolites that can provide new chemical structures [19].

One of the microorganisms, fungi, is a group of organisms that has the most important sources of new bioactive compounds [20]. Fungi are known to grow in wet environments suitable for biofilm development, but fungi protect themselves by synthesizing compounds that block biofilm formation [21]. Endophytic fungi derived from mangroves promise to obtain unprecedented drug structures with excellent bioactivity [22].

Many researchers worked on bioactive compounds from plants for the discovery of novel natural anti-biofilm compounds. For example, the anti-biofilm properties from mangrove found that ethyl acetate extract from *Avicennia marina* leaves showed a highly promising antibiofilm activity [23]. Besides that, microbes originating from mangrove mud are known to inhibit the formation of *S. aureus* biofilms at 1/8 MIC (1.5625 mg/mL) which is the lowest test concentration [24].

In view of the facts mentioned above, our research has focused on the discovery of compounds for the treatment and cure of biofilm formation. In the subsequent step of our investigation, a reliable methodology is needed to measure biofilms. There are several methods that have been used for the detection and measurement of microbial biofilms in response to agents such as quantitative PCR, checkerboard assay, plate counting, and microtiter plate [25]. In this study, we have evaluated the anti-biofilm compound effect against *S. aureus* by the microtiter-plate test to measure the effect of agents against biofilm production, in which the optical density (OD) stained bacterial film is determined using a spectrophotometer. Staining was carried out using crystal violet (CV) to measure the total biomass consisting of bacteria and extracellular polymeric substances (EPS) in the biofilm [26].

The diversity of microorganisms in soil and mangrove plants, especially fungi, and its potential is still under-explored. Therefore, this study evaluated the ethyl acetate extract prepared from fungi associated with mud and mangrove plants as a source of anti-biofilm agents. In this study, eight isolates of fungi were associated with mangrove fermentation on shrimp shell waste selective media and screened for *S. aureus*. The extracts tested showed potential as anti-biofilm agents which will be followed up with large-scale development and purification using a bioassay separation guide to obtain the structure of the compound.

**Material and Methods**

**Biomaterials**

***Isolate Mangrove-Derived Fungi***

The isolates used came from mud and mangroves which had been isolated from the Sriminosari mangrove area, East Lampung in November 2020. The isolates selected were from the roots (20AA03RF, 20BA04RF, 20BA0502RF), stems (20BB04RF, 20BB0501RF), leaves (20CD01RF), and mud (20CL0102RF, 20CL02RF) deposited at UPT-LTSIT, Lampung University. All isolates were rejuvenated using MEA TSB media.

***Clinical Pathogenic Bacteria***

The clinical pathogenic bacteria *Staphylococcus aureus* used in this study came from Abdul Muluk Hospital, Bandar Lampung, Indonesia. Bacterial maintained was carried out using Tryptic Soy Agar medium (TSB 3%, agar 2%, and distilled water 100 mL) [27].

***Cultivation and Extraction***

Mangrove derived-fungi were cultivated on shrimp shell waste media that had been blended into smaller pieces. The fungal inoculum was prepared in sterile artificial seawater (ASW) supplement with ME TSB medium and incubated for 4-7 days under static conditions at room temperature. Then, a total of 1:10 (v/v) inoculum was transferred to the shrimp shell medium and incubated for 14 days under static conditions. Cultivation was carried out on 2 L Erlenmeyer flask containing 100 g small pieces of shrimp shell waste. The results of the cultivation were extracted using EtOAc solvent to extract secondary metabolites present in the sample. Each process was done with 3 repetitions. The filtrate was filtered and concentrated using a BUCHII vacuum rotary evaporator at the temperature 40°C and pressure 95 mbar. The sample stock solution was prepared in a concentration 0.5 mg/mL for anti-biofilm screening.

***Screening of Anti-biofilm Assay***

Anti-biofilm screening was carried out to determine the ability of the extract to inhibit the adhesion of *S. aureus* bacteria cells using micro titer 96-well plate. The assay was done using crystal violet (CV) indicator method with several modifications [28]. First, *S. aureus* was prepared which had been cultured on Tryptic Soy Broth (TSB) agar containing 3% (w/w) TSB and 2% (w/w) agar which was incubated in an incubator at 37°C for 24 hours. The inoculum was prepared by culturing 3-4 ose of bacteria into 3 mL of Tryptic Soy Broth (TSB) for 18 hours using a shaker. The suspension was diluted 1:100 on TSB medium with the final concentration 1% (contained 40% glucose). A total of 100 μL bacterial inoculum of approximately 5 × 106 cells/well and 100 μL of growth medium TSB 1% was added to the well as growth control. To the test compound, 100 μL of the compound with a concentration 250 µg/mL was added. MeOH pa 12.5% solvent was used as a negative control. In the contamination control, 200 μL TSB medium 1% was added. Plates were incubated for 18 hours at 37°C. After incubation, the media was emptied and the nonadherent bacteria were removed by washing each well with 3 repetitions using Phosphate Buffer Saline (PBS) solution. The plate is turned over to speed up the drying process. The plate was dried in the oven for 1 hour to repair the damaged cells during the washing process. The remaining bacteria were stained with the addition 200 μL of 0.1% (w/v) crystal violet (CV) at room temperature for 10-15 minutes. Then, CV washing was carried out using distilled water with 5 repetitions to remove excess stains and let the plate wells dry for 15 minutes. The stage ended with the addition of 200 μL 95% ethanol (w/v) to dissolve crystal violet and the optical density (OD) value was measured at absorbance of 630 nm using a Hospitex Diagnostics instrument. Each test was done in triplicate.

The anti-biofilm activity of test substance was calculated using the following formula:

Anti-biofilm activity% =

**Characteristics of Selected Isolate**

***Macroscopic***

The selected fungus was observed visually appearance by observing the color of the surface fungus that had been inoculated on Potato Dextrose Agar (PDA) media for 4-7 days.

***Microscopic***

The fungus was placed on a coverslip and covered with a preparation and then identified morphologically using a light microscope Observer Axio Zeiss Imager A1. ME and TSB agar media were poured on sterile petri dishes and allowed to solidify. Fungi were streaked on it at an angle of 45° and incubated at temperature 37°C for 3-4 days [29].

***Thin Layer Chromatography (TLC) of Selected Isolate***

Thin Layer Chromatography (TLC) was carried out to determine the characteristics of the components present in the compound. The crude extract of CD01 was examined using silica plate F254 as the stationary phase and a combination of *n*-Hexane and Isopropyl alcohol (IPA) (7:3) as the mobile phase. After elution, spots or stains were seen under a UV lamp at 254 nm. The TLC plate was dipped in cerium sulfate reagent to reveal the TLC results stain, then dried on a heater. Cerium sulfate reagent is used to determine the content of organic compounds in the sample by marking the appearance of blackish-brown stains. Further observations were made using Dragendorff's reagent and ninhydrin. Dragendorff reagent was used to determine the presence of alkaloid compounds (N-tertiary group) which was characterized by the formation of orange stains on the TLC results. Ninhydrin reagent was used to determine the presence of amino acid compounds (NH2 group) which was characterized by the formation of purple spots. The heated TLC plate was observed and the Rf value was calculated to determine the components of the compounds present in the extract.

**Results and Discussion**

***Screening of Anti-biofilm Assay***

All fungal isolates were cultivated on shrimp shell fermented media and screened for *Staphylococcus aureus* which was resistant to antibiotics amoxicillin, chloramphenicol, and clindamycin (multidrug-resistant). This study successfully carried out the anti-biofilm screening of crude extract of EtOAc 8 isolates of mangrove-derived fungi. The presence of compounds that have anti-biofilm activity can be observed by staining with crystal violet which will bind to bacterial cells and the extracellular matrix [28]. Wells containing anti-biofilm compounds showed a significant reduction in the intensity of crystal violet staining. Based on the quantitative test results, all isolates had the potential to inhibit biofilm growth with varying percentages of inhibition. The extract of 20CDO1RF (250 µg/mL) showed the largest crystal violet reduction with an OD 630nm value of 0.28 (Figure 1) and a percentage of inhibition of 24.30% (Figure 2). This indicates that the 20CD01RF isolate has the potential to inhibit the growth of *S. aureus* biofilms.

Figure 1. Anti-biofilm screening using a 96-weels microtiter plate. The bars on the graph represent the mean ± SD.

Figure 2. Percentage Inhibition Anti-Biofilm Extract Fungi against *S. aureus* (%)

***Characteristics of Selected Isolate***

Visualization fungus cultured on Potato Dextrose Agar (PDA) medium for 3 days showed white mycelium with greyish-white surface colonies (Figure 3a). Microscopic identification of sporangium 20CD01RF fungus forms filaments (hyphae) that resemble elongated rod-like structures and the tip looks like a head shape. As shown in Figure 3b, the isolate showed hyphae, conidiophores, vesicle, conidia head globos, which are characteristics of the genus *Aspergillus* [30]. Based on the characteristics, 20CD01RF has the same characteristics as fungus from the genus *Aspergillus*.

 

1. (b)

Figure 3. (a) Isolate 20CD01RF on PDA media (b) visualization 20CD01RF magnification 400x

***Thin Layer Chromatography (TLC) of Selected Isolate***

The 20CD01RF extract was checked for compound components using the Thin Layer Chromatography (TLC) method. The eluent *n*-hexane: IPA (7:3) was used as the mobile phase and the silica gel plate as the stationary phase. The isolates were checked for their components using a UV lamp at 254 nm, cerium sulfate reagent, Dragendorff's specific reagent, and ninhydrin. Dragendorff's reagent was used to detect the presence of alkaloid compounds, anisaldehyde/sulfuric acid for steroids and terpenes. Ninhydrin reagent was used to detect the presence of amines and amino acids [31]. Based on the TLC stain pattern obtained using Ce(SO4)2 reagent, there are brown spots in the non-polar region with Rf values of 0.8 and 0.7 (Figure 4a). Furthermore, visualization using UV light 254 nm showed that the compound component of crude 20CD01RF detected a conjugated double bond marked by the black spot in Rf value 0.7 (Figure 4b). Dragendorff's specific reagent showed two orange stains, this indicates that there is a tertiary N group in the compound component detected as Rf alkaloid (Figure 4c). Visualization using ninhydrin reagent did not show a purplish stain, this indicates the absence of NH2 groups in the extract (Figure 4d). Based on the visualization of TLC results and Dragendroff's Rf value, it showed that 20CD01RF contains alkaloid compound.

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| (a) | (b) | (c) | (d) |

Figure 4. Results TLC of 20CD01RF using (a) Ce(SO4)2 (b) UV lamp 254 nm (c) Dragendorff reagent (d) ninhydrin reagent.

**Conclusion**

Based on the data above, it can be seen that fungi from mangroves are a potential source of bioactive compounds with varying levels of biofilm activity. Based on this study, the 20CD01RF isolate extract had the strongest activity in inhibiting the growth of *S. aureus* biofilms at concentration 250 g/mL. The results of microscopic identification showed that the 20CD01RF is a type of fungus from the genus *Aspergillus*. Further studies will be carried out on a large-scale fermentation. Purification of the resulting compound will be carried out using a bioassay guidance separation to the elucidate the structure of the compound.

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