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Antibacterial activity of two isolated endophytic fungi extracts associated with Indonesian mangrove plant *Rhizophora mucronata*

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ABSTRACT

Endophytic fungi are microorganisms reside in the living tissues of the host plant. This fungi can contribute to provide protection to the host from the infection caused by another microorganism. The one reasonable strategy to discover a new antibacterial agent from endophytic fungi are from the plant which lived in special condition such *Rhizophora mucronata*. The aim of this research is to determine antibacterial activity of the endophytic fungi extracts associated with *Rhizophora mucronata* from Sagara anakan. The isolated endophytic fungi was identified as *Neopestalotiopsis* sp. and *Peniophora lycii* using molecular analysis method. The antibacterial activity was carried out by using microdillution method. Antibacterial properties from mycelium extracts showed moderate to low antibacterial activity with Minimum Inhibitory Concentration (MIC) values of 125 up to >500 µg mL^{-1} against *Eshcerichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 bacteria. The *n*-hexane fraction of *Peniophora lycii* showed strongest antibacterial activity against *Staphylococcus aureus* with MIC values of 125 µg mL^{-1}.

Keywords: endophytic fungi, *Rhizophora mucronata*, antibacterial activity

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INTRODUCTION

Endophytic fungi known as microorganism that live in internal tissues of living plants with symbiotic interaction (Alvin *et al*., 2014; Reinhold-Hurek *et al*., 2011). Endophytic fungi can contribute to their host plant by producing substances that provide protection to the plant, for instance, from pathogenic infection (Strobel, 2004). Becaused of that, no wondering if the endophytic fungi can play a role as a new source as antibacterial agents (Radić and Štrukelj, 2012). It is important to determine reasonable plant used to provide isolate endophytes prone to produce antibacterial agents (Yu *et al*., 2010). One reasonable startegy is to choose plant which growing in special habitats like *Rhizophora mucronata* mangrove plant.

Rhizophora mucronata distributed in Indonesia mangrove forest area and locally known as bakau hitam (Suciatmih, 2015; Prihanto *et al*., 2011; Halidah, 2010; Setyawan *et al*., 2006; Sukardjo *et al*., 1992). Traditionally, people used the parts of this plant to threat some disease such as fungal infection, diarrhea, dysentery and tuberculosis (Puspitasari *et al*., 2012; Bandaranayake, 2002). Therefore, many studies have examined the usefulness of this plant as antibacterial agents either in the plant extract or isolated active compounds. For an example, the *R. mucronata* leaves extracts has been reported to show potent antibacterial activity against both Gram positive and Gram negative pathogenic bacteria with in vitro antibacterial assays (Manilal *et al*., 2015; Kusuma *et al*., 2011).

The studies of *R. mucronata* plant with antibacterial properties which were reported not only from the plant extract but also from the endophytic fungi which lives associated with this plant (Buatong *et al*., 2011). From previous report, the ethyl acetate extract of endophytic fungi isolated from *R. mucronata* which collected in Jakarta were reported had antibacterial and insecticidal properties (Abraham *et al*., 2015; Tarman *et al*., 2013). The antibacterial activity from endophytic fungi isolated from *R. mucronata* from another Indonesian mangrove forest like Sagara Anakan Lagoon has not been studied. However, Sagara anakan is known as the largest of remnant mangrove forest in Cilacap, Central Java (Djohan, 2014).

In continuation of a study of antibacterial agents screening from Indonesian mangrove plant especially *R. mucronata*, we wish to report antibacterial activity of fungal endophytes extracts from leaves of *R. mucronata* which collected in Sagara Anakan Lagoon. The bacteria which used in this study of were *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923. Determining the antibacterial activity of the extract using microdillution metods to obtain the value of minimal inhibitory concentrations (MIC).

METHODS AND METHODS

Materials and Instruments

The materials were used in this research are *R. mucronata* leaves from Tritih Lor Village, Cilacap, Central Java, chloramphenicol, amoxicillin, ethanol 70%, sodium hipochlorite 5.25%, ethanol 96%, methanol, *n*-hexane, ethyl acetate, vanillin, sulfuric acid, Dragendorff's reagent, precoated silica gel plates (Merck Kieselgel 60 GF254), Whatman No.1 filter paper, Potato Dextrose Agar (PDA) media, Potato Dextrose Broth (PDB) media, Mueller Hinton Agar (MHA) media, dimethyl sulfoxide (DMSO), McFarland 0.5 standard solution, aquadest, isolate of *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 bacteria. Meanwhile, the instrument which used in this research were general laboratory glass ware, Laminar Air Flow (LAF), micro pipette, autoclave (Hirayama), incubator (Memmert), oven (Memmert), water bath, analytical balances, microplate with 96 wells and Spectrophotometer Microplate Reader (Bio-Rad xMarkmicroplate).

Experimental Methods

Isolation and identification of endophytic fungi

The leaves were rinsed with running water and cut into small pieces (0.5 x 0.5 cm) with sterile pinch cutter and immersed. The samples were immersed in ethanol 95% for 30 s, NaOCl (5.3%) for 5 min, ethanol 95% again for 30 s, before final rinse in sterilized distilled water. Four segment of tissues sample were placed on potato dextrose agar (PDA) medium, and culture at 25^{o}C for 1 weeks. Chloramphenicol (1 mg/mL) was used in PDA media to control the bacterial contamination (Buatong *et al*., 2011). The endophytic fungi were identified with molecular analysis method of the internal transcribed region (ITS) on the locus of ribosomal DNA as described by previous methods (White *et al*., 1990; O`Donnell, 1993; Hiraishi *et al*., 1995). The DNA of the isolated fungi was compared wih DNA data bank from DDBJ (DNA Data Bank of Japan) and NCBI (National Center for Biotechnology Information).

Fungal cultivation and extraction

The two Isolated fungi was grown in 250 mL Erlenmeyer flasks containing 100 ml Potato Dextrose Broth (PDB) medium. The flask were incubated at 25^{o}C for 3 weeks. The fungal culture was filtered by vacuum filter using Buchner funnel to separate the mycelia and filtrate. Dried and powdered mycelia was macerated with methanol and, after solvent evaporation under reduced pressure, afforded a dark yellowish crude extract. Methanol extract was gradually extracted with *n*-hexane and EtOAc. The fractions were evaporated and obtained *n*-hexane and EtOAc extract respectively (Buatong *et al*., 2011).

Phytochemical analysis of extracts

Thin Layer Chromatography (TLC) methods was used to analyse the active compound that present in extracts. The test extract solution was applied on a precoated TLC plate. The different solvent systems of different polarities were prepared and choosed to select which capable of showing better resolution. After drying the plates, they were sprayed with detection reagent. Vanillin/sulfuric acid reagent was used as universal reagent. Tha alkaloids present was detected with Dragendorff's reagent. The plates were visualized directly after drying with the help of UV at 366 nm (Deepa and Padmaja, 2014).

Antibacterial assay

The Minimum Inhibition Concentration (MIC) was determined by using broth microdilution method according to the Clinical and Laboratory Standard Institute (CLSI, 2012). The samples were tested against *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922. Chloramphenicol were used as positive control. The samples were dissolved in dimethylsulfoxide (DMSO) to achieve 500 μg/mL in the first well. Two-fold dilution of samples was performed in a 96-wells microplate over the range 500 μg/mL to 3.9 μg/mL. The lowest concentration of extract that inhibited visible growth was recorded as the MIC value.

RESULTS AND DISCUSSION

In this study, we successfully isolated two isolated endophytic fungi (T1 and T4) from the leaves of *R. mucronata*. The ribosomal DNA of the isolated fungi showed similarity sequences with genome data in DDBJ/NCBI and identified as *Neopestalotiopsis* sp. (T1) and *Peniophora lycii* (T4) (Figure 1). This is the first report of *Neopestalotiopsis* sp. (T1) and *Peniophora lycii* endophytic fungi which isolated from *R. mucronata* plant. Endophytic fungi of *Pestalotiopsis* sp were reported associated with *R. mucronata* (Xu *et al*., 2011). The species of *Neopestalotiopsis* sp. were found in *Bridelia retusa*, *Careya arborea*, *Cinnamomum malabatrum*, *Cordita dichotoma*, and *Diospyros montana* plants (Reddy *et al*., 2016). From the powdered mycelium of *Neopestalotiopsis* sp. (2 g), the methanol, *n*-hexane, and ethyl acetate extracts that we obtained were 425 mg, 75 mg, and 5 mg

respectively. At the same time, from the *Peniophora lycii* (2 g) we collected methanol extract (455 mg), *n*-hexane extract (2 mg), and ethyl acetate extracts (45 mg).

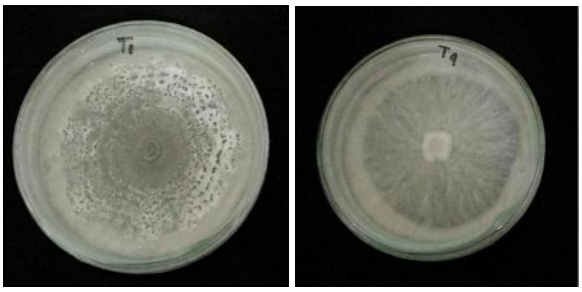
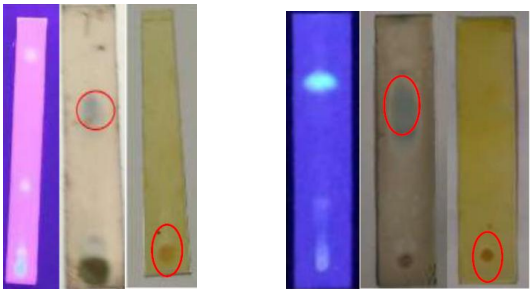


Figure 1. Endophytic fungi *Neopestalotiopsis* sp. (T1) and *Peniophora lycii (T4)* isolated from *R. mucronata*

Thin Layer Chromatography (TLC) method was used for further analysis of the present of active compound. Vanillin/sulfuric acid were used as universal reagent. Dragendorff's reagent were used to detect alkaloid compound (Gibbon, 2006). Based on TLC analysis, terpenoid (blue colours) and alkaloid (orange colours) compound were detected in total extract of both endophytic fungi (Figure 2). Among the extracts, the *n*-hexane fraction of *Peniophora lycii* displayed the strongest antibacterial activity against *Staphylococcus aureus* with MIC value of 125 µg mL^{-1} (Table I). It suggested terpenoids compound play a role to the antibacterial activity in *n*-hexane fraction. Some terpenoids, especially sesquiterpenes, were proved to have antibacterial activity. Beside that, sesquiterpenes, diterpenes and triterpenes are found as the major terpenoids compound from microbial endophytes (Yu *et al*., 2010).



T1 **T4**

Figure 2. TLC analysis of MeOH extract of *Neopestalotiopsis* sp (T1) and *Peniophora lycii* (T4). UV light detection at 366 nm (left), detection with vanillin/sulfuric acid reagent (center), and detection with Dragendorff's reagent (right)

Up to now, the isolated active compound from *Peniophora lyci* has not been reported yet. Meanwhile, a decalin-type tetramic acid derivative and furanone derivative were reported as isolated compound from *Neopestalotiopsis* sp. (Zhao *et al*., 2015). The biological activities of this compound were reported can inhibit bacterial DNA gyrase (Schobert and Schlenk, 2008). The antibacterial properties of isolated endophytic fungi extracts showed more active against Gram-positive bacteria (*Staphylococcus aureus*) than Gram-negative bacteria (*Escherichia coli*). In contrast to extracts of fungal endophytes *Peniophora lycii* isolated from *Juniperus* trees in Saudi Arabia, it can inhibited bacterial growth of Gram-positive and Gram-negative bacteria (Gherbawy and Elhariry, 2014).

Table I. MIC values (µg mL^{-1}) from mycelium extracts of T1 and T4

Endophytic Fungi Mycelium Extracts	*S. aureus* ATCC 25923	*E. coli* ATCC 25922
***Neopestalotiopsis* sp. (T1)**		
Methanol extract	250	250
n-hexane extract	250	>500
Ethyl acetate extract	250	250
***Peniophora lycii* (T4)**		
Methanol extract	250	250
n-hexane extract	125	500
Ethyl acetate extract	250	250
Amoxicillin	3.9	7.8

CONCLUSION

In this study, two endophytes fungal have been successfully isolated from *Rhizophora mucronata* leaves which identified and molecular characterized as *Neopestalotiopsis* sp and *Peniophora lycii*. The extracts from isolated endophytic fungi displayed antibacterial activity with MIC values of 125 up to >500 µg mL^{-1}. The *n*-hexane fraction from *Peniophora lycii* fungi showed the strongest antibacterial activity against *Staphylococcus aureus* with MIC value of 125 µg mL^{-1} than the other fracitons. Terpenoids compound in *n*-hexane fraction suggested plays a role to antibacterial activity.

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