Antibacterial Potential of Endophytic Fungi

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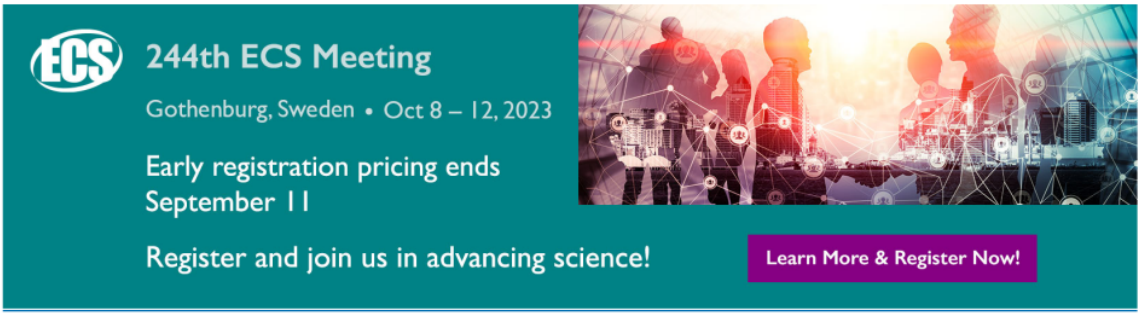

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Antibacterial Potential of Endophytic Fungi Isolated from Mangrove *Rhizophora apiculata* Blume Species at Tanjung Api-Api, South Sumatra, Indonesia

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Abstract. Endophytics fungi are found in *Rhizophora apiculata* mangrove. They are known to produce bioactive compounds that act as an antibacterial, which can be a solution for sustainable use without destroying existing resources. This study aims to determine the types of endophytic fungi on the species of *R. apiculata* as antibacterial. The method used was the isolation and identification of endophytic fungi from the species of *R. apiculata* and the antibacterial activity test using the Kirby Bauer method. The results obtained three types of fungi from five pure isolates, namely *Aspergillus* sp., *Cladosporium* sp., and *Penicillium* sp. Three genera of endophytic fungi were found to have antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa* bacteria. The rate of inhibition against *S. aureus* and *P. aeruginosa* from *Cladosporium* sp. extract respectively were 17.45 ± 1.15 mm and 2.85 ± 0.08 mm, *Aspergillus* sp. was 14.61 ± 1.07 mm and 2.6 ± 0.20 mm, and *Penicillium* sp. was 14.11 ± 0.08 mm and 2.7 ± 0.20 mm. These three isolates showed inhibitory activity against both bacteria. However, the level of inhibition was stronger against *S. aureus* (gram positive) than *P. aeruginosa* (gram negative), especially by marine *Cladosporium* extract found in this study.

Keywords: Antibacterial Activities, Endophytic Fungi, Mangrove Species, *Rhizophora apiculata*, Tanjung Api-api

1. Introduction

Mangrove communities grow in brackish waters or estuaries with a wide range of salinity (1–3). Several species such as *Rhizophora apiculata*, *Avicennia alba*, *Avicennia marina*, and *Sonneratia caseolaris* are the dominant species on the coast of South Sumatra (4,5). *R. apiculata* has been reported to contain secondary metabolites, such as flavonoids, steroids, phenol hydroquinone, and also tannins, which can be used as a potential antibacterial ingredient (6,7). Antibacterial activity of *R. apiculata* was found in the strong inhibition category (8). Bioactive compounds reported including flavonoids, steroids, alkaloids, and saponins (8,9).

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Using *R. apiculata* as an antibacterial can increase the added value of the ecosystem, but it can also have an impact on damaging the environment on a large scale. Isolation of endophytic fungi is the way because it can produce the same bioactive compounds as their host (11,11). Important bioactive compounds as antioxidant, antimicrobial, and anticancer were found from the endophytic fungi *Penicillium sumatrense* isolated from *Lumnitzera racemosa* and *Aspergillus luchuensis* isolated from *Ceriops tagal* leaves (10,12). The strategic value in the field of science in this research is to report antibacterial activity on endophytic fungi in *R. apiculata* species.

Several previous studies have reported potential endophytic fungi as antibacterial originating from marine and coastal organisme. The research from (13) reported endophytic fungi that inhibited bacterial pathogens *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Bacillus subtilis*, and *Escherichia coli*. In addition, endophytic fungi also have the potential for antiviral and anticancer uses (14). In general, the isolation of endophytic fungi from several mangroves has proven its potential as an antibacterial (10). Several compounds were identified using mass spectrophotometry, such as ayamycin, essramycin, benzopyrones, and coumarins derivatives. They were important as antibacterials (15).

Study on the role of endophytic fungi in mangroves is still very limited, especially their potential benefits as marine natural products. This study is important to find the potential active compounds from endophytic fungi isolated from *R. apiculata* mangrove species. This study will be an effort to increase the added value of the mangrove ecosystem locally.

2. Materials and Methods

2.1 Study area

The Tanjung Api-Api mangrove area is wide with various species that dominate, including *R. apiculata*, *A.* alba, *A. marina*, and *S. caseolaris* (3,4). Based on geographic location, the sampling location was located in the intertidal area with coordinate positions S 2.3715833 and E 104.8041083 and had a fairly busy domestic port activity (Figure 1). This area has thick mud with fertile mangrove habitat and another benthic organism (4,16,17). This area is also influenced by the dynamics of water quality parameters, where it is a mixing area between a large mass of fresh water from the Banyuasin River and a mass of salt water from the Bangka Strait, forming a very large brackish area (18–20). This condition certainly supports the mangrove habitat's growth in this area.

Figure 1. Mangrove sampling location in Tanjung Api-api, South Sumatra

Based on the measurement of environmental parameters directly in the Tanjung Api-api mangrove area, pH value was 7.20, salinity was 12 psu, temperature was 25°C, and dissolved oxygen

was 8.04 mg L^{-1}. These parameters could be stated in good condition. Water parameters are very influential on the growth of mangroves. If an estuary has too high a salinity, it will cause excessive osmotic pressure. Besides, the pH range, which tends to be neutral, can impact primary productivity. Water quality impacts mangrove vegetation. Certainly, it will also greatly affect the biochemical processes in the body and its growth (4,12,21).

2.2 Description of mangrove Rhizophora apiculata and isolation of endophytic fungi

The mangrove of *R. apiculata* was found in the Tanjung Api-api. The samples taken were stems, roots, and leaves. The mangrove species *R. apiculata* could be distinguished from other species based on their morphology (Figure 2).

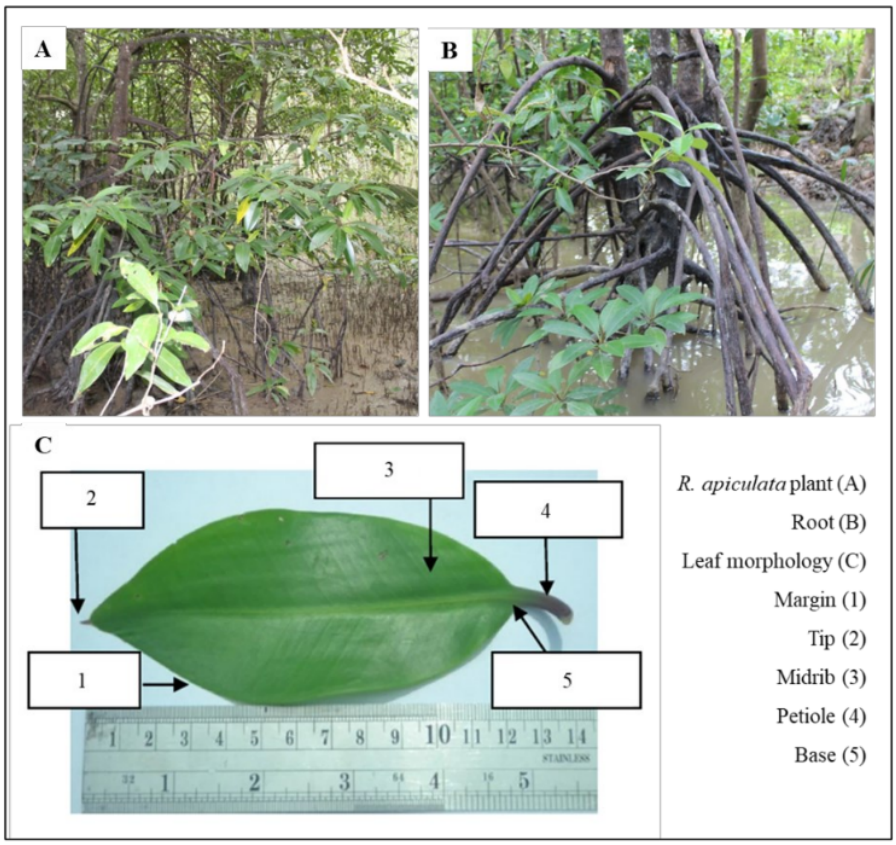

Figure 2. Morphology of *R. apiculata* mangrove

The *R. apiculata* species in the Tanjung Api-api mangrove area had elliptical leaves with red leaf shoots, gray stems, and stilt-shaped roots. According to (22), the leaf of mangrove *R. apiculata* was narrow and elliptical and measured 9-18 cm. The front of the leaves had a yellowish-green color and had small black spots scattered on their surface, and the tip of the leaves was red. The bark of *R. apiculata* had a mosaic-like pattern with a dark gray color. These characteristics proved that the mangrove obtained from the Tanjung Api-api mangrove area was indeed the *R. apiculata* species.

Samples of *R. apiculata* leaves were taken as much as 100 g (wet weight), washed with sterile distilled water, soaked with 70% alcohol for three minutes and 2% NaClO for five minutes, and rinsed again with distilled water for a few seconds. The samples were dried, roughly chopped, and then placed

on Potato Dextrose Agar (PDA) media (39 g per liter compositions). Samples were incubated for five days at a temperature of 28°C (23,24). Isolation was carried out on each growing fungal colony until pure isolates were obtained. The isolate code used the labels P1, P2, etc.

2.3 Morphological and physiological characterization of isolates

The shape, color, elevation, and margin observation was made on each pure fungal isolate. Microscopic observations was using the slide culture method. PDA media was cut into 1 x 1 cm squares and then placed on sterile glass slides. Fungi on PDA media were scraped and covered with a sterile cover glass before incubation. Cover glass of PDA media that had been overgrown by fungi was taken and moved to a new sterile glass preparation with Lactophenol Cotton Blue. Fungi observations were carried out with a light microscope with a magnification of 40X. The fungi was identified according to the fungi identification guide (25,26).

2.4 Antibacterial test of endophytic isolates against pathogen bacteria

Endophytic fungi culture used Potato Dextrose Broth (PDB) media. The composition of PDB was 24 g L^{-1}, where only 500 mL was needed for one type of fungi. The culture was carried out for 21 days. Then the media was macerated with ethyl acetate at a ratio of 1: 1 (w/v) for 24 h. The media was separated with filter paper and then extracted using a rotary evaporator at 47°C (27). The fungi extract was weighed at 0.1 g and then added 1 ml of ethyl acetate, equivalent to a concentration of 10,000 mg L^{-1}. The concentrations were diluted to make solutions of 2,000, 1,000, and 500 mg L^{-1} for each type of fungi extract. *S. aureus* and *P. aeruginosa* as test bacteria were isolated into sterile test tubes containing 10 ml of 0.9% NaCl and homogenized (28,29). A sterile cotton swab was dipped in a solution of suspended bacteria and then scratched on the surface of the media three times on three sides until evenly distributed. The antibacterial test used the Kirby-Bauer method (30). Disc paper measuring 6 mm was immersed in the extract solution to be tested. The extract-soaked disc paper was placed on the surface of the test medium. The negative control used ethyl acetate as the solvent for the extract, while the positive control used disc paper containing 30 g of chloramphenicol. The media was incubated in an incubator at 37°C for 24 h (31).

2.5 Data analysis

The data were analyzed using Microsoft Excel 2019, and the significant value at ($P < 0.05$) was calculated. The inhibition rate was measured based on the diameter of the clear zone of the disc paper using a caliper. Inhibition rate measurement using the formula (1):

$$Inhibition\ rate = \frac{d1+d2}{2} - x \quad (1)$$

Information:
d1 : vertical diameter
d2 : horizontal diameter
x : disc paper diameter

3. Result and Discussion

3.1 Morphological and physiological characteristics of endophytic fungi isolates

The endophytic fungi obtained were five isolates, where there two isolates were marked as P1-1 and P1-2, two isolates marked P2-1 and P2-2, and one isolate marked P3-1. Macroscopic and microscopic observations of each isolate were presented in Table 1.

The endophytic fungi of *R. apiculata were* obtained from the Tanjung Api-api mangrove area. Five isolates derived from three types of endophytic fungi, such as *Aspergillus* sp., *Cladosporium* sp., and *Penicillium* sp. Identification of the type of endophytic fungi based on macroscopic and microscopic observations. Media markers P1-1, P1-2, and P3-1 were *Aspergillus* sp., P2-1 was *Cladosporium* sp., and P2-2 was *Penicillium* sp. Isolates P1-1, P1-2, and P3-1, identified based on macroscopic and microscopic, were believed to be *Aspergillus* sp. It had a black color with white edges and black to

greenish. Microscopically *Aspergillus* sp. had conidia, vesicles, conidiophores, and phialides that filled the vesicles (32). P2-1 isolate, based on microscopic observations, had ramoconidia, conidia, and conidiophores. Morphology characteristics of isolate P2-1 were a dense and smooth texture like velvet. Microscopically, there were ramoconidia, conidia, and conidiophores. Based on these, it was believed to be the fungi *Cladosporium* sp. (33). Isolate P2-2 had circular conidia with chains on the phialid, supported by metulae. Isolate P2-2 had branched conidiophores. Isolate P2-2 was believed to be a fungi *Penicillium* sp., which had chained conidia, conidiophores, metulae, and phialids. Colonies of this type of fungi were orange to reddish (34).

Table 1. Characteristics of endophytic fungi isolated from *R. apiculata*

Isolates Characteristics		Isolate code				
		P1-1	P1-2	P2-1	P2-2	P3-1
Macroscopic	Colour	Black	Black	Black	Pink	Black
	Texture	Smooth	Smooth	Bulky	Smooth	Smooth
	Form	Irregular	Irregular	Irregular	Irregular	Irregular
	Elevation	Flat	Flat	Raised	Flat	Flat
	Margin	Entire	Entire	Undulate	Undulate	Entire
Microscopic	Hyphae	+	+	+	+	+
	- Colour	Hialin	Hialin	Hialin	Hialin	Hialin
	- Bulkhead	+	+	+	+	+
	- Branch	+	+	+	+	+
	Conodia	+	+	+	+	+
	- Colour	Dark	Dark	Hialin	Hialin	Dark
	- Form	Chain rounded	Chain rounded	Chain oval	Chain rounded	Chain rounded
	Conidiophore	+	+	+	+	+
	Ramoconidia	-	-	+	+	-
	Metulae	+	+	-	+	+
	Phialide	+	+	-	+	+
	Vesicle	+	+	-	-	+
	Species	*Aspergillus sp.*	*Aspergillus sp.*	*Cladosporium sp.*	*Penicillium sp.*	*Aspergillus sp.*

There were five isolates of three fungi species, such as three isolates of *Aspergillus* sp., one isolate of *Cladosporium* sp., and one isolate of *Penicillium* sp. Fungi were identified by observing microscopically and macroscopically based on (25,26). Fungi of the genus *Aspergillus* sp. had benefits in the fields of biotechnology and industry (35,36). One species of *Aspergillus* fungus, *Aspergillus niger*, has been used in the food industry and medicinal production (37,38). The fungi *A. niger* produced enzymes that could remove mycotoxins for food product safety (39) and produced organic acids in the form of citric acid from the fermentation process of glucose and sucrose (40). The *Penicillium* sp. fungi were also called antagonist fungi because they contained secondary metabolites that could inhibit the growth of pathogenic bacteria (41,42). This species was a biopesticide and biofertilizer in industry and antibiotics in the health sector (43,44). *Aspergillus* and *Penicillium* fungi were known as important microbes in various needs. However, in this study, *Cladosporium* sp. usually had pathogenic properties that caused damage to the host (45,46). Several studies discussing *Cladosporium* sp. stated that there were differences in properties between those found on terrestrial and marine (47). The tendency of pathogenic properties was owned by terrestrial *Cladosporium*, but marine *Cladosporium* was very different. It had bioactivity with a spectrum of activities such as antibacterial, anti-insecticide, and anti-inflammatory (48,49). The ability of marine *Cladosporium* bioactivity was produced due to environmental dynamics factors that were more supportive in triggering the production of bioactive compounds (50,51).

3.2 Antibacterial ability of fungi isolate extracts

The antibacterial activity of three isolates of endophytic fungi had a stronger inhibition rate against *S. aureus* than *P. aeruginosa*. The detailed inhibition value was presented in Table 2, while the appearance of antibacterial activity on the test media was presented in Figure 3.

Table 2. Inhibition rate of antibacterial activity of endophytic fungi extract

Isolates	Extract Concentrations (mg L^{-1})	Inhibiton rate ± SD (mm)	
		S. aureus	*P. aeruginosa*
Aspergillus sp.	2,000	14.61 ± 1.07	2.6 ± 0.20
	1,000	10.61 ± 0.50	1.38 ± 0.02
	500	7.05 ± 0.91	0.46 ± 0.10
Cladosporium sp.	2,000	17.45 ± 1.15	2.85 ± 0.08
	1,000	16.04 ± 0.83	1.43 ± 0.25
	500	9.46 ± 1.10	0.36 ± 1.60
Penicillium sp.	2,000	14.11 ± 0.08	2.7 ± 0.20
	1,000	11.79 ± 0.24	1.7 ± 0.05
	500	9.08 ± 0.32	0.85 ± 0.21

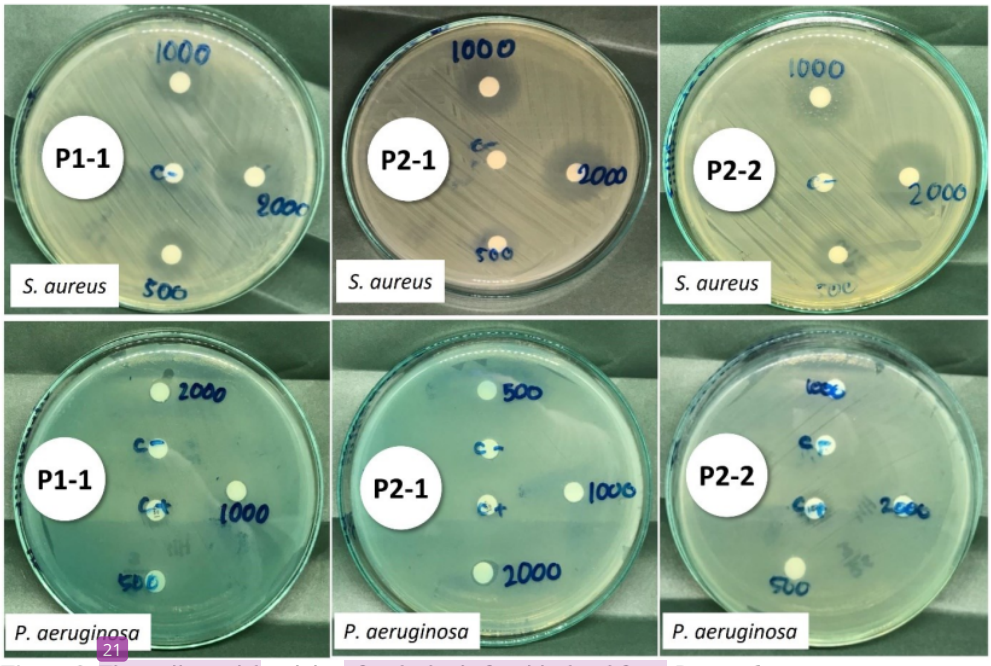

Figure 3. The antibacterial activity of endophytic fungi isolated from *R. apiculata*

Antibacterial activity of endophytic fungi, *Aspergillus* sp., *Cladosporium* sp., and *Penicillium* sp. against *S. aureus* and *P. aeruginosa* bacteria showed the ability to inhibit the growth of pathogenic bacteria. *Cladosporium* sp. had the greatest ability to inhibit the growth of *S. aureus* and *P. aeruginosa* bacteria compared to extracts of *Aspergillus* sp. and *Penicillium* sp. The *Cladosporium* sp. fungi were known to have strong antibacterial properties (52,53).

In this study, *P. aeruginosa* bacteria were more difficult to inhibit growth by various extracts. Several studies had reported that these bacterial strains were very strong in adapting to the environment, so they were quite resistant to various environmental conditions (54,55). This strain was classified as gram-negative bacteria, meaning the cell wall structure was more complex and could form a biofilm

system in self-defense. It was made by polysaccharides under extreme environmental conditions (56,57). Based on this, *P. aeruginosa* was one of the microbial strains that had been studied further to find its weaknesses and reduce the problems caused by this strain. This study resulted in important information for overcoming the growth of *S. aureus* bacteria normally found on the skin and digestive system (58,59). In addition, *S. aureus* was also present in the human environment and caused several health problems. In short, the extract of *Cladiosporium* sp. from mangroves could be useful as an agent to inhibit the growth of *S. aureus* (60,61).

Conclusion

Endophytic fungi isolated from *R. apiculata* mangrove found three isolates from *Aspergillus* sp., one isolate from *Cladosporium* sp., and one isolate from *Penicillium* sp. The three genera of endophytic fungi from *R. apiculata*, such as *Aspergillus* sp., *Cladosporium* sp., and *Penicillium* sp., had antibacterial activity against *S. aureus* and *P. aeruginosa* bacteria. The widest zone of inhibition was obtained from the endophytic fungi extract *Cladosporium* sp., with an inhibition rate of 17.45 ± 1.15 mm against *S. aureus* and 2.85 ± 0.08 mm against *P. aeruginosa* bacteria at a concentration of 2,000 mg L^{-1}. The antibacterial ability in this study would greatly assist in developing potential endophytic fungi in the future.

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