

Isolation and Characterization of Dryland Microfungi From Northern Insana as Potential Sources of Antibacterial Agents

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ABSTRACT

Microfungi are one of the most extensively studied groups of microorganisms due to their remarkable ability to produce a diverse range of unique bioactive compounds. This study aimed to explore and characterize microfungi isolated from the drylands of North Insana as potential sources of sustainable antibacterial agents. The research methods included the isolation of microfungi, morphological identification, and evaluation of antibacterial activity using the disc diffusion method. Based on morphological characterization, microfungi isolated from the North Insana dry soil showed similarities to three genera, namely Mucor, Penicillium/Aspergillus, and Aspergillus. Antibacterial assays revealed that all three isolates exhibited varying degrees of activity, ranging from moderate to strong, against Staphylococcus aureus. Isolate 1 produced an inhibition zone of 7.6 ± 0.20 mm (moderate activity), isolate 2 produced 10.17 ± 0.06 mm (strong activity), and isolate 3 produced 10.77 ± 0.06 mm (strong activity). According to the Tukey test ($p < 0.05$), isolate 3 showed no significant difference from the standard antibiotic chloramphenicol (11.23 ± 0.15 mm), indicating a comparable antibacterial effectiveness. These results suggest that dryland-endemic microfungi possess adaptive biosynthetic capacities, enabling them to produce antibacterial secondary metabolites in response to extreme environmental stress. This potential reinforces the role of dryland microfungi as promising new sources of natural antibacterial compounds for sustainable biotechnological and health applications.

Introduction

The emergence of bacterial resistance to conventional antibiotics has become a major global challenge in the fields of health and biotechnology[1]. The increasing incidence of infections caused by antibiotic-resistant *Staphylococcus aureus* underscores the urgent need to discover new, effective, and environmentally friendly antibacterial sources[2]. One promising source of such bioactive compounds is microorganisms, particularly microfungi, due to their remarkable ability to produce a wide range of secondary metabolites with diverse biological activities, including antibacterial, antifungal, and antitumor properties[3].

Microfungi play a crucial role in soil ecosystems and possess high adaptive capacity to environmental stress. Extreme conditions, such as drought, nutrient limitation, and high

temperatures—commonly found in dryland ecosystems—can stimulate the biosynthesis of unique secondary metabolites as part of their physiological defense mechanisms[4]. This makes dryland-endemic microfungi attractive candidates for the exploration of novel antibacterial compounds that may not be found in fungi from moist or fertile environments.

The North Insana region, located within the dryland zone of Timor Island, represents a distinctive and underexplored ecosystem. Unlike other Indonesian drylands, North Insana experiences prolonged dry seasons lasting up to eight months, while the broader North Central Timor (TTU) region records relatively low annual rainfall—CHIRPS-based analyses indicate average precipitation ranging from approximately 1,155 to 1,893 mm per year, with substantial areas receiving less than 1,398 mm annually[5]. Its soils in Insana have been shown

to contain very low organic matter (C-organic $\approx$ 0.57%, range 0.09–1.4%) based on recent field surveys in Desa Sekon, Insana District, consistent with nutrient-poor Entisol conditions. The regional geology is dominated by calcareous lithologies and frequent rock outcrops, while the combination of extended dry periods, erratic rainfall, shallow soils, and high diurnal temperature fluctuations results in severe nutrient and water stress[5][6].

Such extreme and fluctuating environmental pressures are expected to drive the evolution of specialized metabolic pathways in soil microorganisms. In particular, microfungi inhabiting these conditions may develop unique biosynthetic responses, potentially leading to the production of secondary metabolites with novel chemical structures and biological activities[7]. However, to date, no comprehensive study has investigated the diversity and antibacterial potential of microfungi from the North Insana drylands. Therefore, this study was conducted to explore and characterize microfungi from this distinctive ecosystem as potential sources of natural antibacterial compounds. The findings are expected to contribute scientifically to the discovery of novel bioactive metabolites from the drylands of Timor Island and to support the sustainable utilization of local biological resources for future biotechnological applications.

Materials and Methods

The materials used in this study included soil samples (10 cm depth) collected from the drylands of Fatumtasa Village, North Insana District; 0.1% Tween 80; 70% alcohol; distilled water; Potato Dextrose Agar (PDA), Czapek Dox Agar (CDA), Yeast Extract Sucrose-Broth (YES-Broth); chloramphenicol antibiotic, Ethyl acetate; lactophenol cotton blue; sterile cotton buds; and sterile cotton. The equipment used included a vortex mixer, glassware, rotary evaporator, a laminar air flow cabinet, an incubator, and a microscope.

Isolation and Identification of Microfungal

A total of 1 gram of soil sample, previously cleaned from roots and plant debris, was placed into a sterile test tube and mixed with 9 mL of

0.1% Tween 80 solution[8]. The mixture was vortexed for 5 minutes to detach fungal hyphae or spores from soil particles. Serial dilutions were then prepared up to 10^{-4} . The 10^{-2} , 10^{-3} , and 10^{-4} dilutions were inoculated onto Potato Dextrose Agar (PDA) medium supplemented with the antibiotic chloramphenicol, using a sterile cotton bud streaked in a zigzag pattern. The plates were incubated at 27 °C for 7 days[9]. Fungal colonies that appeared were subsequently transferred to fresh CDA medium for purification and re-incubated for 12 days.

Morphological identification was carried out microscopically on unique pure colonies using the Lactophenol Cotton Blue staining method. A small portion of the colony mycelium was placed on a glass slide, stained with a drop of the dye, and covered with a cover slip. Observations were performed using a light microscope to identify the main morphological structures, including the shape and branching pattern of conidiophores, the presence of vesicles, phialides, and metulae, the shape and arrangement of conidia, as well as hyphal characteristics (septate or non-septate, hyaline or pigmented). The identification of fungal genera was based on the identification key of microscopic fungi by Barnett and Hunter (1998)[10].

Preparation of Microfungal Cultures

The microfungal isolates used in this study were selected based on their unique and distinctive morphological characteristics, which were considered promising for further exploration, with the preliminary assumption that the isolates were non-pathogenic. This selection aimed to minimize laboratory safety risks and ensure the sustainability of the research. Each selected microfungal isolate was aseptically cultured in Yeast Extract Sucrose Broth (YES-Broth) medium, which is known to support optimal fungal growth while stimulating the biosynthesis of secondary metabolites [11]. The liquid cultures were incubated on a shaker at 110 rpm for 21 days to provide adequate aeration, promoting uniform mycelial growth and maximizing secondary metabolite accumulation[12]. The resulting cultures were subsequently used as the source

of crude extracts for evaluating antibacterial activity against the target bacteria.

Isolation of Microfungal Secondary Metabolites

Secondary metabolites from microfungi were isolated through a two-step separation process. The liquid culture was centrifuged at 4000 rpm for 15 minutes at room temperature to separate the fungal biomass from the medium, and the resulting supernatant was transferred to a new sterile tube. The supernatant was then filtered through a 0.25 μm sterile membrane filter to remove any remaining biomass or spores, yielding a clear filtrate. Extraction was performed using ethyl acetate (1:1, v/v), and the organic phase was concentrated using a rotary evaporator at 40 °C to obtain the crude extract. The dried extract was then redissolved in a minimal volume of ethyl acetate, which was subsequently used for antibacterial activity testing[13].

Antibacterial Activity Test

The antibacterial activity was evaluated using the paper disc diffusion method against

Staphylococcus aureus. Each extract represented a distinct antibacterial material derived from morphologically different microfungal isolates. Bacterial cultures (24 h old) grown on Mannitol Salt Agar were suspended in sterile 0.9% NaCl solution and adjusted to the 0.5 McFarland standard ($\approx 1.5 \times 10^8$ CFU/mL)[14]. The bacterial suspension was evenly spread on Nutrient Agar plates, and sterile paper discs (6 mm in diameter) impregnated with 10 μL of each extract were placed on the surface. Each Petri plate contained three discs—one for each extract—with chloramphenicol (30 $\mu\text{g}/\text{disc}$) used as the antibiotic standard control. The plates were incubated at 37 °C for 24 h. Antibacterial activity was assessed by measuring the inhibition zones around the discs using a digital caliper[15]. All treatments were conducted in triplicate ($n = 3$). Data are presented as mean $\pm$ standard deviation (SD) and were analyzed using one-way ANOVA followed by Tukey's post hoc test ($p < 0.05$) with **Minitab 17** software.

Results and Discussion

Isolation and Identification of Microfungi

Isolation of potential microfungi producing antibacterial compounds was carried out using a soil suspension method using a 0.1% Tween 80 solution, which acts as a surfactant to help release spores from soil particles[16]. The suspension was vortexed to ensure even distribution and optimal release of spores. Next,

serial dilutions were performed to reduce microbial density and facilitate colony separation. This step is crucial for obtaining a single micro fungal colony that is separate from other colonies, allowing for a more efficient purification process[17]. The results of the micro fungal culture after 7 days of incubation at 27°C are shown in Figure 1.

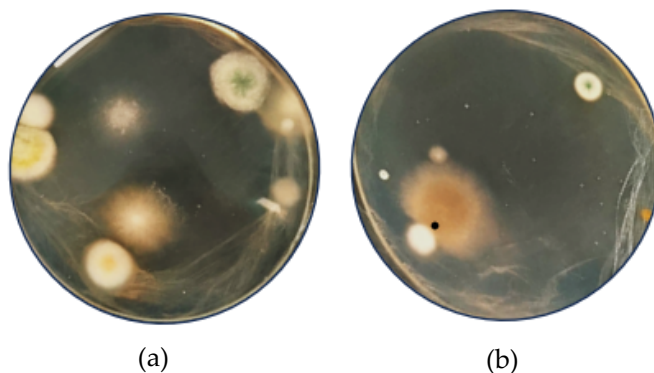


Figure 1. Microfungi colonies resulting from dilution (a) 10^{-3} (b) 10^{-4} .

Based on Figure 1, microfungi colonies grown in PDA media at a 10^{-3} dilution exhibit macroscopic morphological diversity with quite clear differences in colony density. At a 10^{-3} dilution, microfungi colonies still grew very densely and were predominantly white. Most colonies were round, with flat edges and a fuzzy surface. Some colonies exhibited unique characteristics, such as colonies with orange centers and white edges, white colonies with fuzzy centers with light green centers, and deep dark green colonies that formed a star-like pattern with white edges. This morphological diversity indicates differences in the characteristics of the microfungi isolates grown at this dilution. Meanwhile, at a 10^{-3} dilution, colonies grew more sparsely and were clearly separated. Some colonies appeared to be

still in the growth phase, indicated by the appearance of white spots in the media. Colonies that were clearly observable included light brown colonies with fuzzy surfaces, white colonies, and colonies with deep dark green centers. This dilution level provided the best conditions for the selection of isolates based on their distinct macroscopic morphology. Further separation and purification were performed to obtain potential microfungi isolates. After a series of repeated cultures, three candidate isolates were obtained, which were then further examined for their macroscopic and microscopic morphology to determine the suspected species of the microfungus. The morphology of the successfully isolated microfungus is shown in Figure 2.

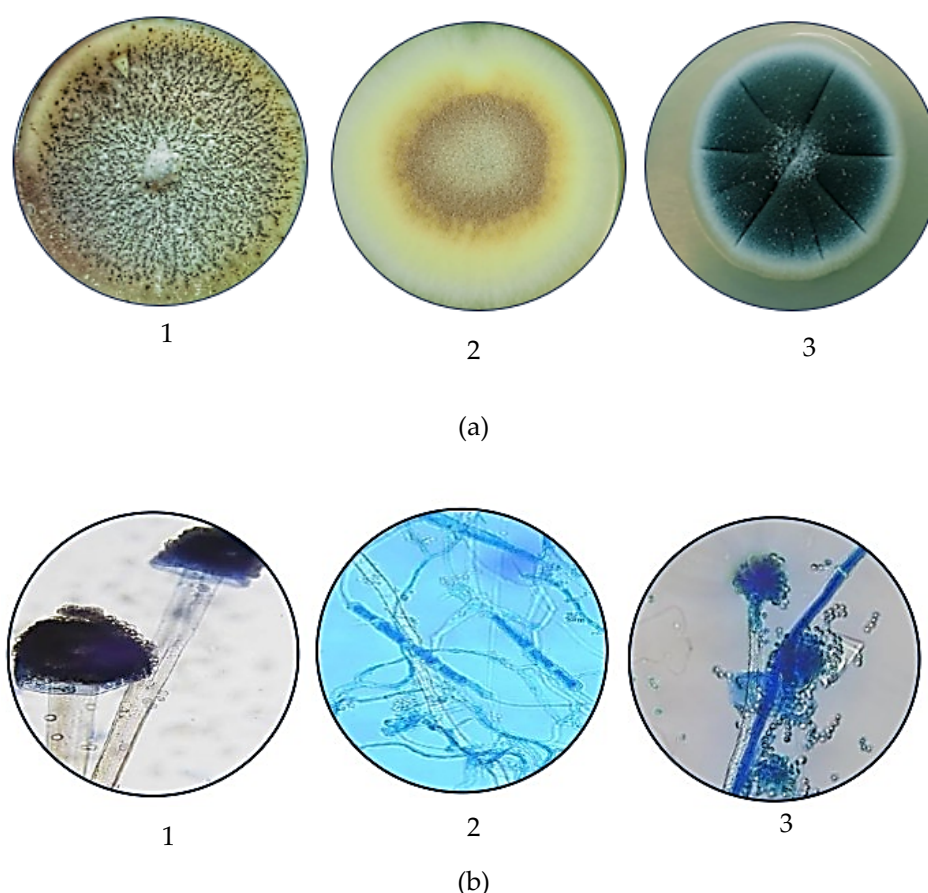


Figure 2. Morphological Appearance of Microfungi Isolates: (a) Macroscopic; (b) Microscopic

Based on the results of macroscopic and microscopic identification of macrofungi that have been carried out, the suspected

species of each isolate are described in Table 1.

Table 1. Presumed Species of Macrofungi Isolates from the North Insana Drylands

Isolate	Macroscopic Morphology	Microscopic Morphology	Presumed Genus
1	Grayish white colonies, cottony texture, radial growth[18].	Sporangiophores with dark round sporangia, non-septate hyphae[18][19].	<i>Mucor</i> sp.
2	Yellowish brown colonies, darker center, fading edges[20].	Septate hyphae, phialids formed around vesicles, conidia in chains[21].	<i>Penicillium</i> sp./ <i>Aspergillus</i> sp.
3	Dark green to black colonies, clear radial pattern[20].	Conidiophores with vesicles and chains of small round conidia[22].	<i>Aspergillus</i> sp.

The microfungi identification results in Table 1 remain presumptive, based on morphological references. To obtain more accurate results, molecular analysis is necessary, as it allows comparison with genetic databases of previously identified microorganisms. Morphological identification is often inadequate given the millions of microfungi species that have been discovered, so matching with genetic databases is necessary to improve accuracy[23]. If the molecular analysis results do not match the existing data, the isolated microfungi could potentially be categorized as a new species[24].

Antibacterial Activity of Microfungi

The antibacterial potential of each microfungi isolate was evaluated using the disc diffusion method. This method is one of the most commonly used techniques for assessing antibacterial activity[25]. This method relies on the diffusion of bioactive compounds from the disc into a medium inoculated with the test

bacteria, where antibacterial activity is indicated by the formation of a clear inhibition zone surrounding the disc[26]. The diameter of the inhibition zones was measured to assess the strength of antibacterial activity[27]. Based on the size of the inhibition zones, antibacterial activity was classified into four categories: weak, moderate, strong, and very strong. Activity was considered weak if the inhibition zone diameter was <5 mm, moderate if 5–10 mm, strong if 10–20 mm, and very strong if >20 mm[28]. After three repetitions, the results showed that all three isolates exhibited antibacterial activity against *Staphylococcus aureus*, with varying degrees of effectiveness. The third isolate demonstrated the strongest activity, with an inhibition zone comparable to the standard antibiotic chloramphenicol. The inhibition zones observed for each microfungi isolate are illustrated in Figure 3. Detailed measurements of the inhibition zone diameters, along with their corresponding antibacterial activity categories, are summarized in Table 2.

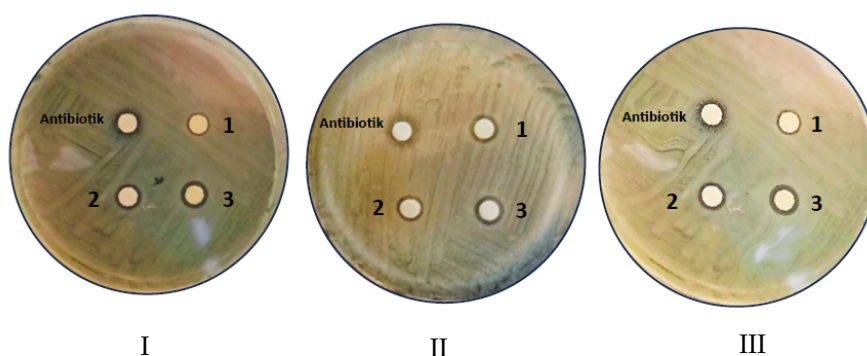
**Figure 3.** Antibacterial Test Results of Ethyl Acetate Extract of Microfungi

Table 2. Inhibition Zone Diameters of Ethyl Acetate Extracts of Microfungal

Extract	Inhibition zone Diameter (mm)			Mean $\pm$ SD
	I	II	III	
1	7,4	7,8	7,6	7,6 $\pm$ 0,20 ^c
2	10,2	10,1	10,2	10,17 $\pm$ 0,06 ^b
3	10,8	10,8	10,7	10,77 $\pm$ 0,06 ^{ab}
Antibiotic	11,2	11,4	11,1	11,23 $\pm$ 0,15 ^a

Note: Numbers followed by different letters indicate significant differences according to Tukey's test ($p < 0.05$), while the same or shared letters (e.g., a and ab) indicate no significant difference.

Based on Tukey's test ($p < 0.05$), the three microfungal extracts exhibited significantly different antibacterial effects against *Staphylococcus aureus* (Table 2). Extract 1 displayed moderate activity with an average inhibition zone of 7.6 $\pm$ 0.20 mm, whereas Extracts 2 and 3 showed strong activity with inhibition zones of 10.17 $\pm$ 0.06 mm and 10.77 $\pm$ 0.06 mm, respectively. The standard antibiotic produced 11.23 $\pm$ 0.15 mm inhibition zone, also classified as strong activity. Extract 1 was significantly less effective than the other treatments (superscript c), while Extract 2 (superscript b) exhibited improved inhibitory activity yet remained below the antibiotic. Extract 3 (superscript ab) did not differ significantly from the antibiotic (superscript a), indicating comparable efficacy.

The observed differences in inhibition zones are likely due to variations in the composition and properties of bioactive compounds, including their diffusion capacity and interaction with bacterial cells[29]. The moderate activity of Extract 1 may be attributed to lower levels or less effective bioactive metabolites. In contrast, the strong activity of Extracts 2 and 3 suggests the presence of highly potent metabolites capable of interfering with bacterial metabolism or disrupting cell structures.

The antibacterial effects are probably related to secondary metabolites such as polyketides, cyclic peptides, and alkaloids, which can damage bacterial cell walls, inhibit protein synthesis, or modulate metabolic pathways[30]. Similar findings have been reported in *Penicillium* sp. isolated from peat

soil, which produced secondary metabolites with antibacterial activity[31]. Additionally, *Aspergillus* isolates from semi-arid ecosystems exhibited significant antibacterial activity against clinical pathogens[32]. These observations support the idea that soil-derived microfungi from extreme environments can produce unique bioactive metabolites. The strong efficacy of Extract 3, comparable to the standard antibiotic, indicates its potential to be further developed as a natural antibiotic candidate.

Dryland microfungi, exposed to prolonged drought and temperature fluctuations, may produce secondary metabolites as adaptive mechanisms to environmental stress[33]. This highlights dryland ecosystems as valuable reservoirs of novel and environmentally friendly antibacterial compounds. Therefore, further chemical characterization and bioactivity studies are warranted to identify the specific metabolites responsible for the observed effects and to evaluate their safety and therapeutic potential.

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Conclusion

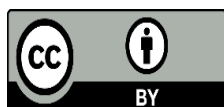
Three microbial isolates from dryland areas exhibited distinct antibacterial activities. Isolate 3, tentatively identified as *Aspergillus* sp., demonstrated the strongest activity, showing no significant difference compared to the standard antibiotic ($p > 0.05$). Isolate 2 (*Penicillium/Aspergillus* sp.) exhibited strong activity, while isolate 1 (*Mucor* sp.) showed moderate activity. These results confirm that endemic dryland microorganisms possess considerable antibacterial potential. Although the isolates have not yet been molecularly identified, further characterization is necessary to determine their species and to elucidate the bioactive antimicrobial compounds they produce. Such investigations are critical for supporting the development of novel, environmentally sustainable antibacterial agents with potential applications in medicine and biotechnology.

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