BIODIVERSITAS
Volume 26, Number 2, February 2025
Pages: 963-970

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d260245

Isolation and evaluation of the antimicrobial activity of endophytic fungi from *Orthosiphon aristatus*

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Manuscript received: 18 October 2024. Revision accepted: 21 February 2025.

Abstract. *Satriawan H, Zaimi NA, Eriadi A, Efdi M, Tallei TE, Andayani R, Handayani D. 2025. Isolation and evaluation of the antimicrobial activity of endophytic fungi from* Orthosiphon aristatus. *Biodiversitas 26: 963-970.* O*rthosiphon aristatus*, known as cat's whiskers or Javanese tea in Indonesia, has been used in traditional medicine to enhance urination, treat kidney inflammation, mitigate kidney stones, regulate diabetes, address albuminuria and syphilis, alleviate rheumatism, reduce blood glucose levels, and function as an antibacterial agent. The purpose of this study was to investigate the endophytic fungal resources of *Orthosiphon aristatus* and evaluate its potential as a source of bioactive compounds, particularly against antibiotic-resistant pathogens like Methicillin-resistant *Staphylococcus aureus* (MRSA). Eleven fungal isolates were obtained from the plant's leaves, stems, and roots. The ethyl acetate extract of isolates was subjected to comprehensive antimicrobial screening at a concentration of 5%. Three isolates-OAA1, OAA3, and OAD1-exhibited considerable antibacterial activity, with OAD1 displaying the most significant efficacy, as evidenced by a 12.57 ± 1.09 mm inhibition zone against MRSA. Nevertheless, none of the extracts could suppress the proliferation of *Candida albicans*. Subsequent molecular identification verified that OAD1 was *Aspergillus niger*. The phytochemical analysis revealed that the triterpenoid and flavonoid groups exhibited a positive reaction, whereas the alkaloid and phenol compound groups exhibited a negative response. The findings highlight the potential of endophytic fungi from *O. aristatus* as promising sources of novel bioactive compounds, especially for addressing antibiotic-resistant pathogens like MRSA. The identification of *A. niger* as a powerful antibacterial-producing organism presents promising prospects for the creation of novel pharmaceuticals, particularly regarding multidrug-resistant infections. This study emphasizes the pressing necessity for additional research to identify and characterize the specific compounds that confer antibacterial activity and to investigate their extensive therapeutic applications.

Keywords: Antimicrobial activity, *Aspergillus niger*, endophytic fungi, *Orthosiphon aristatus*, phytochemical test

INTRODUCTION

The rapid emergence and spread of antimicrobial resistance (AMR) have become a major global health crisis, making conventional antibiotics much less effective. AMR is one of the ten biggest threats to global public health. It directly causes about 1.27 million deaths a year and is linked to 4.95 million deaths around the world in 2019. Misuse and overuse of antibiotics in the human, animal, and agricultural sectors are the main causes of AMR. Misuse of antibiotics, self-medication, and the widespread use of antibiotics on livestock to boost growth and keep them from getting sick have all sped up the development of antibiotic-resistant microbes. This kind of abuse not only helps "superbugs" evolve but also makes it easier for them to spread through the environment, for example, through polluted water systems (WHO 2023).

In the past, natural products, especially those made from microorganisms, have been very important in finding new drugs and making them better. Bioactive secondary metabolites, which are the building blocks of many drugs, have been found in fungi. Endophytic fungi, which live inside plant tissues without causing any symptoms, have become an interesting and relatively unknown source of new bioactive compounds that could be used in medicine (Singh et al. 2021).

Indonesia renowned for its extraordinary biodiversity, is home to more than 30,000 plant species, with approximately 7,000 recognized for their medicinal properties (Ameer et al. 2012; Novaryatiin and Indah 2019). These plants have been integral to traditional medicine for centuries, providing remedies for various health issues and potential solutions to modern pharmacological challenges. Among these plants, *Orthosiphon aristatus* (Blume) Miq., commonly known as Java tea or cat's whiskers, holds a prominent position due to its diverse therapeutic applications. Indigenous to Southeast Asia, *O. aristatus* has been traditionally used to treat urinary disorders, kidney stones, and other ailments. The plant's pharmacological properties, including its diuretic, antioxidant, antibacterial, hepatoprotective, antiinflammatory, and antihypertensive effects, have been extensively documented, establishing its importance in modern scientific research (Malahubban et al. 2013: Ashraf et al. 2018; Abd Aziz et al. 2021; Ambursa et al. 2021). Despite the extensive knowledge of *O. aristatus's* pharmacological benefits, one crucial aspect of its biology remains underexplored: its relationship with endophytic fungi. They form symbiotic relationships with their host plants and are known to produce bioactive secondary metabolites. These metabolites often mimic or enhance the therapeutic properties of the host plant, making endophytic fungi a promising area of study for drug discovery. For instance, the discovery of penicillin from *Penicillium chrysogenum* highlights the transformative potential of fungal-derived compounds in addressing major health challenges (Stierle et al. 1993; Böhm et al. 2013).

The association between *O. aristatus* and its endophytic fungi has not been systematically studied, leaving a significant gap in understanding its full therapeutic potential. While the pharmacological properties of *O. aristatus* itself are well-characterized, the bioactive potential of its fungal symbionts remains largely unexplored. This is particularly relevant in the global health crisis posed by antimicrobial resistance. Multidrug-resistant pathogens, such as Methicillin-resistant *Staphylococcus aureus* (MRSA), are a growing threat to public health, underscoring the urgent need for new antimicrobial agents (Guignard et al. 2005). Endophytic fungi have shown great promise as sources of novel bioactive compounds, including triterpenoids, known for their potent antibacterial properties (Chung 2020; Wen et al. 2022). Studies on endophytic fungi associated with medicinal plants have demonstrated their potential to produce diverse secondary metabolites with antimicrobial, antifungal, and anticancer activities (Ancheeva et al. 2019; Gakuubi et al. 2021). However, no comprehensive research has been conducted to isolate and characterize the endophytic fungi of *O. aristatus* and to evaluate their antimicrobial activity against key pathogens. This study aimed to isolate and investigate endophytic fungi from *O. aristatus* and assess their potential as a source of bioactive compounds, particularly against antibiotic-resistant pathogens like Methicillin-resistant *Staphylococcus aureus* (MRSA). This gap represents a missed opportunity to discover natural products that could serve as alternatives to conventional antibiotics, particularly in combating drug-resistant infections. This study addresses the knowledge deficit concerning the endophytic fungal community of *O. aristatus* and their antimicrobial capabilities. This research seeks to systematically isolate, identify, and evaluate the bioactivity of novel microbial resources that could aid in drug discovery and provide sustainable solutions for microbial infections. The global pursuit of novel antimicrobial compounds prioritizes the exploration of endophytic fungi from medicinal plants as a promising and eco-friendly strategy to combat antibiotic resistance challenges.

MATERIALS AND METHODS

Plant material collection and identification

Orthosiphon aristatus specimens were collected from the Medicinal Plant Garden (*Kebun Tanaman Obat* [KTO]) at Universitas Andalas in Padang, West Sumatra, Indonesia, as shown in Figure 1.

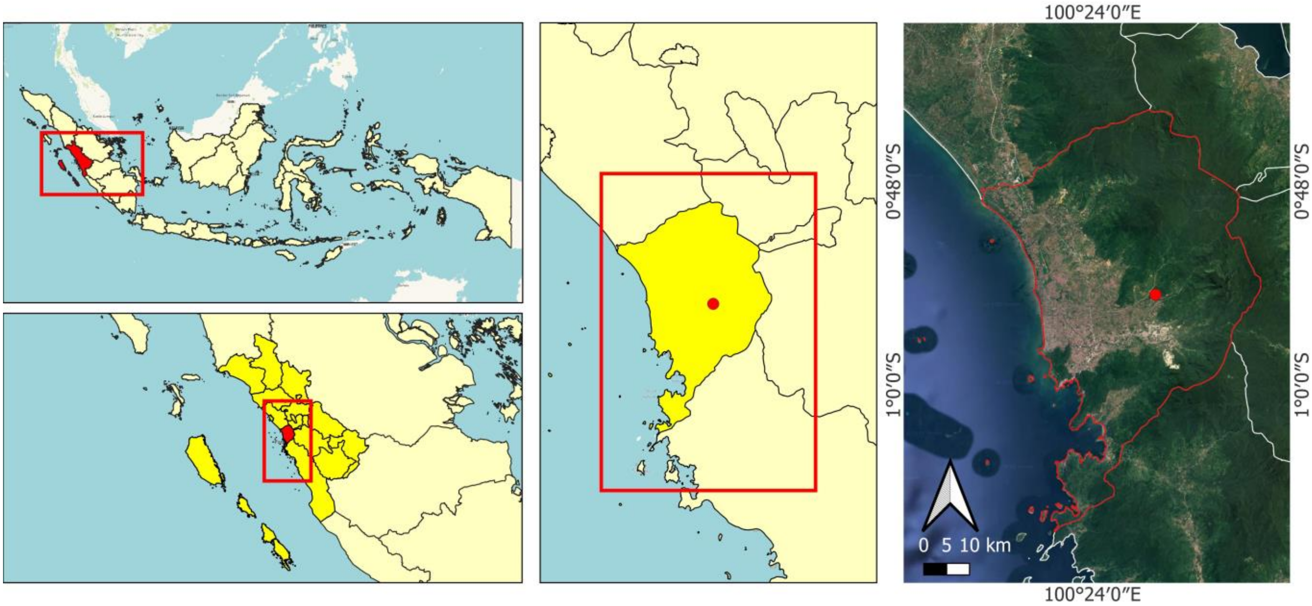


Figure 1. Sampling location of *O. aristatus* in the Medicinal Plant Garden (Kebun Tanaman Obat [KTO]) at Universitas Andalas, Padang, West Sumatra, Indonesia (Herwina et al. 2021)

The plant material, specifically the leaves, stems, and roots, was meticulously selected to ensure that only healthy specimens were used. The selected parts were carefully inspected to confirm they were free from microbial contamination and physical damage. After collection, the plant material was transported to the Herbarium of the Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Andalas. Here, the species was formally identified and authenticated as *O. aristatus*, a member of the Lamiaceae family. The authentication process was documented under letter 461/K-ID/ANDA/III/2022, and the plant material was assigned the voucher number NAZ0012022.

Isolation of fungi

The endophytic fungus *O. aristatus* was isolated through direct cultivation and pour culture technique. The direct cultivation method commences with the sample immersion in 70% ethanol for one minute to sterilize the surface, followed by its placement on Sabouraud Dextrose Agar (SDA). This method enables direct interaction between the plant tissue and the agar, thereby enhancing the proliferation of the fungal isolate. The plates were incubated at 25°C for 5 to 7 days. In the pour culture method, the plant material was washed to eliminate surface contaminants and chopped into small fragments. 10 g of this material was ground and dissolved in distilled water, then diluted to a concentration of 10^{-6} g/L through serial dilutions. A concentration of 10^{-6} g/L was selected as it is a standard in microbiological research to diminish microbial load and promote the growth of distinct colonies. The diluted samples were inoculated onto SDA and incubated at 27°C to 29°C for 5 to 7 days. Colonies displaying unique morphological traits, such as differences in size, texture, and pigmentation, were isolated and purified to yield pure fungal isolates (Kjer et al. 2010).

Production and extraction of pure fungal isolates using rice medium

Pure fungal isolation was cultivated on rice to increase secondary metabolite production. Fragments of fungal colonies were inoculated into 1,000 mL Erlenmeyer flasks containing autoclaved rice medium (100 g rice and 110 mL distilled water). The cultures were maintained at 20-25°C for 4-6 weeks until the fungi thoroughly colonized the rice. After total growth, it was extracted through maceration with ethyl acetate (EtOAc) in a 1:1 ratio for 24 hours, followed by filtration. The EtOAc macerate was evaporated using a rotary evaporator to yield the fungal extract. Subsequently, the extract was prepared to evaluate the antimicrobial activity (Kjer et al. 2010).

Antimicrobial activity screening

The bacterial strains employed for assessing antibacterial activity included *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, and Methicillin-resistant *S. aureus* (MRSA), sourced from the Microbiology Laboratory at the Faculty of Medicine, Andalas University. Bacterial suspensions were prepared to a turbidity standard of 0.5 McFarland and uniformly spread on the surface of nutrient agar plates. Bacterial cultures were grown on nutrient agar (NA), while fungal cultures were grown on SDA. The bacterial cultures were incubated at 37°C for 24 hours, while the fungal cultures were incubated at 27°C for 72 hours. Sterile paper disks were soaked with 10 µL of a 5% preparation of each fungal extract and placed on agar plates inoculated with the corresponding microbes.

The 5% concentration was chosen based on standard practices in antimicrobial testing to ensure a balance between extract potency and solubility. Chloramphenicol (30 µg) and nystatin (100 units) served as positive controls, while Dimethyl Sulfoxide (DMSO) served as a negative control. DMSO was chosen because it is commonly used in antimicrobial assays to dissolve nonpolar compounds while remaining inert in biological systems, ensuring that any observed antimicrobial activity was due to the fungal extracts and not the solvent. While other solvents could have been considered, DMSO is a widely accepted standard for this type of assay. After incubation, the zones of inhibition around the disks were measured to evaluate the antimicrobial activity of the extracts (Handayani and Aminah 2017; Handayani et al. 2018; Sandrawati et al. 2020).

Phytochemical screening of endophytic fungus extract

Phytochemical screening was conducted on the ethyl acetate (EtOAc) extract of the endophytic fungi, demonstrating the greatest antibacterial activity in determining the presence of various classes of secondary metabolites. The qualitative method identified alkaloid, phenol, terpenoid, and steroid compounds in each endophytic fungus extract (Harborne 1984, Handayani and Artasasta 2017; Handayani et al. 2018).

Alkaloid detection

1 mL of the extract was dissolved in 0.5 mL of 10% dilute hydrochloric acid, which was then filtered. Furthermore, three to four drops of Mayer's reagent were introduced to 1 mL filtrate. The presence of yellowish-white precipitate in filtrate indicated the presence of alkaloids.

Flavonoid detection

Flavonoids were detected in 1 mL of extract in 2-4 mL of ethanol, followed by adding 5-10 mg of magnesium powder and 2-4 drops of concentrated hydrochloric acid; a red or pink color appears.

Steroid and terpenoid detection

1 mL of the extract was dissolved in chloroform and subsequently filtered. The filtrate was treated with one to two drops of acetic anhydride (99%) and concentrated sulfuric acid (H_2SO_4). A blue or purple hue in the filtrate showed the presence of steroids, whereas a red coloration of the filtrate indicated terpenoids.

Phenolic detection

1 mL of the extract was dissolved in 0.5 mL of 10% dilute hydrochloric acid and then filtered. Three to four drops of ferric chloride solution were added to the filtrate. A blue color in the filtrate indicates the presence of phenolic compounds.

Macroscopic, microscopic, and molecular fungal identification

Potential antimicrobial endophytic fungi were identified molecularly at the Environmental Biotechnology Laboratory, Indonesia Center for Biodiversity and Biotechnology, Bogor, West Java, Indonesia. The identification process involved macroscopic and microscopic examinations of all endophytic fungal isolates. Macroscopic identification included visual observation of colony morphology, focusing on characteristics such as color, surface texture, and edge appearance. Isolates with identical macroscopic features were considered the same, while those with differing features were treated as distinct, with each unique colony separated as an individual isolate. Microscopic examination involves the observation of fungal structures under a microscope. Fungal mycelia were placed on a glass slide, stained with lactophenol cotton blue solution, covered with a glass coverslip, and examined under the microscope to observe detailed morphological features.

Molecular identification was conducted utilizing the ITS1 primer (F5'-`TCC GTA GGT GAA CCT GCG G`-3') and the ITS4 primer (R5'-`TCC TCC GCT TAT TGA TAT` GC-3') through a two-step process involving DNA extraction and PCR amplification. DNA extraction was performed by Saitoh et al. (2016). The PCR procedure comprised 34 cycles, which included denaturation at 95°C for 5 minutes, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute (Ferrer et al. 2001). The PCR products were sequenced at First Base Malaysia and trimmed and assembled using BioEdit V.7.0.5. Additionally, the sequences were analyzed for identification utilizing the BLAST program on NCBI. The Neighbor-Joining (NJ) phylogenetic tree was constructed utilizing MEGA 7.0 software (Kumar et al. 2016), employing the Kimura-2-Parameter model with 1,000 bootstrap replications.

RESULTS AND DISCUSSION

Antimicrobial activity of endophytic fungi isolated from *O. aristatus*

In this study, 11 endophytic fungi were successfully isolated from various parts of *O. aristatus*, specifically from the roots, stems, and leaves. The code OAA was assigned to six isolates of endophytic fungi obtained from the roots, OAKB label was applied to one stem isolate. The four different leaf-derived isolates were referred to together as OAD1-4. The ethyl acetate extract yields from each fungal isolate showed considerable variation, as detailed in Table 1, with yields ranging from 2.2 g to 14.9 g. The highest yields were obtained from root isolates, particularly OAA5 at 14.9 g, OAA4 at 12.8 g, and OAA3 at 11.7 g. Furthermore, the root samples yielded the most significant number of fungal strains, indicating a potentially higher diversity of endophytes within this part of the plant, as illustrated in Figure 2.

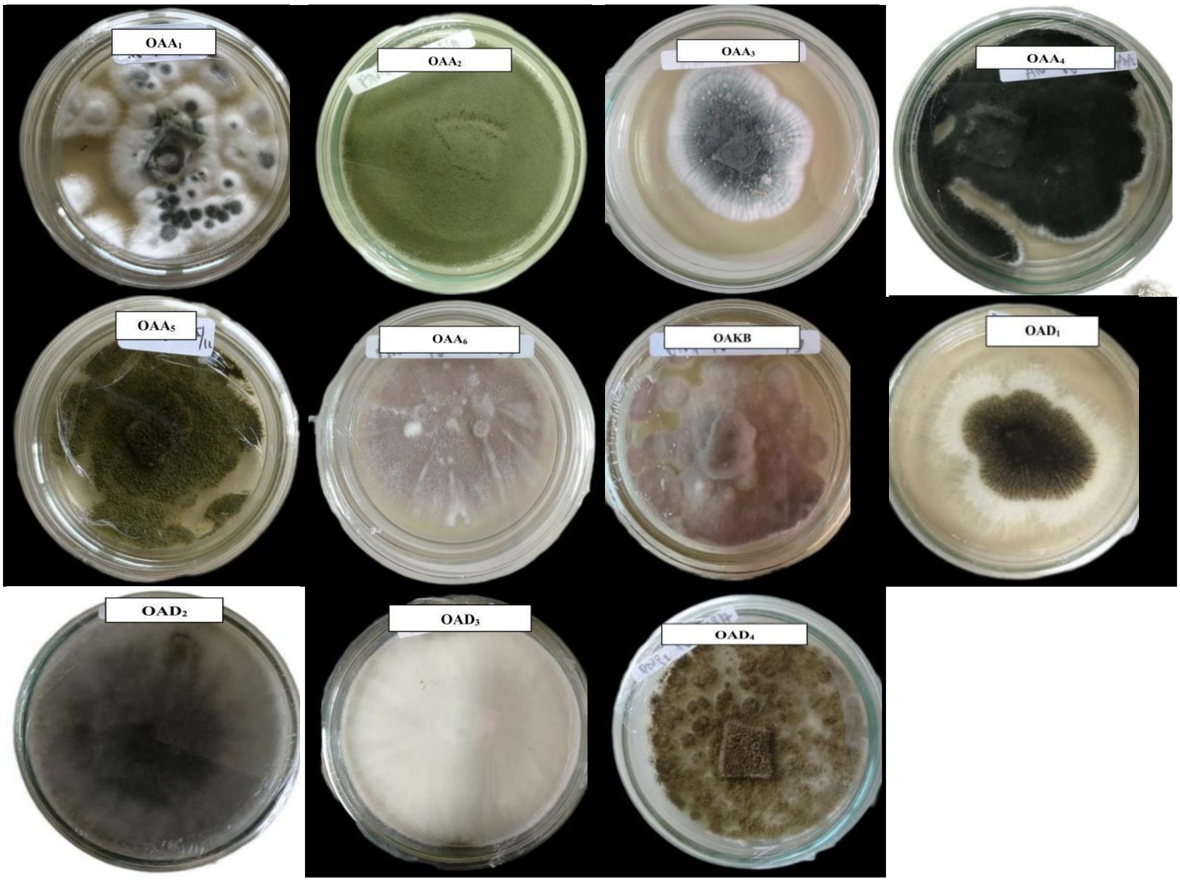


Figure 2. Pure culture of fungal endophytes isolated from *O. aristatus* on SDA medium

These results indicate that the roots of *O. aristatus* are particularly rich in secondary metabolites, contributing to higher extract yields. Endophytes that thrive in roots frequently contribute significantly to the plant's defense system by synthesizing bioactive compounds as a protective strategy, enhancing the quantity and concentration of the produced extract (Chaudhary et al. 2022). The substantial yields from isolates such as OAA3 and OAA5 suggest significant potential for developing new antimicrobial agents, as these extracts are likely rich in triterpenoids, compounds known for their antimicrobial properties. In contrast, lower yields from other isolates, such as OAD2, may reflect a lower production of such metabolites or the need for different extraction techniques. In conclusion, the root-associated endophytic fungi from *O. aristatus* show considerable promise as sources of bioactive compounds.

Antimicrobial activity screening results indicated eleven isolated endophytic fungal strains exhibited varying inhibitory activities against *S. aureus*, *E. coli*, and MRSA. Nevertheless, none of the extracts could suppress the proliferation of *C. albicans* (Table 2 and Figure 3). Davis and Stout (1971) classified the inhibition strength as very strong (≥20 mm), strong (10-20 mm), moderate (5-10 mm), and weak (≤5 mm) depending on the inhibition diameter. The fungal extract exhibiting the most significant inhibitory efficacy against the proliferation of *S. aureus* and MRSA was OAD1, yielding average inhibitory diameters of 10.157 ± 0.79 mm and 12.57 ± 1.09 mm, respectively. OAA1 exhibited an average inhibitory diameter of 12.35 ± 1.03 mm against MRSA, whereas OAA3 demonstrated average inhibitory diameters of 9.42 ± 0.57 mm and 10.16 ± 0.60 mm against *S. aureus* and MRSA, respectively (Table 3 and Figure 4).

The ethyl acetate extract OAD1 exhibits a robust inhibitory effect on MRSA, whereas its inhibitory effect on S. aureus is deemed moderate. The OAA1 extract exhibits significant inhibitory efficacy against MRSA, while the OAA3 extract demonstrates moderate inhibitory activity against both *S. aureus* and MRSA. Ethyl acetate extracts from other isolates, including OAA2, OAA4, and OAA5, exhibited no significant antimicrobial activity, suggesting a lack of antimicrobial compounds. The pronounced inhibition of OAD1 and OAA3 extracts, particularly against MRSA, suggests that these extracts contain potential antimicrobial compounds. Additional research is required to isolate and examine the properties of secondary metabolite compounds that contribute to these antibacterial effects.

Table 1. The EtOAc extract weight (g) of endophytic fungi from *O. aristatus*

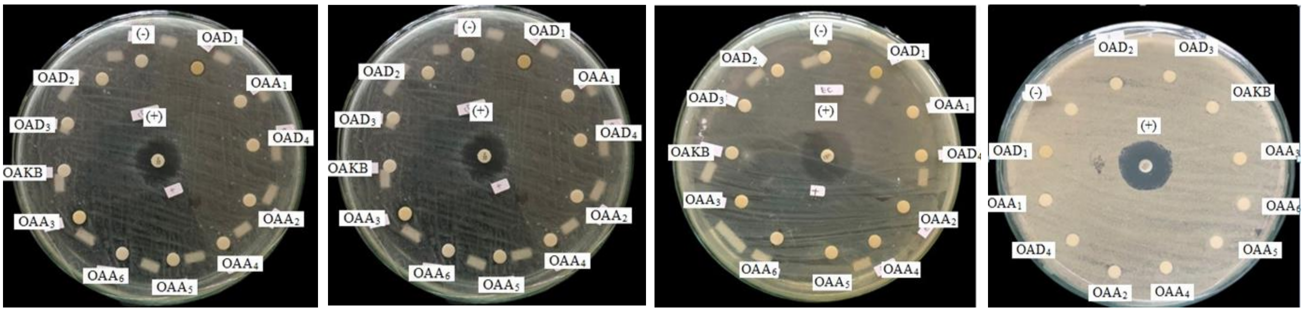
Fungal codes	**EtOAc extract weight (g)**
OAA1	7.2
OAA2	5.3
OAA3	11.7
OAA4	12.8
OAA5	14.9
OAA6	2.9
OAKB	9.4
OAD1	4.3
OAD2	2.2
OAD3	2.9
OAD4	5.5

Table 2. The antimicrobial activity screening results of EtOAc extracts of endophytic fungi isolated from *O. aristatus*

Fungal codes	**Mean inhibition zone (mm)**			
	S. aureus	***E. coli***	**MRSA**	***C. albicans***
OAA1	-	-	8.76	-
OAA2	-	-	-	-
OAA3	7.63	-	8.86	-
OAA4	-	-	-	-
OAA5	-	-	-	-
OAA6	8.47	-	-	-
OAKB	-	-	-	-
OAD1	8.50	-	8.23	
OAD2	-	-	8.71	-
OAD3	-	-	9.04	-
OAD4	-	-	-	-
Positive control	22.00	23.93	31.65	26.65

Table 3. The antimicrobial activity of EtOAc extracts of fungal endophytes OAA1, OAA3, and OAD1.

Fungal codes	**Mean ± Standard deviation**			
	MRSA	***E. coli***	***S. aureus***	***C. albicans***
OAA1	12.35 ± 1.03	-	-	-
OAA3	10.16 ± 0.60	-	9.42 ± 0.57	-
OAD1	12.57 ± 1.09	-	10.157 ± 0.79	-
Positive control	29.33		31.13	



A **B** **C** **D**

Figure 3. The antimicrobial activity screening results of EtOAc extracts of endophytic fungi. A. MRSA; B. *S. aureus*; C. *E. coli*; D. *C. albicans*

Phytochemical analysis

The phytochemical analysis of ethyl acetate extracts is detailed in Table 4. The findings indicated the existence of triterpenoids in all three isolates-OAA1, OAA3, and where flavonoids were identified solely in OAA1. All extracts generally contain triterpenoids; however, only OAA1 contains flavonoids. The extracts' triterpenoids and flavonoids are significantly associated with antibacterial activity. The variation in secondary metabolite composition among the three endophytic fungal extracts (OAA1, OAA3, and OAD1) can be affected by numerous factors, including fungal strain variability, host plant influence, metabolic regulation and gene expression, environmental conditions, culture media, and growth conditions, as well as fungal-fungal or fungal-microbial interactions (Harborne 1984; Wang et al. 2014).

Macroscopic, microscopic, and molecular identification of fungal endophytes OAA1, OAA3, and OAD1

Figure 5 presents the phylogenetic tree generated using the neighbor-joining method, illustrating the evolutionary relationships between fungal isolate OAD1 and related fungal species. This analysis confirmed that OAD1 was closely related to *A. niger*, a species renowned for producing various bioactive secondary metabolites, including antimicrobial agents. The high bootstrap values on key branches reinforce the confidence in OAD1's phylogenetic placement within the *Aspergillus* genus. Macroscopic, microscopic, and molecular techniques were also used to identify the fungal isolate OAD1, which possesses antibacterial properties. The identification of fungi requires both morphological and molecular methods. Microscopic observation of *A. niger*, as shown in Figure 6, reveals structural details of the fungus, highlighting key features involved in its reproductive process. Fungi were classified morphologically based on colony characteristics, color, spore size, texture, shape, growth rate, and conidial attachment (Ordynets et al. 2021).

Aspergillus niger produces several secondary metabolites with potential medicinal applications, including pyranonigrin A and aspergillomarasmine A. Pyranonigrin A is known for its potent antioxidant properties and potential to protect against neurodegenerative diseases, making it a promising candidate for drug development (Tang et al. 2018). In contrast, aspergillomarasmine A serves as an effective metalloprotease inhibitor and has shown promise in combating antibiotic-resistant bacterial infections, particularly those resistant to beta-lactam antibiotics, underscoring its potential in the fight against antimicrobial resistance (Albu et al. 2016). These compounds exemplify the metabolic potential of fungal isolates like OAD1, making their inclusion in the study both relevant and necessary.

Table 4. Phytochemical analysis of EtOAc extracts of OAA1, OAA3, and OAD1 fungal isolates

Fungal codes	Chemical constituents				
	Alkaloid	Flavonoid	Triterpenoid	Steroid	Phenol
OAA1	-	+	+	-	-
OAA3	-	-	+	-	-
OAD1	-	-	+	-	-

Note: (+): Presence; (-): Absence

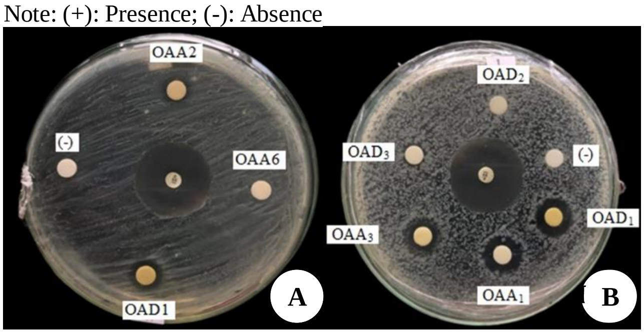


Figure 4. The antimicrobial activity of EtOAc extracts of fungal endophytes OAA1, OAA3, and OAD1. A. MRSA; B. *S. aureus*

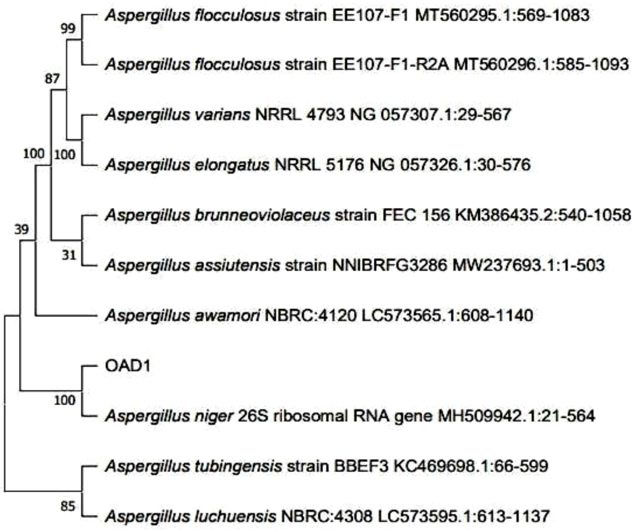


Figure 5. The phylogenetic tree of the fungal isolate OAD1 and its related taxa was inferred using the neighbor-joining method of internal transcribed spacer sequences

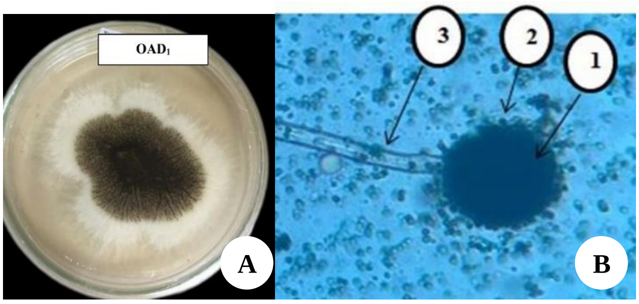


Figure 6. A. Microscopic images of fungal isolate OAD1; B. Showing 1. Vesicle; 2. Conidia; and 3. Conidiophore

Aspergillus sydowii part of the *Aspergillus* genus, is closely related to other fungi that produce secondary metabolites, such as aflatoxins and lovastatin. The phylogenetic placement of *A. sydowii* within this genus emphasizes the importance of marine-derived fungi as reservoirs of novel compounds with significant pharmacological properties

(Boruta et al. 2019; Sandrawati et al. 2020). Therefore, integrating *A. sydowii* into phylogenetic studies enhances our understanding of fungal biodiversity and supports identifying new candidates for drug discovery, particularly in the ongoing battle against antibiotic-resistant pathogens and cancer.

In conclusion endophytic fungi from *O. aristatus* represent a promising source for synthesizing secondary metabolites exhibiting antibacterial properties. This research effectively isolated 11 endophytic fungi from the plant. The EtOAc extract of isolate OAD1, identified as *A. niger*, showed the most significant antibacterial activity, especially against MRSA. The results underscore the importance of *O. aristatus* endophytic fungi in the pursuit of antimicrobial agents, particularly against antibiotic-resistant bacteria. The identified bioactive compounds may be essential in addressing the global challenge of multidrug-resistant pathogens. Future research must focus on isolating and identifying these bioactive compounds utilizing advanced techniques, including mass spectrometry and nuclear magnetic resonance spectroscopy.

ACKNOWLEDGEMENTS

The Ministry of Research and Technology, Indonesia, funded this through the Fundamental Research program (grant number 68/UN16.19/PT.01.03/PL/2024).

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