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The Activity of Extract from *Rhizophora mucronata* Endophytic Fungi Solid Fermentation Against *Staphylococcus aureus* and *Enterobacter aerogenes*

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ABSTRACT

Endophytic fungi, such as *Rhizophora mucronata*, can produce bioactive compounds from secondary metabolites that have the potential to become alternative antibiotics. This study aims to determine the activity of solid fermentation extract of mangrove endophytic fungi *Rhizopora mucronata* on the inhibition of bacteria *Staphylococcus aureus* and *Enterobacter aerogenes*. The extracts were fermented using rice media for 21 days and tested for antibacterial activity using disc diffusion method. Two of the five fermented extracts inhibited the growth of *Staphylococcus aureus* and *Enterobacter aerogenes* bacteria, namely codes ARM1M1 and DRM3M3. KLT-Bioautography was performed on isolate ARM1M1 to identify compounds that have antibacterial activity. Fermentation time variations were conducted at 0, 4, 8, 11, 14, 16, 18, 20, and 21 days. The disc diffusion method antibacterial test was carried out again to find out on which day of fermentation the extract actively inhibited bacterial activity and HPLC analysis was carried out to determine the retention time of the compound. As a result, *Aspergillus* sp. from *Rhizophora mucronata* code ARM1M1 showed the best inhibition against *Staphylococcus aureus* and *Enterobacter aerogenes* bacteria on day 20 of fermentation, with a retention time of 35 minutes, and an Rf value of 0.4.

Keywords: Endophytic fungi, Rhizophora mucronata, antibacterial, mangrove, solid fermentation

Introduction

Endophytic molds are a group of microorganisms that exist in the tissues of various plants, including leaves, stems, roots, and seeds. However, endophytic fungi are not parasitic or harmful to the host plant. This characteristic allows the symbiotic mutualism that occurs between plants and endophytic fungi due to secondary metabolite compounds (Melliawati & Sunifah, 2017).

Endophytic fungi can produce the same compounds as their hosts even though they have different derivative forms. There are some compound results obtained from endophytic fungi that have higher activity when compared to their host plant compounds. Thus, secondary metabolite compounds produced by endophytic fungi can be developed into alternative antibiotics.

Endophytic fungi can produce the same compounds as their hosts even though they have different derivative forms. In fact, there are some compound results obtained from endophytic fungi that have higher activity when compared to their host plant compounds. Thus, secondary metabolite compounds produced by endophytic fungi can be developed into alternative antibiotics. One of the various plant species rich in bioactive compounds is mangrove plant (Pasappa et al., 2022).

Mangroves are plants with several types of endophytic fungi that produce secondary metabolite compounds in their tissues. Endophytic fungi in mangroves are also part of the second-largest group of marine microbes (Zhou et al., 2018). Mangrove ecosystems are mixed, causing endophytic fungi to have certain ecological, morphological, biological, and physiological

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adaptations. So that it can activate certain metabolic pathways and produce biomolecules (alkaloids, phenolics, saponins, avonoids, terpenoids, steroids, etc.) to survive in a unique ecosystem (Sari & Hasibuan, 2017).

Indonesia has the largest mangrove forest in the world with 202 types of mangrove species spread throughout the country (Wardani et al., 2016). There is one type of mangrove plant that will be the focus of this research, which is *Rhizophora mucronata*. *Rhizophora mucronata* is a mangrove plant that contains alkaloids, tannins, saponins, phenolics, flavonoids, terpenoids, steroids, and glycosides that can be extracted from leaves, stems, roots, and fruit (Maulani et al., 2019).

Material and Methods

Cultivation of endophytic fungi isolate

There are 5 types of isolates obtained from the leaves and roots of R. mucronata with the codes ARM1M1, ARM3L1, DRM2K51, DRM3M3, and SRM6T4. Modified from Bakhtra et al. (2020), the cultivation of endophytic fungi isolates from *Rhizophora mucronata* mangrove plants was grown on Potato Dextrose Agar. Each of the five pure isolates was inoculated on 1000 g sterile rice and then incubated at room temperature for 21 days in a 2000 ml Erlenmeyer flask.

Identification of endophytic fungi

Identification of endophytic fungi was done macroscopically and microscopically. Macroscopic observations were made based on the color, surface, colony edges, and growth of each fungi isolate. Endophytic fungi isolates were grown on a glass slide using a PDA medium for 5 days at room temperature for microscopic observation.

Solid fermentation

Fermentation was carried out by solid-state fermentation using sterilized rice. Modified from Dewi et al. (2011), 1000 g of rice washed thoroughly with running water. Rice was sterilized using an autoclave at 121oC for 15 minutes with water 1:1. Room temperature rice was put in a 2000 mL erlenmeyer. Each endophytic mold isolates in the petri dish was cut by 3 mm and inoculated into the rice. Incubated for 21 days at room temperature, and observed until the fungi distributed evenly on the rice before extraction.

Extraction of endophytic fungi

Modified from Yulianingtyas and Kusmartono (2016), fermented fungi soaked for 24 hours using 1000 mL of ethyl acetate distillate solvent. Sonicated for 45 minutes, then filtered using filter paper and a glass funnel to separate the rice with the macerate. Concentrated using a rotary evaporator with a water bath temperature of 40oC. Using nitrogen, the concentrated extract from the endophytic mold fermentation was dried and stored in a refrigerator for further assay.

Disc diffusion

Measure out 31.5 g of Mueller Hinton Agar medium dissolved in 1500 ml of distilled water. Sterilized at 121^{o}C for 15 minutes. Poured into 20 cm petri dishes, 150 ml each. Mueller Hinton Broth media measured 2.1 g, dissolved with 100 ml of distilled water. Sterilized at 121^{o}C for 15 minutes. Poured into test tubes, 6 ml each. One loop needle of S. aureus and *E. aerogenes* bacterial isolates was inoculated into each test tube of MHB medium, and incubated for 24 hours at 37°C. Bacterial turbidity compared to McFarland standard of 1.5 x 10^{8} CFU/mL (Ogofure, et al., 2022).

Bacteria were streaked evenly on MHA media. 20µL of each control was dripped onto the disc paper. Positive controls (chloramphenicol and cefoperazone), negative control (methanol), and fermented fungi extract. Incubated for 24 hours at 37°C.

Preparative TLC and bioautography

Modified from Rabel and Sherma, (2017), the diluted extract was dripped on a 1 x 10 cm KLT plate using a capillary tube. The eluent was toluene: methanol 9:1. The plate was eluated and air-dried, UV lighted at 254 nm and 366 nm, fluorescent spots were marked. Fluorescent spots were identified using HPLC by scraped and dissolved using HPLC methanol.

Bioautography method modified from Choma and Grzelak, (2011) the eluate KLT plate will be dipped into liquid media containing bacteria, and incubated for 18 hours at 37°C. The KLT plate was sprayed using TTC reagent, and incubated for 1 hour. The Rf value of the inhibition zone was measured.

Time variation of solid fermentation

The active isolate (ARM1M1) was fermented in a 250 mL Erlenmeyer flask. Variation of extraction time using ethyl acetate distillate solvent on days 0, 4, 8, 11, 14, 16, 18, 20, 21, and 22. The extract was concentrated in an evaporator and dried using nitrogen.

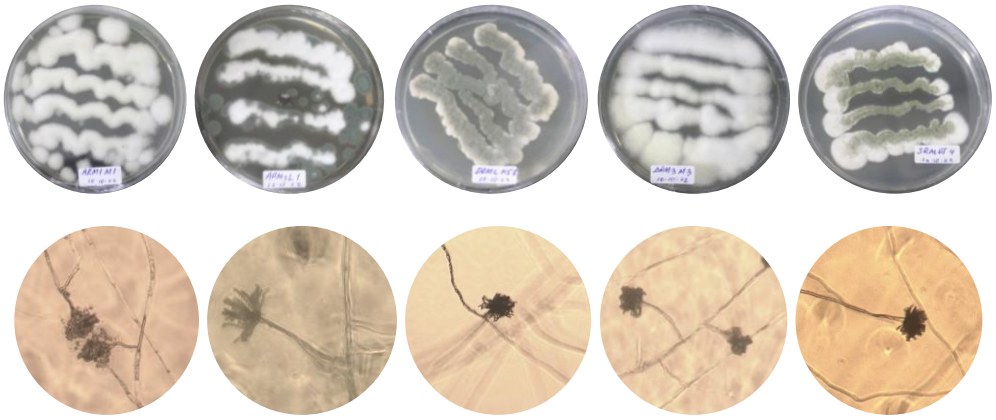
HPLC

The column used in this study was GL Sciences Inertsil ODS - 3, 4.6 x 250 mm, and particle size 5µm. Before injection, the extract was dissolved using methanol HPLC with a concentration of 1 mg/mL and filtered using a 0.22µm PTFE syringe filter. The mobile phase used at minutes 0 and 5 was methanol: 0.1% TFA 5: 95, while minutes 37 and 38 used methanol: 0.1% TFA 100: 0. The flow rate used was 1 mL/minute and detected using UV light 254 nm.

Results and Discussion

Characterization of endophytic fungi isolates

Based on the Figure 1, the species of fungi from the five isolates of *Rhizophora mucronata* mangrove plants is an *Aspergillus* sp. It has macroscopic characteristics of fine velvet white or greenish. In microscopic, it has hyphae fibrous with branches, and spherical conidia.



(a) (b) (c) (d) (e)

Figure 1. Characterization of Endophytic Fungus Isolates (a) ARM1M1, (b) ARM3L1, (c) DRM2K51, (d) DRM3M3, (e) SRM6T4 Macroscopically and Microscopically

The active endophytic fungi extracts

A fermented extract of isolate ARM1M1 can inhibit *E. aerogenes* bacteria by 6.51 mm and S. aureus bacteria by 25.04 mm. The fermentation extract of isolate DRM3M3 can inhibit *E. aerogenes* bacteria by 12.09 mm and by 22.82 mm in S. aureus bacteria. Control (+) Cefoperazone

can inhibit by 8.94 mm and Chloramphenicol by 15.44 mm against S. aureus bacteria. Meanwhile, the (+) control could not inhibit *E. aerogenes* bacteria, possibly due to antibiotic resistance (Table 1).

Compared to research conducted by Kumala and Prawiti (2014) endophytic fungi of mangrove roots of *Rhizophora apiculata* species tested for antibacterial activity on S. aureus and E. coli bacteria with positive control of Cifoprofloxacin antibiotics produced inhibition zones of 20 mm and 17 mm at a concentration of 2000 ppm. The difference in inhibition zone might be affected by the type of antibiotic, bacterial resistance, and concentration.

Table 1. Inhibition Zone Diameter of Solid Fermentation Extracts of Isolates ARM1M1, ARM3L1, DRM2K51, DRM3M3, and SRM6T4

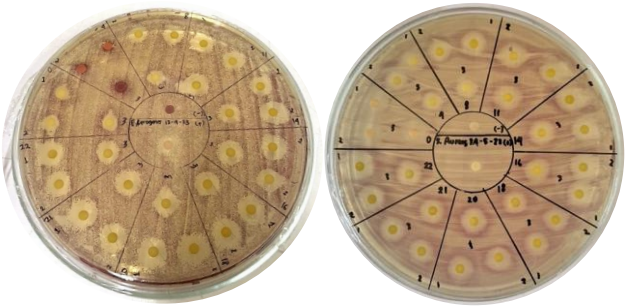
No.	Bacteria	Inhibition Zone Diameter (mm)							
		(-)	Control (+)		Sample				
			CFZ	CRP	1	2	3	4	5
1.	*E. aerogenes*	-	-	-	-	-	-	6,51	12,09
2.	*S. aureus*	-	8,94	15,44	-	-	-	25,04	22,82

The zone of inhibition in the second assay was smaller when compared to the first assay because no dilution was done (Table 2). Extracts number 4 and 5 were chosen because there was inhibition against bacteria at low concentrations. Extract number 4 (ARM1M1) was used as the focus of this study and isolate number 5 (DRM3M3) was kept for further research.

Table 2. Inhibition zone diameter of solid fermentation extracts of isolates ARM1M1 and DRM3M3

No.	Bacteria	Inhibition Zone Diameter (mm)								
		Control			Code					
					1000 ppm		2000 ppm		3000 ppm	
		-	CFZ	CRP	4	5	4	5	4	5
1.	*E. aerogenes*	-	-	-	7,15	7,14	7,24	7,41	7,25	7,49
2.	*S. aureus*	-	11,36	15	7,43	7,08	6,76	7,26	7,27	7,25

Disc Diffusion Assay of Time-variation Fermentation Fungi Extracts



(a) (b)

Figure 2. Antibacterial Activity Test Results of Endophytic Fungi Isolate ARM1M1 with Variation of Fermentation Time 0, 4, 8, 11, 14, 16, 18, 20, 21, and 22 Days. (a) E. aerogenes bacteria; (b) S. aureus bacteria

The diameter of the inhibition zone produced by time-variation fermentation isolates on extract code ARM1M1 against Gram-negative bacteria *E. aerogenes* ranged from 0.00 mm (day 0) to 22.39 mm (day 22) (Figure 2a). The average inhibition zone formed against *E. aerogenes* bacteria on day 0 was 0.00 mm, day 4 was 10.54 mm, day 8 was 16.73 mm, day 11 was 15.59 mm,

day 14 was 16.83 mm, day 16 was 17.91 mm, day 18 was 18.58, day 20 was 22.22 mm, day 21 was 21.34 mm, and day 22 was 22.39 mm. The largest inhibition zone obtained from the fermentation of ARM1M1 extract against *E. aerogenes* bacteria on day 22 can be categorized as a very strong inhibition. Quoted by David and Stout (1971) in Siahaya (2015), there are several classes of strength of antibacterial activity, including diameter <5 mm categorized as weak criteria, diameter 5-10 mm as a medium category, diameter 10-20 mm strong category, and >20 mm into a very strong category.

The inhibition zone of ARM1M1 extract against S. aureus bacteria ranged from 5.60mm (day 0) to 15.38 mm (day 22). The average zone of inhibition against S. aureus bacteria was 5.60 mm on day 0, day 4 was 7.51 mm, day 11 was 14.28 mm, day 14 was 13.17 mm, day 16 was 14.47 mm, day 18 was 14.61 mm, day 20 was 17.31 mm, day 21 was 15.23 mm, and day 22 was 15.38 mm. The largest inhibition zone was measured on the 20th day of fermentation, which amounted to 17.31 mm. According to Tangapo, et al. (2022), the difference in diameter may be affected by the ability of each isolate to produce secondary metabolite compounds. In addition, differences in the cell wall composition of each bacteria affect the inhibition of endophytic fungi fermentation extract.

Graph of the relation between fermentation time variation on the inhibition zone diameter and HPLC peak area

Graphs were made between the variation length of fermentation time to the diameter of inhibition produced along with the area on the HPLC chromatogram profile at a retention time of 35 minutes. Based on the data in Figures 3, both the diameter of the inhibition zone and the area produced at a retention time of 35 minutes appear to increase significantly and optimally until the 20th day of fermentation time variation.

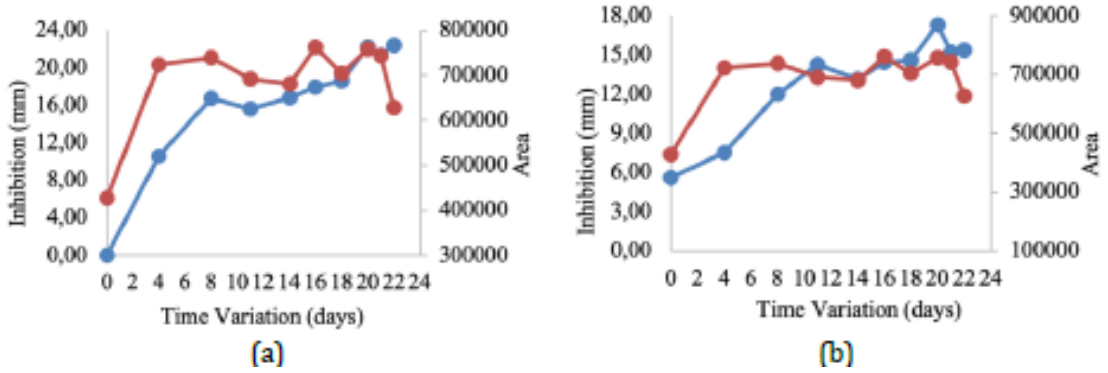


Figure 3. The relation between fermentation time variation on the diameter of E. aerogenes (a) and S. aureus (b) inhibition zone and the area of retention time 35.1 minutes

Conclusion

There are 5 isolates used in this study coded ARM1M1, ARM3L1, DRM2K51, DRM3M3, and SRM6T4. The five isolates were selected through the screening process of the antibacterial activity test using the disc diffusion method, the results showed that 2 of the 5 isolates (ARM1M1 & DRM3M3) were active in inhibiting Gram-positive bacteria S. aureus and Gram-negative *E. aerogenes.* The variation of fermentation time carried out on ARM1M1 endophytic fungi extract has an effect on the diameter of the inhibition zone, the longer the fermentation time, the wider the diameter of the inhibition zone. However, the best time variation was carried out on day 20. The active compound of endophytic fungi Aspergillus sp. from *Rhizophora mucronata* code ARM1M1 which is thought to have inhibition against bacteria S. aureus and E. aerogenes appears at a retention time of 35,1 minutes and has an Rf value of 0.4. Further research, can be done to

determine the type of secondary metabolite compounds produced by the endophytic fungi Aspergillus sp extracted from mangrove *R. mucronata* and the Minimum Inhibitory Concentration test to determine the minimum level of antibacterial substances that can inhibit bacterial growth.

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