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Research article

Endophytic fungi isolated from the root bark of sungkai (*Peronema canescens*) as Anti-bacterial and antioxidant

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ABSTRACT

Traditional medicine is still used by the community to prevent and treat various diseases, including infectious diseases and exposure the free radicals. The part of the medicinal plant that is often used as traditional medicine is root bark of sungkai. This study explored endophytic fungi from root bark of sungkai and determined their antibacterial and antioxidant activities. Endophytic fungi were isolated from fresh root bark tissue in PDA (Potato Dextrose Agar) media and identified morphologically. The DPPH (2,2-diphenyl-1-picrylhydrazyl) and diffused-disc paper methods were used for antioxidant and antibacterial. The endophytic fungi extracts showing the best bioactivity were identified molecularly. A total of 6 isolates of endophytic fungi were found in this study (RNA1-RNA6). Antibacterial and antioxidant activity test showed the best activity for RNA4 isolates. Molecular identification results describe it as *Penicillium janczewskii*. This research can be used as a basic reference for isolating pure compounds that have antibacterial and antioxidant properties through chromatography techniques.

Keywords: Antibacterial, Antioxidant, Endophytic Fungi, Root Bark of Sungkai

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INTRODUCTION

The natural resources in Indonesia stores various types of plants, including medicinal plants. The use of plants as medicines is still ongoing today [1,2]. The use of medicinal plants is often used by the community because those are believed to have relatively mild side effects, easy to obtain, and cheaper than synthetic drugs [3,4]. The plant that is often used as a medicinal plant is sungkai. The root bark is used to cure infectious diseases. Infectious diseases are diseases caused by infectious pathogens. Infectious disease is one of the diseases that greatly impacts the entire population and economy globally [5-7]. Corona virus, one of the infectious diseases, is a problem that is still being faced by the global community today. To prevent its spread, foods or substances that contain antioxidant and antibacterial compounds are needed to increase immunity [8–10]. Immunostimulants are closely related to antioxidant and antibacterial activity. Foods, such as fruits, vegetables, spices, and various medicinal plants are the sources of antioxidants and antibacterial substances [11–13]. Antioxidants and natural antibacterials contain many important secondary metabolites, namely polyphenols, alkaloids, flavonoids, and various vitamins, especially vitamins E and C [14–17]. Unfortunately, this natural resource is not always sufficient due to the decreasing of natural resources so other alternatives are needed, such as endophytic fungi. Endophytic fungi are fungi that live in symbiotic mutualism in plant tissues. This interaction causes endophytic fungi to produce secondary metabolites that are identical to their host plants [18–20]. The process of isolation, cultivation, and extraction of this endophytic fungi does not take a long time, so this biotechnology is very effective in providing a source of new secondary metabolites, such as phenolic and lacton group. Therefore, this endophytic fungus is the focus of research because it can be used as a source of medicinal raw materials.

MATERIALS AND METHODS

Chemicals used

The chemicals used were aquadest, 70% of alcohol, 1,1-diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid, methanol, dimethyl sulfoxide (DMSO), and extracts of endophytic fungi.

Preparation of plant samples

The fresh and healthy root bark was used for isolation of endophytic fungi obtained from Palembang City, South Sumatra, Indonesia. The plant samples obtained are then determined in the Biosystematic Laboratory, Sriwijaya University (302/UN9.1.7/4/EP/2 021).

Isolation and purification of endophytic fungi

Root bark was washed for ± 3 minutes by using running water. Next, the samples were soaked in 70% alcohol for ± 1 minute, rinsed for ± 1 minute using sterile distilled water, and soaked by using NaOCl 10% for ± 1 minute. After sterilization, the samples were aseptically cut and inoculated into PDA (Potato Dextrose Agar) media in petri dishes. The inoculant was incubated for 3-15 days at room temperature. Purification was carried out on colonies that appeared around the sample by transferring the colonies to a new petri dish containing PDA media and incubating for 2x24 hours [21,22].

Identification of Endophytic Fungi Morphologically

Morphological identification included macroscopic characters (colonial surface color, reverse color of the colony, colony texture, appearance of exudate dots, radial lines, and concentric circles) and microscopic (hyphae (partitioned or not) and spores observed through culture slides under a microscope up to 1000X magnification). The characters that appear during the observation were compared to the relevant references [23–25].

Extraction and Cultivation

Endophytic fungal isolates were cultivated in 15 culture bottles containing 300 ml of PDB (Potato Dextrose Broth) media and incubated for 30 days at room temperature. After the incubation period, the biomass was separated and ethyl acetate solvent was added to the media at a ratio of 1:1. The solvent was evaporated using a rotary evaporator [26].

Test of Antioxidant Activity

Various concentrations of endophytic fungi extract in methanol, namely 1000 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL, 31.25 µg/mL, and 15.625 µg/mL were tested for antioxidant using DPPH. 3.8 ml of DPPH solution (0.05 mM) was homogenized with 0.2 ml of concentrated extract and then incubated (in the dark) for 30 minutes. The UV-VIS spectrophotometer (Shimadzu UVVIS-UV1900) was used to measure absorbance with a wavelength of 517 nm. Antioxidant activity was measured based on the IC_{50} value. The linear regression of inhibitor percent obtained in the form: y = b + ax, was used to find the IC_{50} value (50% inhibitor concentration) for each sample, by stating the y value of 50% and the x value as the IC50. The IC50 value represents the concentration of sample solution required to reduce 50% of DPPH [27,28].

$$\%\ \text{Inhibition} = \frac{A_k - A_s}{A_s} \times 100$$

A_k = Control

A_s = Samples

Test of Antibacterial Activity

The diffused-disc paper method was used to test the antibacterial activity. Four bacteria were used for testing, such as *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus*, and *Bacillus subtilis* as well as MHA (Muller Hinton Agar) as the media. A total of 400 µg/disc of endophytic fungi extract was dripped into the disc paper and tetracycline (30 µg/disc) as a positive control. Paper discs that had been given the extract were placed on the media that has been inoculated with bacteria. Furthermore, It incubated for 1x24 hours at 37°C. The inhibition zone was observed after the incubation period and the criteria for antibacterial activity were determined using the formula [29]:

strong: $\frac{A}{B}$x 100% > 70 %; moderate: 50 % <$\frac{A}{B}$x 100% < 70 %; weak: $\frac{A}{B}$x 100% <50 %

A: Inhibition zone of test samples

B: Inhibition zone of antibiotic

Molecular Identification of Endophytic Fungi

Endophytic fungi extract that showed the best antioxidant and antibacterial results were identified molecularly based on the ITS rDNA area (primers ITS 1 and ITS4). The sample sequences were then BLAST on the http://blast.ncbi.nlm.nih.gov/Blast.cgi. Sample sequences and databases were aligned with the CLUSTAL W method in the MEGA11 program. Phylogenetic tree reconstruction using Neighbor-Joining with a bootstrap value of 1000 [30].

RESULTS AND DISCUSSION

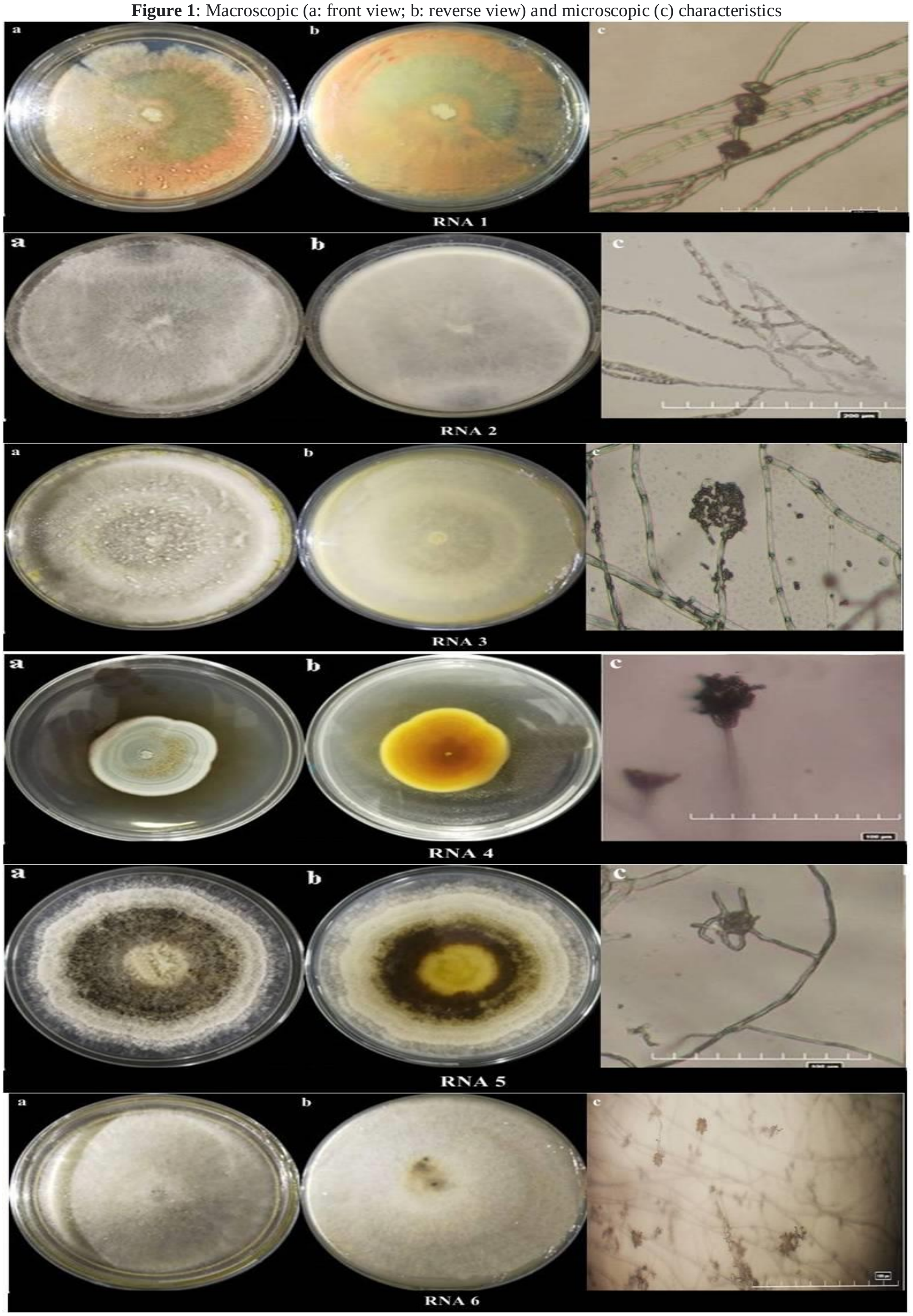
Characteristics of entophytic fungi isolated from Sungkai root bark

The characteristics found in endophytic fungi colonies isolated from sungkai root bark showed variations, both macroscopically and microscopically (Figure 1).

Table 1: Characteristics of endophytic fungi colonies isolated from root bark of sungkai

Code	Surface Colony	Reverse Colony	Structure	Elevation	Pattern	Exudate Drops	Radial line	Concentric circle
RNA1	Orange	Orange	Velvety	rugose	zonate	-	-	√
RNA2	White	White	Cottony	rugose	zonate	-	-	-
RNA3	White greentint	White	Cottony	umbonate	zonate	-	-	√
RNA4	Grayish green	Yellowish brown	Velvety	umbonate	zonate	-	-	√
RNA5	Grayish brown	White soft brown	Cottony	rugose	radiate	-	√	√
RNA6	White	White with greentint	Cottony	umbonate	radiate	-	√	-

Figure 1: Macroscopic (a: front view; b: reverse view) and microscopic (c) characteristics



In this study found six isolates of endophytic fungi. The results of observing the characteristics of the endophytic fungi can be seen in Table 1 and Table 2.

There were 6 species of endophytic fungi found in this study with macroscopic and microscopic characteristics described in Tables 1 and 2. The characteristics that emerged became a reference for identifying the endophytic fungi found. The endophytic fungi found were *Phytophthora megasperma, Pythium Indigofera, Trichoderma pseudokoningi, Penicillium janczewskii, Papulaspora nishigaharanus,* dan *Aspergillus sp.*.

Bioactivity of Endophytic Fungi Extract

The ethyl acetate extract of endophytic fungi isolated from sungkai root bark had antibacterial and antioxidant activity (Figure 2 and Figure 3). Three extracts of endophytic fungi showed strong antioxidant and strong antibacterial activity against several test bacteria. However, only RNA4 revealed strong antioxidant and antibacterial activity against the four tested bacteria.

Tabele 2: Microscopic characteristics of endophytic fungi isolated from root bark of sungkai

Isolate	Spore	Shape	Hyphae	Characteristic	Species of Identification
RNA1	Conidia	sub globose	Septate	Oogonia globose, hyphae often well twisted and knobby	*Phytophthora megasperma*
RNA2	Sporangia	Cylindrical	Septate	Hyphae often slowing dendroid branching, sometimes slightly swollen, often direct connected.	*Pythium indigoferae*
RNA3	sporangia	Globose	Septate	Conidiophores hyaline, branched, phialides short and thick, sub globose	*Trichoderma pseudokoningi*
RNA4	Conidia	SubGlobose	Septate	Conidiophores hyaline, erect, slightly rough, developed from aerial hyphae	*Penicillium janczewskii*
RNA5	sporangia	Globose	Septate	Conidiophores for phialoconida hyaline, erect, simple,rarely branched.	*Populaspora nishigaharanus*
RNA6	sporangia	Cylindrical	Septate	Conidiophores, pale brown, simple, conidia phialosporous, pale green, globose	*Aspergillus sp.*

Figure 2: Percentage of antibacterial activity of endophytic fungi extract isolated from root bark of sungkai compared to tetracycline as a positive control

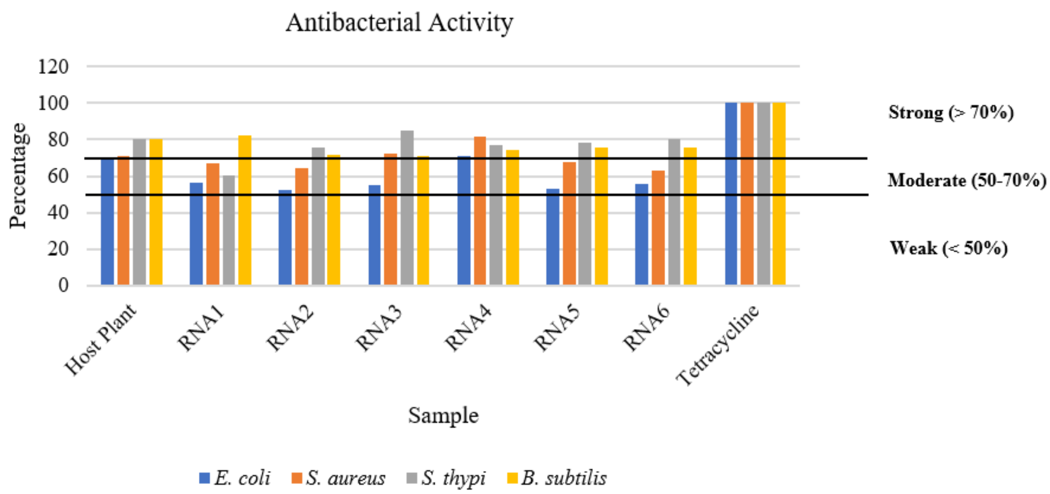


Figure 3: Antioxidant activity (IC_{50} value) of endophytic fungi extract isolated from root bark of sungkai compared to ascorbic acid as a positive control

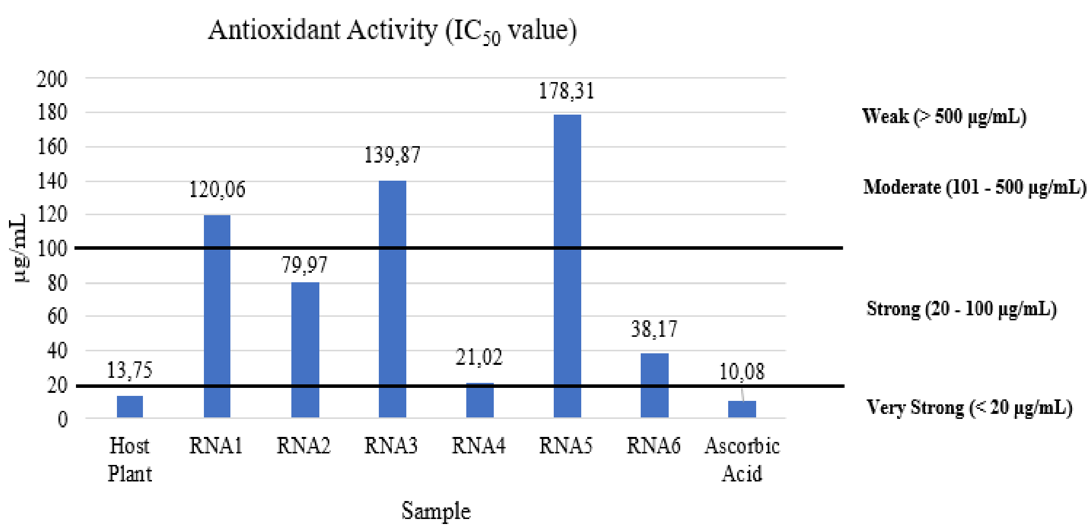


Figure 2 and figure 3 explain that the methanol extract of host plant (root bark of sungkai) inhibited the growth of the four tested bacteria and had very strong antioxidant activity. Ethyl acetate extract of endophytic fungi isolated from host plant also exhibited various categories of antioxidant and antibacterial activity. RNA4 isolates showed strong antioxidant and antibacterial activity with IC_{50} values closest to the positive control, 21.02 µg/mL.

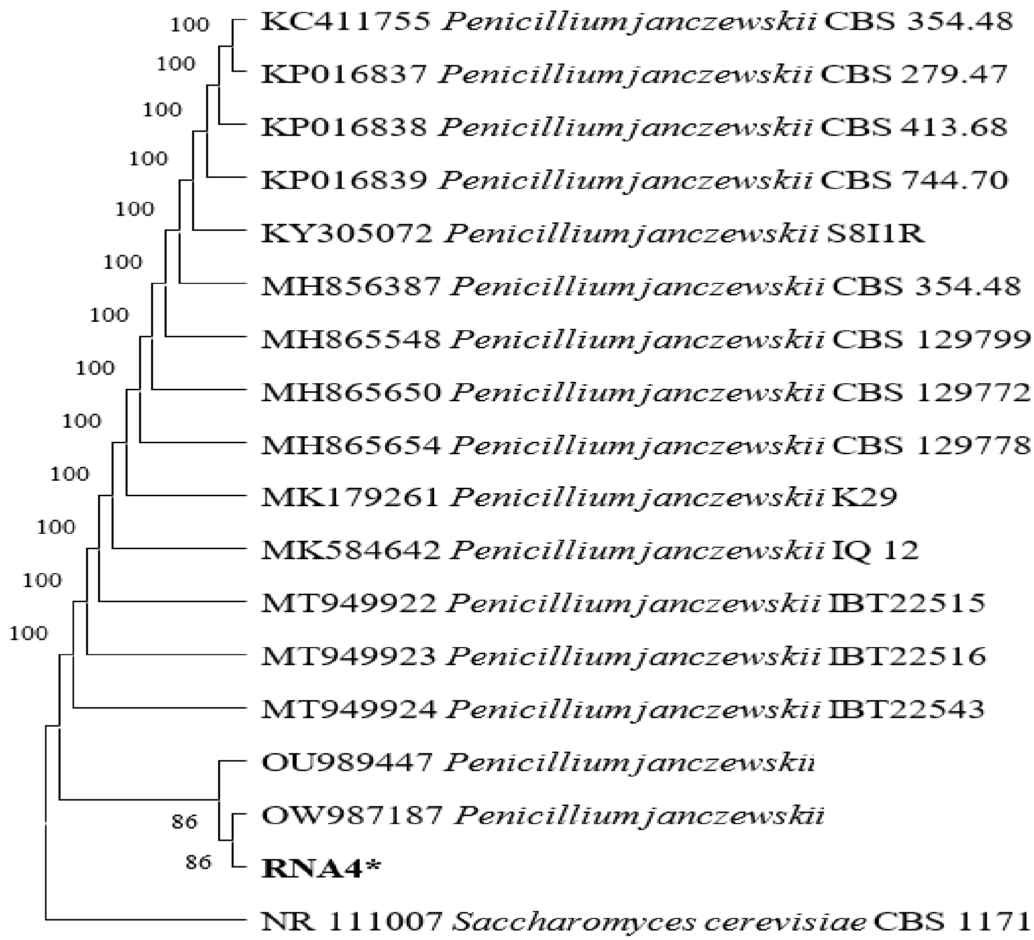
Molecular Identification of Endophytic Fungi

RNA4 isolate was an isolate that has strong bioactivity both antioxidant and antibacterial compared to other isolates. After being tested molecularly, the order of the RNA4 sequence was as follows:

TCATTACCGAGCGAGAATTCTCTGAATTCAACCTCCCACCC
GTGTTTATTGTACCTTGTTGCTTCGGCGGGCCCGCCTCACG
GCCGCCGGGGGGCATCTGCCCCCGGGCCCGCGCCCGCCGA
AGACACCTTGAACTCTGTATGAAAATTGCAGTCTGAGTCTA

AATATAAATTATTTAAAACTTTCAACAACGGATCTCTTGGT
TCCGGCATCGATGAAGAACGCAGCGAAATGCGATACGTAA
TGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACG
CACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCG
AGCGTCATTGCTGCCCTCAAGCCCGGCTTGTGTGTTGGGTC
TCGTCCCCCTTCCCGGGGGGACGGGCCCGAAAGGCAGCGG
CGGCACCGCGTCCGGTCCTCGAGCGTATGGGGCTTTGTCAC
CCGCTCTGTAGGCCCGGCCGGCGCTTGCCGATCAACCAAA
ACTTTTTTCCAG. Based on these sequences, RNA4 isolate showed a similarity of 99.80% and 1 clade with *Penicillium janczewskii*. The phylogenetic tree can be seen in Figure 4.

Figure 4: Reconstructed RNA4 (*) phylogenetic tree by using Neighbor-Joining (bootstrap value = 1000)



Penicillium is a fungus from the Ascomycota class with soil as its natural habitat. This genus is more common in the environment than fungi of other genera and they are also found as endophytes in plant tissues[31-34]. In this study, *Penicillium janczewskii* was found as an endophyte on the root bark of sungkai. At present, there is no specificity of the living tissue of *P. janczewskii* because this species has been reported to be found in the phloem of plants [35,36]. Fungi found in plant tissues, especially medicinal plants, are known to have the same bioactivity as their hosts, such as antioxidants, anti-inflammatories, antibacterials, and anticancer **[37–39]**. The findings in this study indicate that *P. janczewskii* was capable of producing compounds found in its host. Several studies have described that the conidia of *P. janczewskii* have many benefits to their host plants, such as reducing anthracnose disease, activating defense genes, and protecting the lower part of the plant stem. Apart from having these benefits, its nature as an endophyte, the compounds produced by this fungus also have biological activities such as antibacterial and antioxidant [36,40,41].

The antibacterial activity of host plant methanol extract (Figure 2 and Figure 3) was in the strong category against the four test bacteria and very strong antioxidant activity (IC_{50} < 20 µg/mL). This bioactivity is related to the content of secondary metabolites. Several studies reported that Sungkai root bark contains alkaloids, flavonoids, phenolics, tannins, saponins, and peronemin compounds [27,42,43]. These secondary metabolites are known to have antibacterial and antioxidant activities.

Antibacterial and antioxidant activity of endophytic fungi extract isolated also had strong activity. Figure 2 and Figure 3 revealed that there was one endophytic fungi extract that had the best antibacterial and antioxidant activity, namely isolate RNA4. The results of molecular identification described that the RNA4 isolate was *Penicillium janczewskii*. Extraction of the endophytic fungus *P. janczewskii* using ethyl acetate solvent showed strong antioxidant activity (IC_{50} < 100 µg/mL) and strong antibacterial activity against the four test bacteria. However, host plant extracts showed better antibacterial test against several test bacteria and antioxidant activity than the endophytic fungi extracts. The various chemical components contained in host plant extracts lead to better bioactivity. This is as a result of the components synergizing with each other so that the bioactivity is stronger[39,44]. However, extracts of endophytic fungi, especially *P. janczewskii* isolated from root bark of sungkai had potential as antibacterial and antioxidant. With the many benefits produced by this mushroom so that it can be used as a source of raw materials for medicines. The existence of resistance to antibiotics at this time makes this discovery a bioprospect. The bioactivity shown by *P. janczewskii* extract was inseparable from its chemical content.

The secondary metabolites contained in the *P. janczewskii* are griseofulvin, fumagilin, curvulic acid, pseudotin A, various types of terpenoids, and flavonoids which are the dominant substances. These ingredients have been reported as antioxidants and antibacterials[45–48]. Flavonoids are phenolic substances that can be found in various plants. This secondary metabolite is reported to have antioxidant and antimicrobial activity [49–52]. Many studies describe that flavonoids act as hypoallergenic, antiviral, anti-inflammatory, and vasodilating. However, most explain that flavonoids are specialized as antioxidants because of their ability to reduce and even eliminate free radicals [53,54]. Other information states that flavonoids are known as antibacterial agents against various pathogenic microorganisms. With the increasing prevalence of untreatable infections caused by antibiotic-resistant bacteria, flavonoids have been shown to be potential substitutes for antibiotics. The hydroxyl groups in the aromatic ring of flavonoids increase their bioactivity, both antioxidant and antibacterial. However, methylation of active hydroxyl groups generally decreases bioactivity. In addition, the lipophobicity of the A ring is critical for the activity of the chalcones. Hydrophobic substituents such as prenyl groups, alkylamino chains, alkyl chains, and heterocyclic groups containing nitrogen or oxygen usually increase their bioactivity [55].

CONCLUSION

Six endophytic fungi were found in this study, namely *Phytophthora megasperma*, *Pythium indigoferae*, *Trichoderma pseudokoningi*, *Penicillium janczewskii*, *Papulaspora nishigaharanus*, and *Aspergillus* sp. The fungus *P. janczewskii* has antioxidant and antibacterial activity due to the presence of metabolites of the flavonoid class. In future studies, isolation of antioxidant and antibacterial compounds will be carried out which have not been reported so far.

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Conflict of interest:

There is no conflict of interest.

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