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Exploration of Potential Endophytic Fungi from Ironwood (*Eusideroxylon zwageri* Teijsm. & Binn.) as an Antibacterial Agent

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Abstract

This study investigates the endophytic fungi found in ironwood (*Eusideroxylon zwageri* T et. B), a plant valued by the Kalimantan community for its medicinal uses, especially in treating rheumatism and diabetes. The main goal was to identify these fungi and evaluate their potential antibacterial properties. Samples of ironwood, including leaves and roots, were collected from the CV Nursery at Nusantara Asri, Cempaka Baru, Gunung Kupang, Banjarbaru, South Kalimantan, with coordinates -3.4892255, 114.8907642, 723. Using a series of steps—such as sterilizing samples, isolating fungi, conducting secondary metabolic fermentation, and testing for antimicrobial activity against Staphylococcus aureus and Escherichia coli—11 fungal isolates were successfully obtained from the plant's roots, leaves, and seeds. Five isolates were chosen for further study based on their growth rates, representing various genera including *Cladosporium* sp., *Aspergillus* sp., *Mucor* sp., and *Hyphomycetes* sp. Although the antimicrobial tests revealed that none of these isolates inhibited the bacteria, the results offer valuable insights into the interactions between these fungi and the bacterial strains tested.

Keywords: Antibacterial agent, Endophytic fungi, *Eusideroxylon zwageri* Teijsm. & Bin

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Introduction

Kalimantan is the largest island in Indonesia, comprising five provinces: Central Kalimantan, West Kalimantan, South Kalimantan, East Kalimantan, and North Kalimantan. This diverse number of provinces contributes to a rich cultural heritage and traditions. Known internationally as Borneo, Kalimantan is characterized by its vast and beautiful forests. These extensive forests are home to a wide variety of flora and fauna, many of which are endemic to the region. Endemic species originate from and are exclusively found in a specific area, such as the forests of Kalimantan.

The people of Kalimantan rely heavily on the natural products in the region's forests for their essential needs, including food and medicine. They commonly utilize various parts of trees and plants, such as roots, seeds, stems, leaves, and tubers, for medicinal purposes. Over time, many of these beneficial plants have been cultivated on a larger scale for medicinal use. Traditional medicine, particularly among the Dayak tribe of Kalimantan, utilizes these medicinal plants, showcasing their deep connection with nature. The Dayak tribe possesses extensive knowledge about which plants have the potential to be used for healing.

One of the plants that grows in the forest and is used as an ingredient in medicines is ironwood (*Eusideroxylon zwageri* T et B). Ironwood, also known as ulin, is an endemic species found on the island of Kalimantan. This plant thrives in its natural habitat and has significant economic value due to the durability and quality of its Class 1 wood products. Ironwood is referred to by various names in different regions, including onglen, belian, and bulian. Typically, these trees grow in lowland

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forest areas at elevations of up to 400 meters above sea level. Under natural conditions, ironwood trees can live for 50 to 100 years (Ningsih, 2015).

The ironwood plant is valued not only for its use as a building material but also by the people of Kalimantan, who utilize it as a source of traditional medicine for various ailments (Khairiah & Nintasari, 2017; Mariani *et al*., 2020). Specifically, the ironwood plant treats conditions such as rheumatism and diabetes. The seeds of the ironwood tree, which fall to the ground, are commonly used for medicinal purposes. These seeds are boiled, and the resulting water is consumed by individuals suffering from these diseases. Additionally, some people in Kalimantan use ironwood infusions to remedy toothaches (Khairiah & Nintasari, 2017). Plants utilized for traditional medicine often host microorganisms that play a role in their therapeutic properties. These microorganisms, known as endophytes, typically reside in the plant's roots, seeds, leaves, and twigs (Imaningsih *et al*., 2023b)

Endophytes are microorganisms that coexist with plants in a mutually beneficial relationship. These endophytes, which can live within plant tissues, produce secondary metabolites that can be valuable for various uses. One promising type of endophyte is fungi or molds. Endophytic fungi have the ability to generate bioactive compounds that serve as antifungal, antibacterial, antioxidant, antiviral, and anticancer agents (Nwobodo *et al*., 2017; Khan *et al*., 2017; Lutfia *et al*., 2020, 2021; Syarifah *et al*., 2021). For example, the endophytic fungi *Phomopsis* sp., isolated from the *Alpinia shengzhen* fresh leaves produce bioactive components that can inhibit *E. Coli*, *S. aureus* and methicillin resistant *S. aureus* bacteria (Chen *et al*., 2022)), and endophytic fungi from Hiyung chili plants have the potential to produce anti-microbial bioactive substances (Imaningsih *et al*., 2023b).

The potential of endophytic fungi as antimicrobial agents, particularly against bacteria, presents opportunities for developing medicinal compounds derived from plants. *Staphylococcus aureus* and *Escherichia coli* are the most commonly utilized bacteria for antibacterial testing. These bacteria are responsible for various diseases, including heart valve infections, pneumonia, osteoarthritis, and intestinal infections that can lead to diarrhea. Additionally, they represent a group of both gram-positive and gram-negative bacteria (Habibi *et al*., 2022).

Research on antibacterial from endophytic fungi is expected to be an alternative to initial research on discovering new sources of natural medicines. For this reason, this study will review the characterization of the endophytic fungi of ironwood plants, which the people of Kalimantan believe can cure various diseases such as diabetes and rheumatism. In addition, this study will be tested for antibacterial to determine the characterization of ironwood plant endophytic fungi.

Research Methods

Sampling Sites

Sampling was conducted in two different locations. Samples of ironwood plants, including leaves and roots, were collected from the CV Nursery located at Nusantara Asri, Cempaka Baru, Gunung Kupang, Banjarbaru, South Kalimantan, with coordinates -3.4892255, 114.8907642, 723. Additionally, samples of ironwood seeds were sourced from the Dayak community in Kapuas, Central Kalimantan. Due to the challenging conditions of the collection area deep within the forest, direct sampling of ironwood seeds was not feasible. As a result, the seeds were only obtained from the Dayak community in Kapuas, Central Kalimantan.

Sample Sterilization

Sterilization of the sample was carried out on the tissue (roots, leaves, seeds) of ironine (*Eusideroxylon zwageri* T et. B) 2 months old with a height of 1 m and a diameter of 3 cm (stem). Each tissue (roots, leaves, seeds) is washed with running water for 10 minutes. The tissues (roots, leaves, seeds) are soaked using 70% alcohol for 5 minutes. The tissues (roots, leaves, seeds) are then cut first 1 cm (roots), 1 x 1 cm (leaves), and 1 x 0.5 cm (seeds), respectively. Next, surface sterilization is carried out using NaOCI and 70% alcohol consecutively, the roots are soaked with 70% alcohol for 5 minutes and 5% NaOCI for 10 minutes. Then, the leaves are soaked with 70% alcohol for 5 minutes and 1% NaOCI for 10 minutes and the seeds are soaked with 70% alcohol for 5 minutes and 10% NaOCI for 10 minutes (Khairiah & Nintasari, 2017). Pieces of tissue (roots, leaves, seeds) that have been

sterilized are then dried on sterile filter paper. Surface sterilization is all done in laminar air flow to prevent contamination.

Endophytic Fungi Isolation

Endophytic fungi is isolated by growing endophytic mold from ironwood plant tissues (roots, leaves, seeds) in the medium of PDA (*Potato Dextrose Agar*). Pieces of ironwood plant tissue (roots, leaves, seeds) that have been sterilized and dried, then each piece of tissue (roots, leaves, seeds) is placed into a bowl containing PDA medium. The PDA medium used is first given chloramphenicol to prevent bacterial growth during the incubation process. Each petri dish contains three pieces of samples, each of which is about 5 cm apart. The samples are then incubated at room temperature (26^0C - 33^0C) for 5-7 days. The endophytic fungi that have been overgrown are then transferred to a new medium containing PDA medium. Purification is carried out by moving colonies that grow in the medium by looking at their macroscopic characteristics, such as the different colors and shapes of the colonies. Purification will continue to be carried out repeatedly until a single colony is obtained from the endophytic mold (Khairiah & Nintasari, 2017). The purified endophytic fungi are then assessed to identify those with the fastest growth rates, which will subsequently be tested for antibacterial activity. Screening is performed using the growth rate measurement method. Each isolate is regrown on PDA media, and its growth is tracked by measuring the colony surface area every day for four days after inoculation (DAI). The growth area of the fungi is measured using a caliper, with measurements taken in millimeters (Octriana, 2016).

Characterization of Endophytic Fungi

Characterization is carried out in both macroscopic and microscopic ways. It is macroscopic by observing the shape, color edges of endophytic mold colonies on the surface and when flipped. Microscopic characteristics, including conidia shapes, hyphae, and the location of endophytic mold conidiophores, were observed. Microscopic observations were made using the Slide Culture method, by placing filter paper on a petri dish. Then, the U stem is placed on the filter paper. The filter paper on which the U rod has been placed is added to the steris aquaade little by little. The U-rod that has been placed at the top is added to the object glass. A piece of PDA medium with a size of 0.5 x 0.5 cm is placed on the glass of the prepared object, then the isolate is introduced and covered with a glass cover. Incubation of endophytic fungi isolate is carried out for 3-5 years, during which incubation of the isolate is observed under a microscope (Imaningsih *et al*., 2023a). The character is then matched with "the illustrated genera Of Imperfect Fungi Fourth edition*"* (Barnett & Hunter, 1998)..

Production of Crude Extracts of Endophytic Fungi

Inoculation of endophytic fungi isolate on a liquid medium (Potato Dextrose Broth) is carried out to obtain secondary metabolites. Endophytic fungal isolates from working cultures in PDA medium were taken with a size of 1 x 1 cm, then put into a 100 ml PDB medium in Erlenmeyer flasks with a size of 250 ml. An Erlenmeyer containing a liquid medium of PDB without an isolate was used as a control. Erlenmeyer, which had contained PDB and Isolate medium as well as control, was then incubated in *a shaker incubator* with a temperature (27^0C - 29^0C) and a speed of 120 rpm for 7-14 days. Each of the incubated isolates was then filtered using filter paper and centrifuged at a speed of 3000 rpm for 20 minutes at room temperature (Imaningsih *et al*., 2021). Centrifugation aims to separate or eliminate water fractions and biomass to obtain supernatants used for antimicrobial testing (Khairiah & Nintasari, 2017).

Antibacterial Activity Test

Antimicrobial testing of crude extracts of endophytic fungi was carried out using the diffusion method using the paper *disk*. The test bacteria used were S. *aureus* and *E. coli microbes*. One *ose* of *S. aureus* bacteria was inoculated into MHA Broth media and 1 *ose* of E. *coli* bacteria culture was inoculated into NA Broth media. The bacterial suspension is grown and diluted until a dilution of 10-8. The diluted suspension is compared to McFarland's standard suspension. The 0.5 McFarland standard has a turbidity comparable to 1.5 x 10^8 *colony forming units* (CFU)/ml. Paper discs before use are first soaked with endophytic mold supernatant that has been obtained from the results of the fermentation process for approximately over (Elzuhria A *et al*., 2023; Kumala, 2014).

Antibacterial Test Against *Staphylococcus aureus*

The diluted *suspension of S. aureus* is inoculated into MHA medium (Mueler Hinton Agar) as much as 0.5 ml and flattened using a spreader. The disc paper that has been soaked with endophytic mold supernatant is then placed on a medium that has been given a *suspension of S. aureus* using sterile tweezers. Incubation is carried out for 18-24 hours at a temperature of 370C. Clear areas or clear zones around the disk (*paper disk*) indicate the presence of microbial inhibition areas measured in millimeters (mm) (Kumala, 2014). Chloramphenicol is used as a positive control, and for a negative control, sterile aquatics is used (Khairiah & Nintasari, 2017).

Antimicrobial Test Against *Escherichia coli*

The diluted *E. coli* suspension was taken and pipetted into NA medium in a Petri dish, as much as 0.5 ml, and flattened using a spreader. Disc paper that has been soaked with endophytic mold supernatant is then placed on a medium suspended from *E. coli* using sterile tweezers. Incubation is carried out for 18-24 hours at a temperature of 37^0C. Clear areas or clear zones around the disk (*paper disk*) indicate the presence of microbial inhibition areas measured in millimeters (mm)(Kumala, 2014). Chloramphenicol is used as a positive control, and for a negative control is used sterile aquatics (Khairiah & Nintasari, 2017).

Results and Discussion

Endophytic fungi isolate of ironwood plants (*Eusideroxylon zwageri* T et. B) carried out by the direct planting method on PDA media. After the permutation process, as many as 11 endophytic fungi were obtained. Four endophytic fungi are derived from Roots (AU), six are derived from Leaves are assigned codes, and one is derived from seeds (BU). Each of the endophytic fungi was then measured in terms of growth rate. The average growth rate of endophytic mold is presented in Table 1.

The selected isolate is the one with the fastest growth rate. Isolates AU 3, AU 4, DU 4, DU 6, and BU 1 had a growth rate of 1.25, 1,26, 1,52, 2, and 2 cm2/day. In addition, the growth rate of the 10 isolates can be observed in Figure 2. The figure shows the average surface area of the fourth day after isolation that the isolates AU 3, AU 4, DU 4, DU 6, and BU 1 have the largest surface area compared to other isolates

The screening process is carried out by selecting fungi with the best growth to choose fungi that can adapt to new environmental conditions. One of the characteristics of good adaptation is its ability to grow on insulating media. Imaningsih *et al*. (2021) stated that endophytic fungi produced from one isolated plant tissue are usually more than one type. This is the process of adaptation of endophytic fungi to the environment in which they live and the physiological state of each host plant tissue. The purification process is an advanced process to obtain different types of isolates by separating several colonies of isolates that grow around the host's body.

The isolate obtained from one part of the plant tissue used as a host is usually large. However, only a few microorganisms are dominant in a single host. Colonies from endophytic fungi in the plant tissue that is used as the host occur through a process of penetration. The process of colonization penetration in the plant layer by mechanically breaking down the plant protective tissue or through an enzymatic reaction to the cuticle fissures and plant epidermal tissue. The endophytic mold produced by leaf tissue will have more endophytic mold than other parts of the tissue. This is because leaf tissue has a thin cuticle and a wide surface, so endophytic microorganisms are more likely to penetrate (Mora *et al*., 2018).

Table 1. Endophytic Fungi Growth Rate 4 Days After Isolation (DAI)

No	Isolation Code	Average Growth Rate of Fungi (cm2/day)
1	AU 1	0,25 ± 0,35
2	AU 2	0,28 ± 0,42
3	AU 3	1,25 ± 0,51[T]
4	AU 4	1,26 ± 0,19[T]
5	DU 1	0,24 ± 0,37
6	DU 2	0,21 ± 0,35
7	DU 3	0,7 ± 0,35
8	DU 4	1,52 ± 0,49[T]
9	DU 5	0,52 ± 0,32
10	DU 6	2 ± 0,61[T]
11	BU 1	2 ± 0,68[T]

Note: [T] is the selected isolate

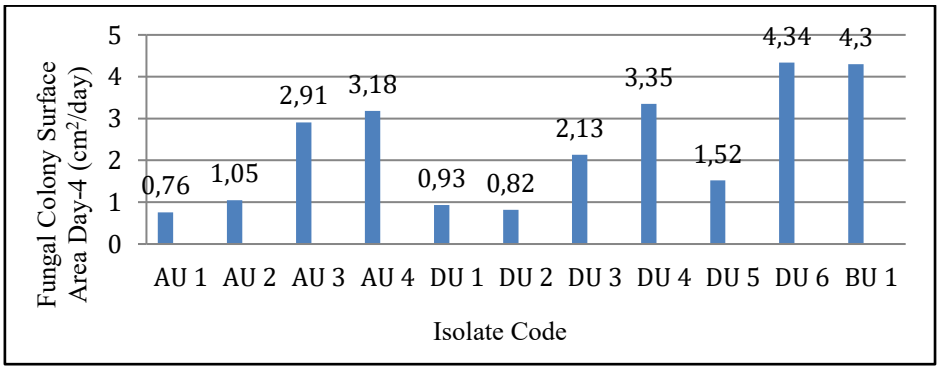

Figure 1. Fungal Colony Surface Area Day 4

DU 6 and BU 1 isolates have very different surface areas compared to other isolates. The surface area of AU 3, AU 4, and DU 4 is also higher than the other isolates (Figure 1).

Morphological Characteristics of Endophytic Fungi from Ironwood

Mold characterization was carried out on five selected isolates with a faster growth rate than other isolates. The fungi selected for use in the follow-up test are AU 3, AU 4, DU 4, DU 6 and BU 1.

The macroscopic and microscopic characteristics of each endophytic fungi isolate are seen in Figures 2 and 3.

Table 1. Macroscopic Character

Isolate code	Surface and inverse color of the colony	Surface	Texture
AU 3	White	Uneven	Wooly
AU 4	Green	Uneven	Granulated
DU 4	Gray	Uneven	Wooly
DU 6	White	Uneven	Wooly
BU 1	Dark gray	Flat	Velvet

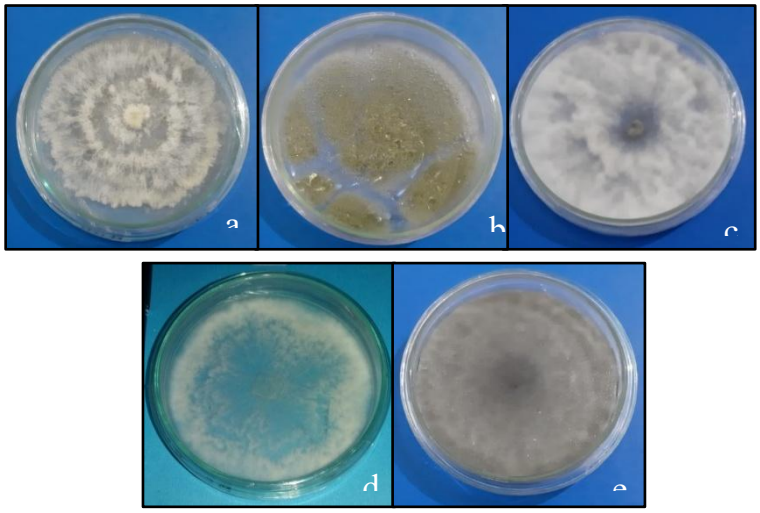

Figure 2. Fungal Colonies Surface Area Day 4 (a) AU 3, (b) AU 4, (c) DU 4, (d) DU 6, (e) BU 1

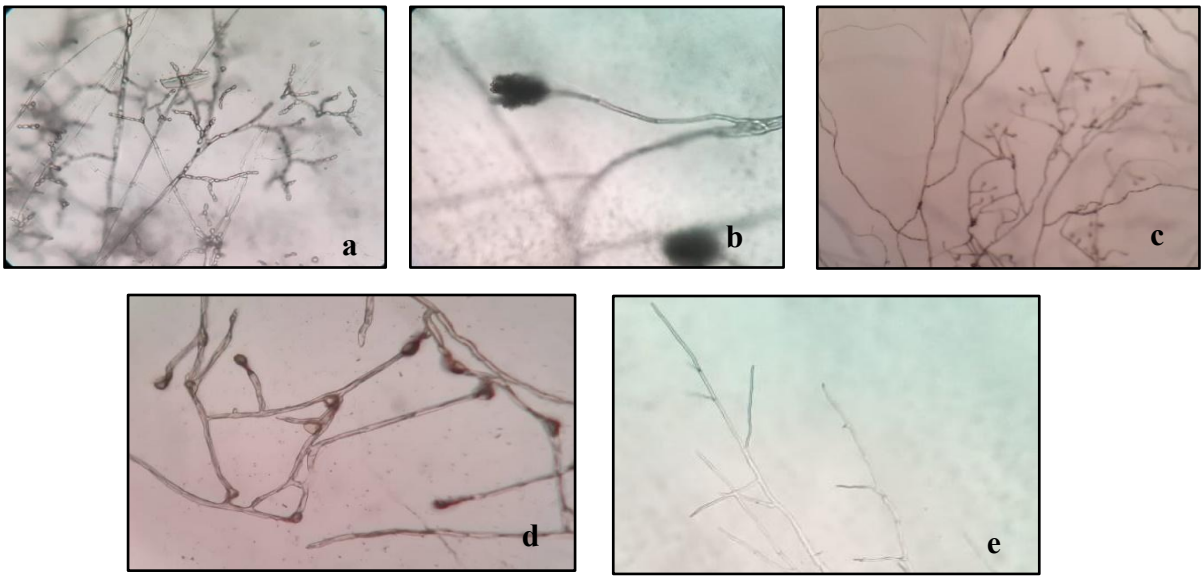

Figure 3. Microscopic cross-sections of conidia isolates AU3, AU4, DU4, DU6, BU1 (a-e) showing the characteristics of the isolates of *Cladosporium* sp., *Aspergillus* sp., *Mucor* sp. (c and d), and *Hyphomycetes* sp.

Antibacterial Activity of Endophilic Fungi Against *Staphylococcus aureus* and *Escherichia coli*

The results of endophytic fungi antibacterial testing against *S. aureus* and *E.coli* are presented in Table 3.

Crude extracts of endophytic fungi isolate tested against *S. aureus* and *E. coli* showed no spectral presence or formation of inhibitory zones, respectively. Testing of crude extracts of ironwood plant endophytic fungi could not inhibit the growth of S. aureus *and* E. coli *bacteria*. Those condition can be due to changes in the structure of the test bacterial cell caused by the speed of diffusion of supernatants from disc paper to the test medium (Bubonja-Šonje *et al*., 2020). Other factors that can affect include the properties of the medium used, the molecular size and stability of the antimicrobial material, the concentration of chemicals, the number of isolated organisms, and the conditions at the time of incubation. Another possibility that causes supernatants not to work well to inhibit is because the secondary metabolite compounds produced include volatile compounds. Volatile compounds themselves are compounds that quickly experience evaporation, especially if there is an increase in temperature (Bubonja-Šonje *et al*., 2020; Widowati *et al*., 2016) .

Table 2. Antimicrobial Effects of Endopytic Fungi Crude Extract against *S. aureus* and *E.coli*

Isolate	Inhibition Zone	
	S. aureus	***E. coli***
Sterile aquades (Negative Control)	-	-
Chloramphenicol (Positive Control)	+	+
Cladosporium sp.	-	-
Aspergillus sp.	-	-
Mucor sp.1.	-	-
Mucor sp.2.	-	-
Hyphomycetes sp.	-	-

Note: + (Formed inhibition zone), - (No Inhibition Zone Formed)

Conclusion and Suggestions

The endophytic mold isolates successfully isolated from ironwood plants in this study included several types of genera, namely *Cladosporium* sp., *Aspergillus* sp., *Mucor* sp., and *Hyphomycetes* sp. The five endophytic isolate filtrates tested did not have the antibacterial activity of *Staphylococcus aureus* and *Escherichia coli.*

The suggestion for the next research is to further test the isolates that have been obtained, especially to see their potential in other fields of study.

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