RESEARCH ARTICLE

Exploration of Endophytic Fungi in the Kelakai (*Stenochlaena palustris*) and Their Antibacterial Effect

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ABSTRACT

The Dayaknese consume *Stenochlaena palustris* for the treatment of various diseases. The plants contain chemical compounds that have the potential to be developed as antibiotics. This study aimed to isolate endophytic fungi from the leaves and stems of *S. palustris*, and further identify and assess the isolated endophytic fungi against microorganisms. The research was carried out in several stages, namely, isolation and purification of endophytic fungi, macroscopic and microscopic identification, activity test on isolates, fermentation and extraction of secondary metabolites, and evaluation of the antibacterial activity of the extracts using the disk diffusion method. This experimental laboratory research was conducted in Palangka Raya City, Central Kalimantan, with a test carried out at the Microbiology Laboratory of the Health Polytechnic Ministry of Health, Palangka Raya. The results showed 3 types of endophytic fungi: SpHtm, SpHjk, and Sporn, identified as *Aspergillus* sp., *Paecilomyces* sp., and *Arthrocristula* sp. The antimicrobial effect of the isolated fungi was assessed at various concentrations, i.e., 15, 30, and 45%, against *S. aureus* and *E. coli*. The results showed that the ethyl acetate extracts of the SpHtm and SpHjk isolates did not demonstrate any inhibitory response to *S. aureus* at concentrations of 15, 30, and 45% and did a small inhibitory response to *E. coli* at an SpHtm concentration of 30% and an SpHjk concentration of 45%, with inhibition zone diameters of 11.63 ± 0.01 and 10.83 ± 0.02 mm, respectively.

Keywords: Disk diffusion, Endophytic fungi, *Stenochlaena palustris*.

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INTRODUCTION

Endophytes are endosymbionts, often fungi, that are conserved inside plants for a certain period of their life cycle with no parasitic effect on the host plant.[1,2] Endophytes grow in inter or intracellular space of plants, in the roots, stems, and/or leaves, and appear to sporulate in mature plant tissues.[3,4]

Endophytic fungi are abundant sources of secondary metabolites. Many endophytic fungi can provide secondary metabolites with various biological activities and chemical structures. Secondary metabolites such as alkaloids, phenolic derivatives, terpenoids, and steroids that are conserved in endophytes are potential for mycotoxin, enzymatic, and antibiotic activities.[5,6]

The Dayaknese generally consume *Stenochlaena palustris* leaves to treat various diseases. *S. palustris* is also empirically used to increase and facilitate breast milk production for mothers. *S. palustris* contains nutrients such as protein (2.36%), crude fiber (4.44%), fat (0.11%), water (89.09%), vitamins, and several minerals such as Ca and Fe.[7-9] The ethyl acetate fraction of *S. palustris* was reported to contain alkaloids, phenolics, flavonoids, saponins, tannins, and terpenoids.[10-12] Alkaloids, saponins, and tannins are secondary metabolites that can provide biological activity and have the potential to be developed as antibiotics.[13,14] Rostinawati *et al.* (2018) demostrated that the ethanolic extract of the kelakai (*Stenochlaena palustris* (Burm.F) Bedd) yielded 9% MIC and 10.8% MBC against *S. typhi*, as well as 10.6% MIC and 11% MBC against *S. aureus*. One mg of tetracycline was found to be equivalent in effect with 23.65 mg of the ethanolic extract of kelakai leaves against *S. typhi* and 28.21 mg against *S. aureus*.

Isolation and identification of endophytic fungi are important as endophytic fungi are capable of producing

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secondary metabolites that have similar pharmacological activities to those of their hosts.[16] This study aimed to identify isolates of endophytic fungi from the kelakai (*S. palustris*) from Palangka Raya City, Central Kalimantan and their antibacterial activity.

MATERIALS AND METHODS

Sample Preparation

Samples of the leaves and stems of *S. palustris* were washed with running water to remove impurities and had their surface sterilized. The sterilization was carried out by immersing the samples in 70% ethanol for 1-minute and in sodium hypochlorite solution (NaOCl 5.3%) for 2 minutes, followed by re-immersion in 70% ethanol for 30 seconds. The samples were dried on sterile paper.

Isolation and Purification of Endophytic Fungus

The sterilized kelakai was cut 1-cm in size for further inoculation on solid potato dextrose agar (PDA) and chloramphenicol media. The inoculated sample was incubated at room temperature for 3–5 days. Observation was carried out for the 3–5 days' period during the fungi's growth. Each fungus that grew was isolated and purified on a new PDA medium and incubated at room temperature for 5 days to obtain pure isolates.[5,17]

Macroscopic Identification of Endophytic Fungus

Macroscopic identification was conducted on the isolates of endophytic fungi, and the morphologies, colors, and surfaces of the colonies formed were observed on the sides and bottom of the petri dishes where they were inoculated.[2]

Microscopic Identification of Endophytic Fungus

The endophytic fungus isolates were inoculated on a sterile glass object, covered with a glass cover, and incubated for 1 day to form spores. The morphologies of the fungi (i.e., colonies, spores, hyphae, conidia, and genera) were observed under a microscope at 400X magnification.[3,17]

Measurement of Endophytic Fungus Isolate Activity

The endophytic fungi obtained were tested for their antibacterial activity against *S. aureus* and *E. coli*. Incubation was conducted at room temperature for 1 day for bacteria and 3 days for fungi. The antibacterial activity was measured based on the inhibition zones formed around the endophytic fungi.[17,18]

Endophytic Fungus Isolate Fermentation

Pure isolates were fermented in a potato dextrose yeast (PDY) medium for 14 days at room temperature to obtain secondary metabolites. The fermentate was separated from the mycelium for further extraction.[17]

Extraction of Secondary Metabolites from the Endophytic Fungus Isolates

A total of 100 mL of fermentate was extracted to be fractionated with ethyl acetate at a ratio of 1:1. The ethyl acetate fraction was separated and evaporated to obtain thick extract. The process was carried out until a clear ethyl acetate fraction was obtained.[18]

Bacterial Strains

S. aureus and *E. coli* were grown at 37ºC for 24 hours in BHI media. The culture's optical density (OD) 600 was adjusted to 0.1, equivalent to 0.5 McFarland standard (the estimated bacterial count in a suspension is 1.5×108 CFU/mL). Furthermore, the culture was diluted in a new growth medium to 0.01 OD600.[19]

Antibacterial Activity Test

An antibacterial activity test of the ethyl acetate extracts of endophytic fungi was carried out against *S. aureus* and *E. coli* using the disk diffusion method. Paper discs were immersed in samples at the concentrations of 15, 30, and 45% w/v (extract in DMSO solvent). The paper discs were then placed in NA media containing bacterial cultures and then incubated at room temperature for 1-day. The diameters of the inhibition zones formed were observed and measured. In this test, controls were used as comparisons for the samples. The controls used were 3 µL of DMSO (negative control) and 3 µL of tetracycline in 1.2 mg/mL (positive control).[15,20]

RESULTS AND DISCUSSION

In this study, we used the leaves and stems (at the top, bottom, and middle) of *S. palustris* as potential plant to provide unique endophytes. The traditional medicinal plant, especially in the leaves and stems, is a good host for the growth of endophytic fungi. Endophytic fungi can be conserved in the internal tissues of the plant. Endophytic fungi produce secondary metabolites that have the potential to be used in the pharmaceutical industry for, among others, antifungal, antibacterial, and cytotoxic activities.[21-23]

After incubation at room temperature for 3 days, some fungal growths were obtained and purified (Figure 1). The isolation and purification of the endophytic fungi on the leaves and stems of *S. palustris* resulted in four colonies of endophytic fungi, which were then coded as SpHtm, SpHjk, SpHjp, and SpOrn (Figure 2).

Furthermore, macroscopic and microscopic characterizations were carried out, and the results would be compared with the Benson method.[2] All fungi had distinctive macroscopic

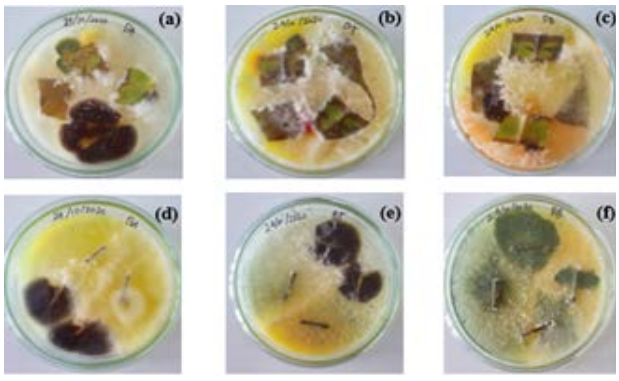


Figure 1: Results of isolation of endophytic fungi on leaves and stems of Kelakai (a: Leaves at top of kelakai; b: Leaves at middle of kelakai; c: Leaves at bottom of kelakai; d: Stem at top of kelakai; e: Stem at middle of kelakai; and f: Stem at bottom of kelakai)

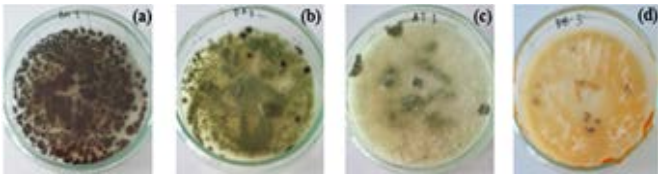


Figure 2: Results of purification of endophytic fungi isolates (a: SpHtm; b: SpHjk; c: SpHjp; dan d: SpOrn)

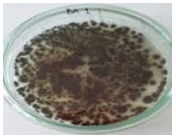
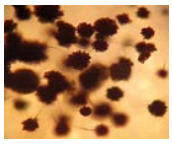
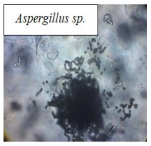
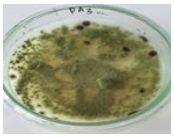
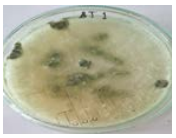
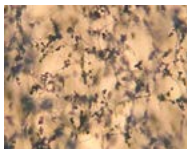
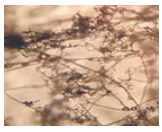
and microscopic characteristics and could be used to identify their respective genera.[23] The macroscopic and microscopic identification results are shown in Table 1. SpHtm isolates were identified as *Aspergillus* sp., SpHjk and SpHjp isolates were *Paecilomyces* sp., and SpOrn isolates were *Arthrocristula* sp.

In the initial screening, antibacterial activity measurements were carried out on the four fungal colonies using *S. aureus* and *E. coli*. These bacteria are gram-positive and gram-negative, respectively, and they can be used to assess the inhibitory activity of endophytic fungus isolates against them.[24] They are microorganisms that cause various infections in humans: *S. aureus* can cause respiratory tract infections, and *E. coli* is the cause of urinary tract and systemic infections.[25] The screening showed that SpHtm and SpHjk had promising inhibitory responses and could be further used for fermentation and extraction processes (Table 2). The results of previous studies showed that the ethanolic extracts of *S. palustris* had good antibacterial activity against *S. aureus* and *S. typhi.*[15]

Pure isolates of SpHtm and SpHjk were fermented and extracted with ethyl acetate as a solvent. The antibacterial activity test of the ethyl acetate extracts of endophytic fungi samples was carried out against *S. aureus* and *E. coli* using the disk diffusion method.[26] The extracts were made at various concentrations, namely, 15, 30, and 45%. DMSO was used as a negative control, and tetracycline as a positive control. The results of this test showed that the ethyl acetate extracts of SpHtm isolates did not demonstrate any inhibitory responses to *S. aureus* at all three concentrations and had weak inhibitory responses to *E. coli* only at the concentrations of 15 and 30%, with inhibitory zone diameters of 10.93 ± 0.02 and 11.63 ± 0.01 mm, respectively. Meanwhile, the ethyl acetate extracts of SpHtm isolates did not demonstrate any inhibitory responses to *S. aureus* at the concentrations of 15, 30, and 45% either, but it had a weak inhibitory response to *E. coli* at the concentration of 45%, with an inhibitory zone diameter of 10.83 ± 0.02 mm. According to Monks *et al.* (2002), bacteria the inhibition zone range of 7–11 mm are classified to have weak activity. Strong activity is shown in inhibitory zones with diameters >16 mm.

The difference in antibacterial activity occurs with the differences in cell wall thickness between gram-positive and gram-negative bacteria. This condition leads to differences in their sensitivity and responses against bacteria. The difference in cell wall thickness is a determining factor in the penetration, binding, and antibacterial activity of compounds. The increase or decrease of compounds in plant extracts can also influence the difference in antimicrobial activity.[5]

Table 1: Macroscopic and microscopic identification of endophytic fungi

Isolate code	*Characteristics of pure isolates of kelakai endophytic fungi*		
	Macroscopic	*Microscopic*	
SpHtm			
	The colony is black. Side shape: *Filamentous*. Surface Shape : *Raised*.	Spores: Aporangium. Hyphae: Aseptate. Conidia: Acrotecha.	
SpHjk			
	Colonies are green-yellow.. Side shape : *Filamentous*. Surface shape : *Convex*.	Spores: Arthospora. Hyphae: Septat Hifa. Conidia : Complex.	
SpHjp			
	Colonies are green-white. Side shape: *Irregular*. Surface shape: *Umbonate*.	Spores: Aporangium. Hyphae: Septat Hifa. Conidia: Complex.	
SpOrn			
	Colonies are orange. Side shape : *Irregular*. Surface shape : *Filiform*.	Spores: Arthospora. Hyphae: Septat Hifa. Conidia: Cladosporium	

The antibacterial activity of secondary metabolites depends on each microorganism's external environment and nature. The ability of microorganisms to degrade certain compounds can generate different results as they have been shown to mediate inactivate antibiotic compounds enzymatically.[28]

Ethyl acetate solvents can dissolve various compounds, including polyphenols, tannins, alkaloids, terpenes, and steroids.[29,30] The ethyl acetate fraction of *S. palustris* was

Table 2: Antibacterial activity test against endophytic fungi isolates

Test Bacteria	*Inhibitory response*			
	SpHtm	*SpHjP*	*SpHjk*	*SpOrn*
S. aureus (ATCC 25923)	+++	+++	+++	++
E. coli (ATCC 25922)	+++	++	+++	+++
+++ : Strong				
++ : Medium				
+ : Low				
- : No				

reported to contain alkaloids, phenolics, flavonoids, saponins, tannins, and terpenoids.[10-12] Among these various compounds, phenolics and flavonoids are available at the highest levels among all compounds, with the latter being greater than the former. It was indicated that flavonoids in the ethyl acetate extract of *S. palustris* are semi-polar.[31,32]

Flavonoid compounds can inhibit carcinogenic processes both *in-vitro* and *in-vivo*. This inhibition takes place in the initiation or progression stage through molecular mechanisms of inactivation of carcinogenic agents, including against tumor cells, inhibition of angiogenesis and cell cycle, apoptosis, and induction of antioxidant activity.[33]

The ethyl acetate fraction had good antioxidant activity as shown by the results of the antioxidant activity test using the DPPH method, with an ED50 value of 0.650 mg/mL, and the results of the cytotoxicity test using the MTT method on HeLa cells, with the lowest IC_{50} value of 8.60 g/mL.[10,34] According to the research by Yanti *et al.* (2021), the ethyl acetate fraction had cytotoxic activity against HepG2 liver cancer cells with an IC_{50} value of 224.12 g/mL. The ethanol extract showed cytotoxicity effect on MCF-7 cells with an IC_{50} value of 493.57 µg/mL.[35] Meanwhile, the ethyl acetate fraction could increase the expression of p53 and caspase-3 proteins in HepG2 liver cancer cells at concentrations in the range 112.06–448.25 g/mL range. The results of the research by Gunawan-Puteri *et al.* (2021)[36] reported that the ethyl acetate fraction of *S. palustris* has good α-glucosidase inhibitory activity, showing that they can be developed as an antidiabetic agent. Further identification of the various potentials possessed by *S. palustris* is needed to find out specifically more about the pharmacological activity of endophytic fungi in *S. palustris*.

CONCLUSION

The isolation of endophytic fungi from the leaves and stems of the kelakai (*S. palustris*) resulted in three types of isolates coded as SpHtm, SpHjk, and SpOrn that were identified as *Aspergillus* sp., *Paecilomyces* sp., and *Arthrocristula* sp. (SpOrn), respectively. The results of the antibacterial activity test showed that the ethyl acetate extracts of SpHtm and SpHjk isolates did not demonstrate any inhibitory responses againts *S. aureus* at the concentrations of 15, 30, and 45% and had lower inhibitory responses to *E. coli* at the SpHtm concentration of 30% and the SpHjk concentration of 45%, with inhibition zone diameters of 11.63 ± 0.01 and 10.83 ± 0.02 mm, respectively. It is necessary to further identify the antimicrobial activity at higher concentrations because several other studies have shown that *S. palustris* has the potential to be antimicrobial.

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