

Molecular Identification of Endophytic Fungi from Bark of Raru (*Cotylelobium melanoxyton*) that Produce the Antibacterial Compounds

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Abstract

The Dipterocarpaceae plant, raru (*Cotylelobium melanoxyton*) is widespread in South-east Asia. The bark of raru has been used by local communities in North Sumatera as antidiarrheal drugs due to its antibacterial compounds. The antibacterial activity of the raru's bark is induced by endophytic fungi that live in the region of the bark. This study aimed to identify the endophytic fungi-producer of antibacterial compounds in the bark of raru (*C. melanoxyton*) by means of molecular analysis. In general, endophytic fungi have the ability to inhibit the growth of pathogenic bacteria. Thirty-eight isolates of endophytic fungi were isolated from the bark of raru. Selection of isolates for antibacterial activity against *Escherichia coli* ATCC 35218 and *Staphylococcus aureus* ATCC 25923 used the dual culture assay. Selection using the dual culture assay yielded 6 endophytic fungal isolates that have the ability to inhibit the growth of test bacteria. EF10A sample was the most powerful isolate inhibiting the growth of both bacteria test. Those six bacteria molecularly identified used a sequence generated from ITS rDNA region. Based on rDNA ITS region sequences, isolate, the producers of the antibacterial compound were identified as *Talaromyces cellulolyticus*, *Penicillium purpurogenum*, *Aspergillus* sp., *Trichoderma harzianum*, and *Aspergillus oryzae*. The results of this study can be used by researchers to explore more potential endophytic fungi in raru plants (*C. melanoxyton*) as a source of medicine. The data obtained need to be supported by further research to isolate the bioactive compounds that can inhibit the growth of microbial pathogens.

How to Cite

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INTRODUCTION

Resistance to microbes become one of the major problems in the world. Therefore, it is necessary for research to find the new sources of bioactive compounds that can overcome the bacterial or fungal infection (Devi & Wahab, 2012). Due to the development of resistance in pathogenic microbes, the need for new antibiotic sources has increased rapidly (Devi *et al.*, 2012). Bioactive compounds can be obtained from several sources, including from plants, animals, microbes and marine organisms (Prihatiningtias & Widayastuti, 2006). Plants are an important source of biologically active compounds in natural product research. Many plant species have been used in traditional healing and have been studied for pharmacological properties (Marcellano *et al.*, 2017). Medicinal plants are known to host multiple endophytic fungi and engage in the production of active metabolite compounds (Alvin *et al.*, 2014).

Indonesia is a country with a high biodiversity and broad tropical rain forests it becomes an advantage in the search for the source of bioactive compounds (Reddell & Gordon, 2000). One source of bioactive compounds derived from microorganisms are endophytic fungi (Strobel, 2003). The endophytic fungus is a rich source of bioactive secondary metabolites (Tan & Zou, 2001) and is one class of endophytic microbes which are most commonly found in nature. These fungi live in a symbiotic mutualism associated with its host plant (Strobel, 2003).

Endophytic fungi infect healthy plants in certain tissues without causing any signs of infection (Bacon & White, 2000), then produce enzymes, secondary metabolites (Tan & Zou, 2001), mycotoxins, and also antibiotics (Carroll, 1988) which can be beneficial for the physiology and ecology of host plants against diseases caused by plant pathogens. Instead, the endophytic fungi can obtain nutrients to complete the life cycle of the host plant (Petrini *et al.*, 1993).

The bark of *raru* (*Cotylelobium melanoxylon*) is one source of antibacterial potential as a medicine and has been used in a long time by the local community. Local communities in North Sumatra have been utilizing the bark of *raru* to treat diarrheal diseases and diabetes (Soerianegara & Lemmens, 1993). The bark of *raru* contains *ampelopsin F*, *isoampelopsin F*, *ε-viniferin*, *vaticanol A*, *E*, *G*, *lyoniresinol* (Matsuda *et al.*, 2009), and *flavonoids* as antidiabetic, *tannins* and *saponins* compounds (Pasaribu, 2010) which are useful as an antimicrobial (bacteria and viruses).

Escherichia coli (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) are two opportunistic pathogens that can cause infection in humans (Bachir & Benali, 2012). *E. coli* is a common pathogenic bacterium toxic to food and causes some diseases in humans that extend from the gastrointestinal tract to the extraintestinal tract such as the urinary tract, bloodstream, and central nervous system (Croxen & Finlay, 2010). *S. aureus* is a dangerous and versatile pathogen that can cause many different diseases, most commonly causing skin infections and respiratory infections (Otto, 2014). Both of these bacteria are commonly used in the bioactivity test of bioactive compounds.

Based on the above background, it is necessary to identify endophytic fungi on plants *raru* (*C. melanoxylon*) that can produce antibacterial compounds so that in the future it can be used as a source of medicine for humans. This study aimed to isolate and identify endophytic fungi on plants *raru* (*C. melanoxylon*) producer of an antibacterial agent using test samples on pathogenic bacteria and molecular analysis of DNA sequences generated from ITS rDNA region.

METHODS

Isolation of endophytic fungi from *raru* plants (*C. melanoxylon*)

The Bark of *raru* plants was obtained from Sitahuis Village, Sibunga-bunga District, Central Tapanuli, North Sumatra Province. *Raru's* bark was in a beam-shaped cut for 15-20 cm long, 3-5 cm wide and 1-2 cm thick to be brought to the laboratory. Bark samples were then cleaned with flowing water. Bark samples were cut to a size of 1.0-1.5 x 0.5-1.0 cm, and placed on filter paper and dried at room temperature. Bark samples were sterilized using Hallman's method (Hallman *et al.*, 2006) that the bark samples were soaked in 96% of ethanol solution for 1 min, in sodium hypochloride solution for 5 minutes, and 70% of ethanol for 1 minute. Bark samples were passed on the through the flame and then rinsed with aqua-bidest twice and dried using sterile filter paper. Samples of bark were placed on PDA media (Potato Dextrose Agar) on petri dishes with the addition of amoxicillin. Each petri dish had 5 sample pieces of bark. Incubation was carried out at room temperature for 5-7 days until the fungal growth occurs.

Isolate Selection for antibacterial activity

Antibacterial activity isolates of *Escherichia coli* ATCC 35218 and *Staphylococcus aureus* ATCC 25923 used the dual culture assay. Endophytic

fungi and test bacteria were maintained in a petri dish with NA medium. Endophytic fungi were incubated for 1-2 days at room temperature while the test bacteria were incubated for 24 hours at a temperature of 30°C. The wells are made on a medium containing test bacteria and filled with endophytic fungal isolates then incubated for 1-2 days at room temperature. Isolates with the clear limpid zone was an indicator of the ability of isolates to inhibit the growth of bacteria. The antibacterial activity was determined by measuring the inhibitory zone (clear zone) around the wells. The inhibition zone (clear zone) was measured and stated in millimeters. Isolates with the ability to inhibit the growth of pathogenic bacteria used for further research.

Molecular identification of endophytic fungi isolates

The endophytic fungi isolate and the potential of antibacterial activity were molecularly identified using the sequence generated from ITS rDNA region. Isolation of DNA was done by GeneJET Specific DNA isolation kit. Amplification of rDNA ITS region of Endophytic fungi applied two universal primers of ITS1 (5'- TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC -3') and by PCR (Polymerase Chain Reaction) machine of SensoQuest Labcyler. The condition of amplified gene fragment was pre-denaturation of the target DNA at 94°C for 5 minutes followed by 30 cycles at 94°C for one minute, primer annealing at 55°C for one minute and primer extension at 70°C for 2 minutes. PCR was ended with the temperature of 72°C for 10 minutes and followed by cooling until the temperature is 4°C. PCR products were visualized by gel electrophoresis of 1.5% and SYBR Safe DNA gel stain for staining then measured by the ladder of 100 bp - 1500 bp utilizing 100-volt electrophoresis machine for 45 minutes. Electrophoresis results were observed using UV Light Gel Documentation System (Biostep, Felix 2040). PCR result showed a clear band followed by sequencing using sequencing service of Genetics Science, First Base, Singapore.

The nucleotide order of ITS rDNA region sequencing results from endophytic fungi was compared with the collection of the ITS rDNA region at the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov). Program Basic Local Alignment Search (BLAST) was used to determine the types of isolates based on the GenBank database. Reconstruction of the phylogenetic tree was created using the MEGA 6 program by comparing the isolates with se-

quential order of endophytic fungi ITS rDNA region obtained from the DNA database at NCBI GeneBank. The root position on the unrooted tree was estimated by using *Rhizopus microsporus* EU798703.1 (GeneBank) as the outgroup strain.

RESULTS AND DISCUSSION

Bioactivity assay of endophytic fungi isolates

A total of 38 isolates of endophytic fungi collected from bark of *raru* (*C. melanoxydon*). Bacteria that were used to determine the antibacterial compound-producing endophytic fungi were *Escherichia coli* (ATCC 35218) and *Staphylococcus aureus* (ATCC 25923). From a total of 38 isolates of endophytic fungi as the isolation result, only 6 endophytic fungi isolates were able to inhibit the growth of both test bacteria. Inhibition zone diameter of 6 isolates of the fungi are shown in Table 1.

Table 1. Zone diameter inhibiting the bacteria growth by fungi

Isolate	Inhibitory Zone	
	<i>E. coli</i> (ATCC 35218) (mm)	<i>S. aureus</i> (ATCC 25923) (mm)
Sample EF2K	10.26	7.1
Sample EF8	7.1	6.96
Sample EF2A	3.93	3.9
Sample EF10A	10.53	7.2
Sample EF10B	7.33	7.13
Sample EF3A	7.3	7.03

Table 1 shows that, six isolates of endophytic fungi have the ability to inhibit the growth of test bacteria. EF10A endophytic fungi isolates had the greatest inhibition zone while isolates EF2A had the smallest inhibitory zone against the *E. coli* and *S. Aureus* bacteria.

Endophytic fungi ability to inhibit microbial growth has been widely reported. Idramsa *et al.*, (2015) reported that the isolated endophytic bacteria from *C. melanoxydon* has the ability to inhibit pathogenic bacteria such as *Escherichia coli* and *Staphylococcus aureus*. The isolated endophytic fungi from *Quercus variabilis* has the ability to inhibit the pathogenic fungi (*Trichophyton rubrum*, *Candida albicans*, *Aspergillus niger*, *Epidermophyton floccosum*, *Microsporium canis*, *Cryptococcus neoformans*) (Wang *et al.*, 2007) and bacteria (*Escherichia coli*, *Bacillus subtilis*, *Pseudomonas fluorescens*) (Phongpaichit *et al.*, 2006). The crude extract of isolated endophytic fungi from *Spondias mombin* inhibits the growth of actinomycetes, *Saccharomy-*

ces cerevisiae, *Geotrichum* sp., *Penicillium canadensis*, *Cladosporium elatum*, and *Mycotypha* sp. (Rodrigues *et al.*, 2000).

Endophytic fungi isolates were able to inhibit the growth of test bacteria because of the activities of secondary metabolites contained in the isolates. The ability of endophytic fungi producing secondary metabolites is a combination of the interaction between the endophytic fungi and the host plant (Tan & Zou, 2001). The ability of isolates in inhibiting the growth of gram positive and negative bacteria showed that the isolates can produce the metabolites as an antibacterial agent (Black, 2002). Various metabolites compound produced by endophytic fungi affected the increase of the host plant resistance against the pathogens and environmental stress (Habibah & Ambar, 2013). Dudeja *et al.* (2012) states that the composition of endophytic fungi is strongly influenced by environmental and host plants. Since the host affects the production of endophytic fungal metabolites, further studies on whether the biosynthesis of active metabolite compounds continues or ceases when the fungus is grown *in vitro* is also necessary to know the main cause of the biosynthesis of the metabolite compound in the fungus (Owen & Hundley, 2004). The composition of endophytic fungi also depends on the interaction of pathogenic fungi and other microorganisms (Rubini *et al.*, 2005).

Environmental conditions affect the diversity of endophyte that lives on its host (Vega *et al.*, 2010). The existence of endophytic fungi is influenced by seasonality (Mahesh *et al.*, 2005), environmental factors (Petriani *et al.*, 1993), and the type of host plant tissues (Mahesh *et al.*, 2005). Variability in the composition of endophyte on a host plant is based on the effect of the physiological condition of the host, even on the same observation area (González & Tello, 2011). According to Arnold & Lutzoni (2007), endophyte will increase the presence, diversity, and regional distribution of host plants if they are at high geographic area. Plant defense responses limit the population of endophytic microorganisms within plants (Rosenblueth & Martinez-Romero, 2006).

The main advantage of endophytic fungi utilization in drug discovery is its diversity offering a new source of abundant metabolites (Marcellano *et al.*, 2017). In addition, endophytic fungi can be used as an alternative compound isolated from plants that can reduce the use of a large number of plants that produce these compounds (Alvin *et al.*, 2014).

Molecular identification of isolates endophytic fungi

The accuracy of molecular biology techniques used for genetic differentiation of species shows a very important advance in the identification of fungal species (Santos *et al.*, 2017). Molecular identification is done by analyzing the isolates that have the ability to inhibit bacterial growth test based on ITS rDNA region sequence comparison (Petti *et al.*, 2005). Furthermore, the obtained rDNA ITS sequence can be compared with the data available in the GenBank database (Magnani *et al.*, 2005).

Phylogenetic analysis demonstrated that EF3A and EF8 isolates sample are the most closely aligned with *Talaromyces cellulolyticus* KM458826.1 (GeneBank) with 99% of homology. EF10B identified sample is closest aligned with *Penicillium purpurogenum* JQ663996.1 (GeneBank) with 91% of homology. Identified EF2A sample is closest aligned with *Aspergillus* sp. KJ934699.1 (GeneBank) with 100% of homology and EF2K sample is closest aligned with *Trichoderma harzianum* JX473720.1 (GeneBank) with 99% of homology. EF10A sample is identified most closely aligned with *Aspergillus orizae* HQ285580.1 (GeneBank) with 99% of homology. Outgroup (*Rhizopus microsporus*) has a separate branch from endophytic fungi isolates (Figure 1).

Identification using ITS sequence analysis is generally used to identify the fungi to species level (Nilsson *et al.*, 2008). Guo *et al.* (2000) use the ITS rDNA region sequences to identify *Valsaceae*, *Mycosphaerellaceae*, *Xylariaceae*, *Clypeosphaeriaceae*, and *Dothideales* of endophytic fungal isolates on *Livistona chinensis* ITS rDNA assay showed that endophytic fungi of 174 medicinal plants are identified as *Altenaria*, *Phomopsis*, *Sporidesmium*, *Paecilomyces* and *Fusarium* (Lin *et al.*, 2007). ITS region has the highest probability of success for identifying the fungi in a broad range with the most obvious gap barcode on interspecific variation and intraspecific (Schoch *et al.*, 2012).

Some members of *Talaromyces* have antibacterial abilities. *Talaromyces islandicus*, *T. radicus*, *T. rugulosus*, and *T. wortmannii* produce rugulosin which inhibits growth in *Staphylococcus aureus* and skyrin which has been reported as antitumor agents (Yamazaki *et al.*, 2010). *Talaromyces coalescens* produces botryodiploidin which is a mycotoxin with antifungal, antibacterial, and insecticidal effects (Cabedo *et al.*, 2007). *Penicillium purpurogenum* has the ability to inhibit the growth of bacterial *E.coli*, *B. subtilis*, *P. aeruginosa*, and *S. aureus* (Sharma *et al.*, 2013). *Aspergillus* sp produc-

es metabolites that exhibit the antibacterial activity against 6 pathogenic bacteria (*Staphylococcus albus*, *Bacillus subtilis*, *Bacillus cereus*, *Sarcina lutea*, *Escherichia coli* and *Micrococcus tetragenus*) and two marine strains (*Vibrio parahaemolyticus* and *Vibrio anguillarum*) (Li *et al.*, 2012). *Aspergillus oryzae* has been used in the food fermentation industry because it produces antibacterial compounds such as kojic acid and aspirochlorine (Leonard *et al.*, 2013). *Trichoderma harzianum* is effective at various concentrations in inhibiting the activity of bacteria *Staphylococcus aureus*, *Proteus*, *E. Coli*, and *Klebsiella* (Leclavathi *et al.*, 2014).

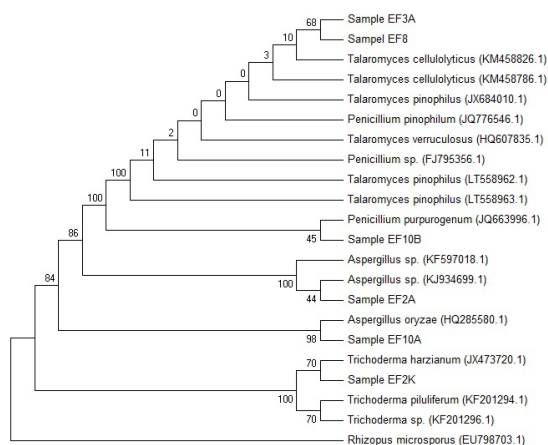


Figure 1. The phylogenetic tree showing the relationship between endophytic fungi isolates of *C. melanoxydon* with various types of endophytic fungi from the GeneBank based on ITS rDNA region sequences.

Indonesia has the world's largest tropical rainforest that offers incredible biodiversity, largely unexplored. Utilizing the traditional knowledge to study a plant that has been used as a medicinal source helps to narrow down the target plant with the potential of new antibacterial compounds. Many bioactive compounds in plants are actually produced by the interactions with their endophytes. Exploring the endophytes of this medicinal plant will help to isolate and produce its bioactive compounds. Efficient identification mechanism are essential for obtaining the endophytic fungi and its potential bioactive compounds. It will help to expand an antibacterial drug discovery from endophytic fungi of traditional medicinal plants.

CONCLUSION

Thirty-eight endophytic fungi were isolated from the bark of *raru* (*C. melanoxydon*). Based on the selection using a dual culture assay, 6 iso-

lates showed their ability to inhibit the growth of gram-negative bacteria (*Escherichia coli* ATCC 35218) and gram-positive bacteria (*Staphylococcus aureus* ATCC 25923). Based on rDNA ITS region sequences, isolates, the producer of antibacterial compounds agent were identified as *Talaromyces cellulolyticus* (EF3A and EF8; homology 99%), *Penicillium purpurogenum* (EF10B; homology 91%), *Aspergillus* sp. (EF2A; homology of 100%), *Trichoderma harzianum* (EF2K; homology 99%), and *Aspergillus oryzae* (EF10A; homology 99%).

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