

Antibacterial Activity of Endophytic Fungi Isolated from Betel Leaf (*Piper betle*) Against *Pseudomonas aeruginosa* and *Staphylococcus aureus*

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Abstract

Betel leaf (*Piper betle*) contains antibacterial compounds such as flavonoids, saponins, and alkaloids. Endophytic fungi within plant tissues can produce similar bioactive metabolites. This study aimed to isolate, identify, and evaluate the antibacterial activity of endophytic fungi from young betel leaves against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. This was experimental research. Endophytic fungi were isolated from young betel leaves on Potato Dextrose Agar (PDA) and identified macroscopically and microscopically. Antibacterial activity was tested using the paper disk agar diffusion method. Three endophytic fungal isolates were obtained: Isolate 1 (*Trichoderma sp.*), Isolate 2 (*Rhizoctonia sp.*), and Isolate 3 (*Fusarium sp.*). Against *P. aeruginosa*, Isolate 2 produced an average inhibition zone of 7.33 mm, and Isolate 3 produced 8.00 mm. Against *S. aureus*, Isolate 1 produced 7.33 mm and Isolate 3 produced 7.33 mm. No inhibition was observed for the negative control (DMSO). Endophytic fungi from betel leaves, particularly isolates resembling *Rhizoctonia sp.*, *Fusarium sp.*, and *Trichoderma sp.*, show weak to moderate antibacterial activity against *P. aeruginosa* and *S. aureus* under the tested conditions. Further studies using molecular identification and purified extracts are recommended.

Keywords: antibacterial, betel leaves, endophytic fungi, *P. aeruginosa*, *S. aureus*

Introduction

Indonesia possesses abundant natural resources, with many plants used in traditional medicine due to their accessibility, low cost, and simple processing methods.¹ Among these, the betel plant (*Piper betle*) is widely utilized.² Betel leaf contains bioactive compounds, including flavonoids, saponins, and alkaloids, which inhibit bacterial growth.^{3,4} Previous research has demonstrated that betel leaf extract effectively inhibits *Staphylococcus aureus* and *Pseudomonas aeruginosa*.⁴

S. aureus is a Gram-positive bacterium found on human skin and mucous membranes, capable of causing abscesses, necrosis, and inflammation. *P. aeruginosa* is

an opportunistic pathogen that infects immunocompromised individuals, causing skin, urinary tract, eye, and respiratory infections.⁵

Endophytic microorganisms live within host plants without causing harm and produce secondary metabolites with antioxidant, antimicrobial, and anticancer activities.⁶ Endophytic fungi, in particular, colonize plant tissues (leaves, stems, roots) and often produce compounds identical or similar to those of their host plants. Therefore, endophytic fungi from betel leaves represent a promising source of natural antibacterials.⁷

While betel leaf extracts are known to be antibacterial, to the best of our knowledge, no previous studies have systematically isolated and tested endophytic fungi from *Piper betle* leaves specifically against *P. aeruginosa* and *S. aureus*.⁸ This study addresses this gap. The rationale is that endophytic fungi may produce unique or more potent antibacterial compounds compared to the host plant itself, and they can be cultured independently, providing a sustainable source of bioactive metabolites. This research aims to isolate, identify, and evaluate the antibacterial activity of endophytic fungi from betel leaves against these two pathogenic bacteria.⁹

Method

This study is an experimental research conducted in the Pharmacy Laboratory of Jember University. The research process includes several stages: collecting and preparing betel leaves, culturing endophytic fungi, isolating and purifying endophytic fungal isolates, and testing their antibacterial activity.¹⁰

Sample Collection and Authentication

Fresh young betel leaves (*Piper betle*) were collected from Banjar Village, Licin District, Banyuwangi Regency, East Java Province. Plant identification was performed at the Herbarium of Materia Medica Batu Regency, East Java Province, and a voucher specimen **000.9.3/34/102.20/2025** was deposited.

Test Bacteria

Pseudomonas aeruginosa and *Staphylococcus aureus* were obtained from the Microbiology Laboratory, Faculty of Pharmacy, Jember University, Indonesia. Standard microbiological methods confirmed their identity.¹⁰

Isolation and Purification of Endophytic Fungi

Sterilization of Equipment

Before sterilization, all glassware and heat-resistant equipment are washed with detergent and rinsed with running water. The glassware and heat-resistant tools are then sterilized in an oven at 160°C for two hours. Non-heat-resistant equipment is sterilized in an autoclave at 121°C and 1 atmosphere pressure for 15 minutes. Meanwhile, metal tools are disinfected using a flame sterilization method with a spirit lamp.¹¹

Preparation of Nutrient Agar (NA) Medium

To prepare the Nutrient Agar (NA) medium, 20 grams of NA powder is weighed and dissolved in 1 L of distilled water (20 g/1000 mL) in an Erlenmeyer flask. The mixture is then heated on a stove until it becomes clear. After that, it is sterilized in an autoclave at 121°C for 15 minutes. Once sterilized, the Nutrient Agar medium is poured into sterile petri dishes while still liquid.

Preparation of Potato Dextrose Agar (PDA) Medium

First, 39 grams of PDA powder is dissolved in 1 liter of distilled water (39 g/1000 mL) in an Erlenmeyer flask. The solution is then heated on a stove until it becomes clear. After that, it is sterilized in an autoclave at 121°C for 15 minutes. Once sterilized, the PDA medium is poured into previously sterilized petri dishes, and chloramphenicol antibiotic is added.

Sample Collection and Processing

Fresh betel leaves (*Piper betle*) used in this study were collected from Banjar Village, Licin District, Banyuwangi Regency, East Java Province. The leaves selected were young. Before performing aseptic surface sterilization, the betel leaves were washed thoroughly under running water for 10 minutes.⁴

Isolation and Purification of Endophytic Fungi from Betel Leaves

Before aseptic surface sterilization, betel leaves were thoroughly washed under running water for 10 minutes. Surface sterilization was performed by sequentially immersing the leaves in 75% ethanol for 1 minute, 5% sodium hypochlorite (NaOCl) for 5 minutes, and 75% ethanol for 30 seconds. The leaves were then dried in a sterile Petri dish lined with sterile filter paper. Next, they were cut into ± 1 cm pieces on a sterile object glass. The leaf pieces were then inoculated onto *Potato Dextrose Agar* (PDA) medium supplemented with 0.005% chloramphenicol in petri dishes. The samples were incubated at 25°C for 5-7 days.

The endophytic fungi that grew on the PDA medium were purified by re-inoculating single colonies onto fresh *Potato Dextrose Agar* (PDA) medium and incubating them at 25°C for 5-7 days. Based on observations of colony color and shape, several types of pure fungi were identified on the *Potato Dextrose Agar* (PDA) medium. Each colony with a distinct color or shape was subcultured multiple times until pure endophytic fungal isolates were obtained.

Identification of Antibacterial-Producing Endophytic Fungi

The isolated endophytic fungi were identified using macroscopic and microscopic observations. Macroscopic observations included colony shape and color, while microscopic observations examined spores, conidia, and hyphal shape and size. The fungi were identified by comparing the observed characteristics with reference literature.

Preparation of Test Bacteria

For bacterial preparation, one inoculation loopful (ose) of pure culture from *Pseudomonas aeruginosa* and *Staphylococcus aureus* was used to inoculate slanted NA medium. The cultures were then incubated at 37°C for 24 hours. After incubation, one inoculation loop (ose) of the bacterial culture was transferred into a test tube containing 10 mL of sterile distilled water (Aquadest) and mixed until homogeneous. The bacterial suspension was incubated for another 24 hours and then adjusted to a turbidity level equivalent to the McFarland standard 0.5.

Testing the Inhibition Zone Diameter of Endophytic Fungi from Betel Leaves (*Piper betle*) Against the Growth of *P. aeruginosa* and *S. aureus* Using the Agar Diffusion Method with Paper Disks

0.1 g sample of 5-7-day-old fungal biomass was suspended in 1 mL of dimethyl sulfoxide (DMSO). Sterile blank paper disks were soaked in this suspension for 30 minutes. This method assesses the combined effect of intracellular and extracellular metabolites released from the biomass; however, it does not distinguish between these

fractions. A more standardized approach using filtered culture filtrate or solvent extracts is recommended for future studies.¹¹ Bacterial cultures were adjusted to 0.5 McFarland standard ($\sim 1.5 \times 10^8$ CFU/mL). The bacterial suspension was swabbed evenly onto Nutrient Agar (NA) plates. Fungal-soaked disks were placed on the agar. No positive control antibiotic (e.g., chloramphenicol or ciprofloxacin disk) was included in this experiment. This represents a limitation of the study, as the antibacterial activity cannot be compared to a standard reference. Plates were incubated at 37°C for 24 hours. Inhibition zone diameters (including disk diameter of 6 mm) were measured in millimeters. The test was performed in triplicate (n=3).

Data Analysis

Data was analyzed using the Mann-Whitney U test (non-parametric) to compare inhibition zones between isolates and the control. A p-value < 0.05 was considered statistically significant.

Result

Tables 1, 2, 3, 4, 5, and 6 below present the results of this study.

Table 1. Macroscopic Characteristics of Endophytic Fungal Isolates from Betel Leaves (*Piper betle*)

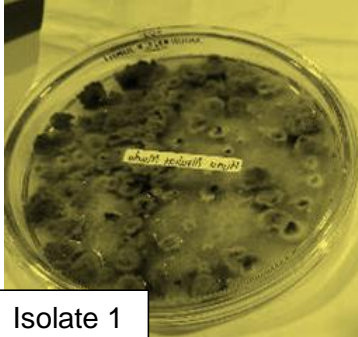
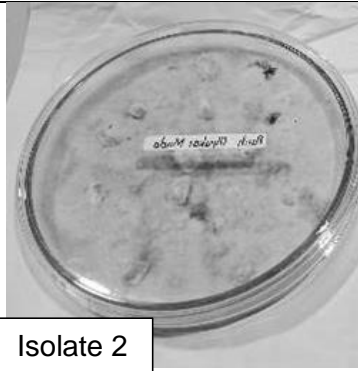
No	Isolate	Description
1		The initial colony color is white, then turns green, and eventually dark green. The colony is round, with a central distribution, and yellowish-brown on the underside.
2		The colony color is white, with a thin, cotton-like surface texture. The colony spreads across the entire agar surface.

Table 1. (Extension)

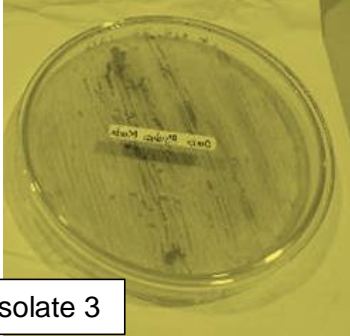
No	Isolate	Description
3		The colony is orange with white edges and has a smooth texture. When streaked, the colony becomes watery and spreads following the direction of the streak.

Table 2. Microscopic Characteristics of Endophytic Fungal Isolates from Betel Leaves (*Piper betle*)

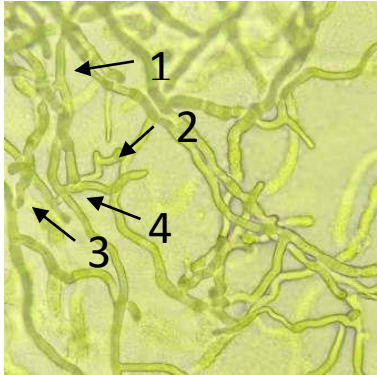

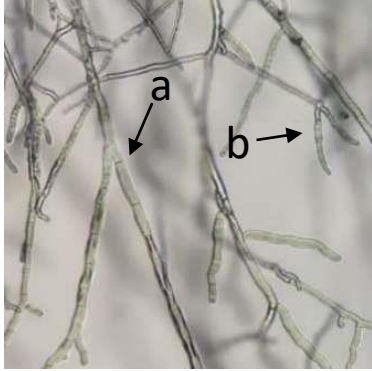
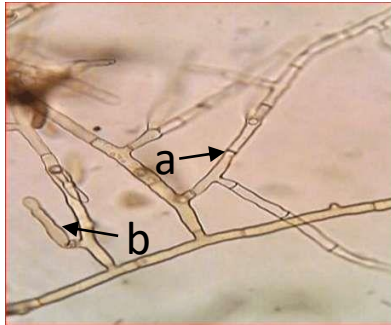
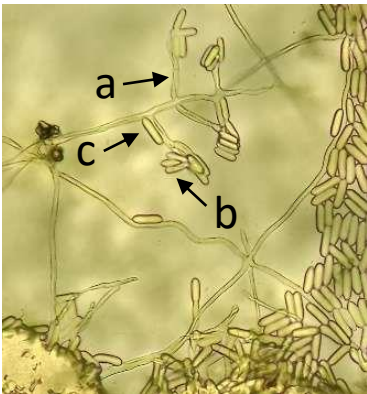
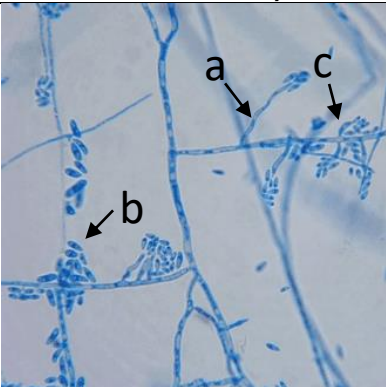
Isolate	Observation Results	Reference (Presumed Identification)	Descriptive
1			<ol style="list-style-type: none"> 1. Conidiophore 2. Conidia / Phialospore 3. Phialide 4. Conidiophore Branches
		<i>Trichoderma sp</i>	
2			<p>Septate hyphae, with hyphal branching at sharp angles, and no conidia.</p> <ol style="list-style-type: none"> a. Septate hyphae b. Hyphal branching
		<i>Rhizoctonia sp</i>	

Table 2. (Extension)

Isolate	Observation Results	Reference (Presumed Identification)	Descriptive
3		 <i>Fusarium sp</i>	a. Conidiophore, long and unbranched b. Microconidia oval-shaped c. Macronidia, long-shaped

From the study on the antibacterial activity of endophytic fungal isolates from betel leaves (*Piper betle*) against the growth of *Pseudomonas aeruginosa* and *Staphylococcus aureus*, conducted for 24 hours at 37°C, the results obtained are as follows:

Table 3. Antibacterial Activity of Endophytic Fungi from Betel Leaves (*Piper betle*) Against the Growth of *Pseudomonas aeruginosa*

Sample	Treatment	Inhibition Zone Diameter (mm) After 24 Hours of Incubation for Each Replication			Total	Average
		1	2	3		
<i>Pseudomonas aeruginosa</i>	Isolate 1 (Green)	0	0	0	0	0
	Isolate 2 (White)	7	8	7	22	7,33
	Isolate 3 (Orange)	8	8	8	24	8
	Negative control	0	0	0	0	0

Table 4. Antibacterial Activity of Endophytic Fungi from Betel Leaves (*Piper betle*) Against the Growth of *Staphylococcus aureus*

Sample	Treatment	Inhibition Zone Diameter (mm) After 24 Hours of Incubation for Each Replication			Total	Average
		1	2	3		
<i>Staphylococcus aureus</i>	Isolate 1 (Green)	7	8	7	22	7,33
	Isolate 2 (White)	0	0	0	0	0
	Isolate 3 (Orange)	7	7	8	22	7,33
	Negative control	0	0	0	0	0

Table 5. Mann-Whitney Analysis Results of Endophytic Fungal Isolates from Betel Leaves (*Piper betle*) Against the Growth of *Pseudomonas aeruginosa*

Sample	Treatment	N	Inhibition Zone of Bacterial Growth				
			Mean	Std.dev	Median	Min.	Max
<i>Pseudomonas aeruginosa</i>	Isolate 1 (Green)	3	0,00	0,000	0,00 ^{cef}	0	0
	Isolate 2 (White)	3	7,33	0,577	7,00 ^{abdg}	7	8
	Isolate 3 (Orange)	3	8,00	0,000	8,00 ^{abdg}	8	8
	Negative control	3	0,00	0,000	0,00 ^{cef}	0	0

Table 6. Mann-Whitney Analysis Results of Endophytic Fungal Isolates from Betel Leaves (*Piper betle*) Against the Growth of *Staphylococcus aureus*

Sample	Treatment	N	Inhibition Zone of Bacterial Growth				
			Mean	Std.dev	Median	Min.	Max
<i>Pseudomonas aeruginosa</i>	Isolate 1 (Green)	3	7,33	0,577	7,00 ^{abef}	7	8
	Isolate 2 (White)	3	0,00	0,000	0,00 ^{cg}	0	0
	Isolate 3 (Orange)	3	7,33	0,577	7,00 ^{abef}	7	8
	Negative control	3	0,00	0,000	0,00 ^{cg}	0	0

Statistical Analysis

The Mann-Whitney U test revealed significant differences between active and inactive isolates ($p < 0.05$). For *P. aeruginosa*, Isolates 2 and 3 produced significantly larger inhibition zones compared to Isolate 1 and the negative control ($p = 0.049$). For *S. aureus*, Isolates 1 and 3 were significantly different from Isolate 2 and the control ($p = 0.049$). There was no statistically significant difference between the inhibition zones of Isolates 2 and 3 against *P. aeruginosa* ($p > 0.05$) or between Isolates 1 and 3 against *S. aureus* ($p > 0.05$).

Discussion

In this study, the antibacterial activity was tested using 100 mg of fresh fungal biomass suspended in 1 mL of DMSO, with each paper disk absorbing approximately 2–3 mg of biomass. However, because the active compounds were not extracted or purified, the concentration of specific antibacterial metabolites remains unknown. Therefore, the results reflect the presence or absence of activity rather than a precise dose-response relationship.

Endophytic fungi are fungi that live inside plant tissues without causing disease to the plant. This study isolated endophytic fungi from betel leaves (*Piper betle*) to determine whether they could act as antibacterial agents against *P. aeruginosa* and *Staphylococcus aureus*. The activity of the endophytic fungi was directly observed against the test bacteria on Nutrient Agar (NA) medium using the diffusion method, in which the antibacterial components diffuse into the agar, preventing microbial growth within it. A clear zone indicates the potential inhibition by the endophytic fungi.

The endophytic fungi successfully isolated from betel leaves numbered 3. For Isolate 1, the initial colony color was white, then turned green, and eventually dark green. The colony was round, with a central distribution, and had a yellow-brown underside. Microscopic observation showed the presence of conidiophores, phialides, and conidia. Based on these characteristics, it was suspected to be *Trichoderma sp.* According to Gusnawaty et al., *Trichoderma sp.* colonies initially appear white and then turn greenish.¹² When the mycelium matures, it turns dark green or yellowish-green, especially in areas with a high number of conidia. The microscopic characteristics include the shape of the conidiophores, conidia, and phialides.

For Isolate 2, the colony color was white, with a cotton-like surface texture. The colony spread across the entire agar surface. Microscopically, there were septate hyphae with sharp-angled branching. Based on these characteristics, it was suspected to be *Rhizoctonia sp.* According to Sari, the macroscopic and microscopic characteristics of *Rhizoctonia sp.* include the absence of conidia and clamp connections.¹³ The mycelium of *Rhizoctonia sp.* develops into a brownish color from its initial white color, and the hyphae are septate with perpendicular branching.

For Isolate 3, the colony color was orange with white edges. The colony texture was smooth, and when scratched, the colony became watery and spread in the direction of the scratch. Based on microscopic observation, it had long macroconidia, oval-shaped microconidia, and long conidiophores, although these did not match the macroscopic appearance. Based on these characteristics, it was suspected to be *Fusarium sp.* This aligns with the statement by Minarni et al., who noted that the morphology of *Fusarium spp.* generally includes long/short macroconidia, oval-shaped microconidia, and long/short conidiophores.¹⁴ According to Sutejo et al., *Fusarium sp.* isolates typically have white colonies, often with purple or red centers. Isolates with many sporodochia tend to have orange-colored colonies.¹⁵

The endophytic fungi isolates from betel leaves were then tested for antibacterial activity against the test bacteria, *P. aeruginosa* and *Staphylococcus aureus*. The aim was to determine whether fungal antibacterial activity could inhibit bacterial growth and to select isolates with potential antibacterial properties. The formation of a clear zone or inhibition zone on the NA medium around the paper disk containing the Betel Leaf fungal isolate was considered a positive result.

The research results showed that the endophytic fungal isolates from betel leaves inhibited the growth of *P. aeruginosa* and *Staphylococcus aureus*, as determined by the paper disk method. For Isolate 2, the average inhibition zone diameter against *P. aeruginosa* was 7.33 mm, while for Isolate 3, it was 8 mm. In contrast, Isolate 1 and the negative control, DMSO, showed no inhibition zone. For Isolate 1, tested against *Staphylococcus aureus*, the average inhibition zone diameter was 7.33 mm; for Isolate 3, it was also 7.33 mm. However, for Isolate 2 and the negative control, DMSO, no inhibition zone was observed.

Previous studies have reported antibacterial activity of direct betel leaf (*Piper betle*) extracts against the same bacterial species. Purnomo and Azzahra found that ethanol extract of betel leaf at concentrations of 10%, 20%, 30%, 40%, and 50% produced inhibition zones against *P. aeruginosa* ranging from 6.5 mm to 14.3 mm, with higher concentrations yielding larger zones.¹⁶ Against *S. aureus*, betel leaf extract has been reported to produce inhibition zones of 10–20 mm depending on the extract concentration and solvent used.¹⁷

In comparison, our endophytic fungal isolates produced inhibition zones of 7.33–8.00 mm using approximately 2–3 mg of crude fungal biomass per disk. While direct comparison is difficult due to different methods (extract vs. crude biomass) and unknown active compound concentrations, our results suggest that endophytic fungi from betel leaves produce antibacterial metabolites that are at least qualitatively similar to those of

the host plant. However, the host plant extract generally produces larger inhibition zones at optimized concentrations. This may be because the host plant contains multiple compounds (flavonoids, saponins, alkaloids) acting synergistically, whereas each fungal isolate likely produces a narrower range of secondary metabolites.

The three isolates showed distinct activity patterns. *Rhizoctonia* sp. (I2) inhibited only *P. aeruginosa* (Gram-negative), *Trichoderma* sp. (I1) inhibited only *S. aureus* (Gram-positive), and *Fusarium* sp. (I3) inhibited both. Several explanations may account for these differences.

First, each fungal genus produces distinct classes of antibacterial compounds. *Trichoderma* species are known producers of peptaibols and glioviridin, which are particularly effective against Gram-positive bacteria by disrupting cell membrane integrity.¹⁴ *Fusarium* species produce a broader range of metabolites, including fusaric acid, beauvericin, and enniatins, some with broad-spectrum activity.¹⁵ *Rhizoctonia* species are less studied, but some strains produce metabolites with specific activity against Gram-negative bacteria.

Second, the cell wall structures of target bacteria differ significantly. Gram-positive *S. aureus* has a thick peptidoglycan layer but no outer membrane, making it susceptible to compounds that interfere with peptidoglycan synthesis. Gram-negative *P. aeruginosa* has an additional outer membrane containing lipopolysaccharide (LPS), which acts as a permeability barrier.¹⁶ *Rhizoctonia* sp. likely produces metabolites capable of penetrating the *P. aeruginosa* outer membrane. In contrast, *Trichoderma* sp. produces metabolites effective against Gram-positive cell walls that cannot cross the Gram-negative outer membrane.

Third, the production of antibacterial compounds depends on growth conditions. The 5-7-day incubation period may have favored the production of certain metabolites over others. *Fusarium* sp. may produce constitutively active compounds, while *Trichoderma* and *Rhizoctonia* may require specific induction signals.¹⁷

A major methodological limitation of this study is the absence of a positive control antibiotic. Without a standard reference such as chloramphenicol or ciprofloxacin, the observed inhibition zones of 7–8 mm cannot be directly interpreted as 'strong' or 'weak' relative to established antibacterial agents. In typical disk diffusion assays, positive controls produce inhibition zones of 20–35 mm depending on the antibiotic and bacterial species. Therefore, the activity reported here should be considered preliminary and indicative only. Future studies must include appropriate positive controls to enable meaningful comparison of potency.¹⁸

The conclusion drawn from this study is that the endophytic fungi isolates from betel leaves exhibit antibacterial activity. Specifically, Isolate 2 and Isolate 3 inhibited the growth of *P. aeruginosa*, while Isolate 1 and Isolate 3 inhibited the growth of *Staphylococcus aureus*, as indicated by the inhibition zones.

Conclusion

Based on the observation data obtained from this study, it can be concluded that: Three endophytic fungal isolates were obtained from young betel leaves (*Piper betle*), presumptively identified as *Trichoderma* sp. (Isolate 1), *Rhizoctonia* sp. (Isolate 2), and *Fusarium* sp. (Isolate 3).

The isolates showed weak to moderate selective antibacterial activity. Isolates 2 (*Rhizoctonia* sp.) and 3 (*Fusarium* sp.) inhibited *P. aeruginosa* with mean inhibition zones of 7.33 mm and 8.00 mm, respectively. Isolates 1 (*Trichoderma* sp.) and 3 (*Fusarium* sp.) inhibited *S. aureus* with mean inhibition zones of 7.33 mm.

Due to the absence of a positive control and the small inhibition zones, these results should be interpreted as preliminary. Further studies with molecular identification,

standardized extracts, and positive controls are required to confirm antibacterial potential.

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