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Research Paper

Antibacterial Activity of Endophytic Fungi from Sembukan (*Paederia foetida L.*) Leaves

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Abstract

The antibiotic resistance of phatogenic bacteria has become a serious health problem and has encouraged the search for novel and effective antimicrobial metabolites. Meanwhile, endophytic fungi have great potential as a natural source for antimicrobial agents. The endophytic fungi that live in plant tissue produces secondary metabolites which potentially act as an antibacterial compound. The isolation of fungi for antibacterial sources reduces the large amount of plant as a source of antibacterial agents. Hence, this study aims to obtain endophytic fungi isolates from *Paederia foetida L.* that are capable of producing secondary metabolites as antibacterial, carry out in vitro tests to verify the antibacterial properties of secondary metabolites of the *Paederia foetida L.* endophytic fungi, and identify the potential of *Paederia foetida L.* endophytic fungi in producing antibacterial compounds. The antibacterial activity was tested against *Escherichia coli* ATCC8739 and *Staphylococcus aureus* ATCC6538 while seven isolates of endophytic fungi that potentially produced antibacterial were obtained from Sembukan (*P. foetida L.*). The results showed that antibacterial activities of SL1, SL4 and SL6 secondary metabolites against *S. aureus* ATCC6538 and *E. coli* ATCC8739 were moderate to strong activities. Furthermore, the Minimum Inhibition Concentration (MIC) of secondary metabolites extract of SL1 against *S. aureus* ATCC6538 value was 250 μg/mL while the values of MIC extract of SL4 against *S. aureus* ATCC6538 and *E. coli* ATCC8739 were 125 μg/mL and 250 μg/mL respectively and MIC extract of SL6 against *E. coli* ATCC8739 value was 125 μg/mL. The secondary metabolites extract of SL1 isolate were alkaloid and tannin, SL4 were phenolic and alkaloid while SL6 isolate were alkaloid and terpenoid. Hence, endophytic fungi SL1 isolate was identified as *Fusarium sp.*, SL4 as *Dematophora sp.*, and SL6 isolate as *Acremonium sp.*

Keywords

Endophytic fungi, *Paederia foetida L.*, Antibacterial Compound, Minimum Inhibition Concentration

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1. INTRODUCTION

Resistance to antimicrobial agents is a significant problem faced by health services and has become a serious concern worldwide (Elisha et al., 2017). Meanwhile, natural products such as fungi from terrestrial, marine and endophytic are considered as an essential source of novel antibacterial compounds due to the abundant of the species diversity, richness in secondary metabolites and the improvements in genetic breeding as well as its relevance with natural products (Calvo et al., 2002).

Endophytic fungi live in plant tissues for a period of time and are capable of forming colonies without damaging the host. Meanwhile, each of the higher level plants contains several endophytic microbes which produces secondary metabolites due to the coevolution or genetic recombination from host plants to endophytic microbes (Tan and Zou, 2001).

The ability of endophytic fungi to produce secondary metabolites in accordance with the host plants is a very large and reliable opportunity of production from endophytic microbes isolated from the host. Almost 300,000 plants species are scattered on the earth with each plant containing one or more endophytic microbes that are made up of bacteria and fungi (Strobel and Daisy, 2003).

When the endophytes isolated from a medicinal plant has the ability to produce the same or higher amount of alkaloids or secondary metabolites as the original plants, there is no need to cut off the original plants to be used as simplicia which takes decades to be harvested (Radji, 2005). Meanwhile, Stierle et al., 1995 stated that the use of endophytic microbes in producing active compounds has several advantages, namely, (1) fast production of uniform quality, (2) large scale production and (3) the possibility of obtaining new bioactive components by providing different conditions.

Plants that are used in traditional medicine have played an essential role in the search for novel endophytic fungi bioactive strains as it is possible that the beneficial properties are due to the metabolites produced by endophytic community (Kaul et al., 2012; Kusari et al., 2013). Previous study showed that *Paederia foetida L.* is a climbing shrub that possesses several ethnomedicinal uses and produces secondary metabolites that are used as antibacterial agents such as essential oils, saponins, tannins and flavonoids (Frederica, 2008).

2. EXPERIMENTAL SECTION

2.1 Materials

Sembukan (*Paederia foetida L.*) leaves, *Staphylococcus aureus* ATCC6538, *Escherichia coli* ATCC8739, alcohol, tetracycline, lactic acid, aquades, $BaCl_2$ 1.175, Dimethyl sulfoxide (DMSO), ethyl acetate, H_2SO_4 1 %, paper disk, Mueller Hinton Agar (MHA), Czapek's Dox Agar (CDA), Malt Extract Agar (MEA), Nutrient Agar (NA), Potato Dextrose Agar (PDA), Potato Dextrose Broth (PDB) media, 0.85 % NaCl and NaOCl.

2.2 Methods

2.2.1 Sampling and Isolation of Endophytic Fungi

Leaves of *Paederia foetida L.* were obtained from Lubuk Selo, Lahat, South Sumatera. Meanwhile, the healthy leaves used as source of endophytic fungi were stored in an ice box at 4 °C which was previously packaged in sealed plastic bags. The leaves were washed thoroughly under running tap water and the samples were sterilized by dipping it into 75 % of ethanol, 3 % of NaOCl, rinsed in a sterile distilled water and dried on sterile filter paper on a petri dish. Furthermore, a piece of each sample was removed with a sterile scalpel and cut into small pieces of approximately 0.5 x 2 cm. Meanwhile, each piece was placed on a petri dish containing Potato Dextrose Agar (PDA) medium and incubated at room temperature (28 °C) to promote fungal growth and sporulation (Katoch et al., 2014).

2.2.2 Cultivation and Extraction

A total of 15 pieces of agar plug were inoculated in 500 ml Potato Dextrose Broth (PDB) medium. The culture was incubated at room temperature (28 °C) for 30 days and sterile PDB was used as a control. Meanwhile, the medium was separated from fungi using filter paper and the dry weight of fungi biomass was measured accordingly (Vinale et al., 2006). Furthermore, the media were mixed with ethyl acetate to extract secondary metabolites and shook properly until the ethyl acetate became layers. The first layer (top) was the medium layer and the second layer (bottom) was the layer of ethyl acetate solvent which contained the secondary metabolites. This ethyl acetate layer was later extracted using a rotary evaporator at 80°C to obtain a thick extract from endophytic fungi (Sharma et al., 2016).

2.2.3 Antibacterial Activity Test

The *Escherichia coli* and *Staphylococcus aureus* suspension approximately 5 × 108 CFU/ml equivalents to 0.5 McFarland units was poured onto MHA medium. Each disc paper was dropped with 10 µL of secondary metabolites extract which had been dissolved using DMSO (1000 µg/mL). Negative control using DMSO and positive control using tetracycline 1000 µg/mL. The plate were incubated for 24 hours at room temperature (Balouiri et al., 2016).

2.2.4 Minimum Inhibitory Concentration(MIC)

MIC of endophytic fungi extract were determined by using agar diffusion method. The extract of the endophytic fungi which have the high percentage of antibacterial activity against *E.coli* and *S.aureus* were made in concentrations of 1000 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL, and 31.25 µg/mL. Each concentration and control were dropped on paper discs that were attached to the MHA medium that had been inoculated with bacteria, and incubated at 37 °C for 24 hours (Salni and Marisa, 2019).

2.2.5 Thin Layer Chromatography

Antibacterial compound extract that showed high potency tested using Thin Layer Chromatogrphy (TLC). The secondary metabolites extract were spotted on a silica gel 60 GF254 plate and eluted with 2 to 1 ratio of n-hexane: ethyl acetate as eluent. Moreover, the plate was sprayed with 5 % H_2SO_4 and heated on hot plate while the Rf value was determined based on the formula below :

$$\text{Rf} = \frac{\text{Distance traveled by component}}{\text{Distance traveled by solven}}$$

2.2.6 Characterization and Indentification

Endophytic fungi were characterized and identified based on macroscopic and microscopic properties of the colonies. The fungi were grown on CDA, MEA and PDA media while the fungal colonies on each medium were observed for colony color and diameter, medium color around the colony and colony reverse color. The results of the fungal characterization identified by compared with the fungal characters in Pitt et al., 2009, Barnett and Hunter, 1972, and Samson et al., 2004.

3. RESULTS AND DISCUSSION

3.1 Isolation of the Endophytic Fungi

Seven isolates (SL1, SL2, SL3, SL4, SL5, SL6, and SL7) of the endophytic fungi were obtained from Sembukan (*Paederia foetida L.*) leaves with different morphological colony characteristics. The diversity of endophytic fungi diversity in a plant occured due to the adaptation mechanism of the fungi to specific microecology and physiological conditions of each host plant (Sopialena et al., 2018).

3.2 Antibacterial Activity

Based on Table 1, the two highest endophytic fungi isolates which have antibacterial activity against *E. coli* ATCC8739 were SL4 and SL6 while SL1 and SL4 were the highest isolates that produced inhibitory zone against *S. aureus* ATCC6538. The percentage difference of antibacterial activity of each isolates occurred due to compounds it contained. Meanwhile, the effectiveness of

antimicrobials is influenced by many factors, namely, (1) bacterial status (susceptibility and resistance, tolerance, persistence, biofilm) and inoculum size, (2) antimicrobial concentrations [mutant selection window (MSW) and sub-inhibitory concentration] (Li et al., 2017). Furthermore, the percentage results were also influenced by the character of bacteria used, this include structure difference and composition of bacteria cell wall as an outer layer which interacted directly with antibacterial compounds. As a gram positive bacteria, *S.aureus* has a thick peptidoglycan structure that makes penetration more difficult for antibacterial than gram negative bacteria. Meanwhile, the gram negative bacteria such as *E.coli* also have advantages in accordance with Madigan et al., 2015, which stated that the cell wall has three layers, namely, the inner membrane, peptidoglycan and the outer membrane. These differences in bacterial defense from the antibacterial compounds showed that each of the compounds has a certain spectrum to inhibit bacterial growth.

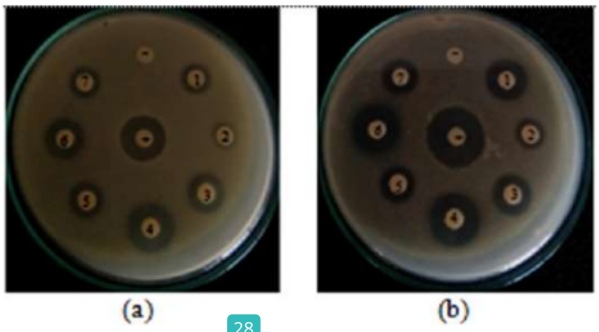

Figure 1. Antibacterial Activity Test of Secondary Metabolites Extract of Endophytic Fungi from Sembukan (*Paederia foetida L.*) Against *Escherichia coli* ATCC8739 and *Staphylococcus aureus* ATCC6538. (+)Tetracyclines, (-) DMSO, (1) SL1, (2) SL2, (3) SL3, (4) SL4, (5) SL5, (6) SL6, (7) SL7, (a) *Escherichia coli* ATCC8739, (b) *Staphylococcus aureus* ATCC6538

Minimum Inhibitory Concentration

The MIC value was determined by lowering the concentration of secondary metabolites extracts that have antibacterial activities to the smallest concentration which has the ability to inhibit bacterial growth. Meanwhile, the results showed that the smallest concentration of secondary metabolites extract of SL4 isolate which formed the inhibitory zone against *Escherichia coli* ATCC8739 was 250 μg/ml and the inhibition zone diameter was 0.5 mm while the SL6 had MIC value of 125 μg/ml against *E. coli* ATCC8739. Furthermore, the SL1 isolate had the smallest concentration of 250 μg/ml for the formation of an inhibition zone against *S. aureus* ATCC6538 while the SL4 had MIC value of 125 μg/ml against *S. aureus* ATCC6538 with 1mm diameter of inhibition zone (Table 2).

Moreover, the ability of secondary metabolites extracts to inhibit bacterial growth decreased directly proportional to the decrease in the extract concentration in MIC test. This showed that the lower the concentration of an antibacterial compound, the lower its antibacterial activity. The inhibition zone that is no longer formed indicated that the concentration of secondary metabolites extract is incapable to inhibit the bacteria growth for the MIC value to be determined. Furthermore, Holetz et al., 2002, stated when the MIC value is <100 μg/ml, the antibacterial activity of the extract is categorized as strong, 100 to 500 μg/ml is categorized as moderate and from 500 to 1000 μg/ml is categorized as weak while when the MIC value is >1000 μg/mL, the extract is categorized as inactive or has no antibacterial activity. Based on these categories, SL1, SL4 and SL6 had secondary metabolites extracts which were categorized as moderate in inhibiting the growth of each bacterium tested.

3.4 Thin Layer Chromatography (TLC) Analyses

Based on Figure 2, the color of the spots formed by each secondary metabolite extract of endopythic fungi isolate was not the same and this showed that the compounds contained in secondary metabolites extract of endophytic fungi were also not the same. Moreover, the SL1 isolate was dark yellow with an Rf value of 0.450 which was presumably to be alkaloid and brown spot as tannin with an Rf value of 0.900. Previous studies by Karmakar et al., 2020, stated that Sembukan (*Paederia foetida L.*) contained alkaloid compounds which showed that SL1 isolate produced secondary metabolites that have the same bioactive compounds as the host plant. Meanwhile, the mechanism of tannins action as antibacterial is to cause lysis of bacterial cells lysis. This happened due to the tannins target on polypeptide wall of the bacterial cell wall for the formation of cell wall to become less perfect and die. Furthermore, the tannins also have the ability to inactivate bacterial enzymes and interfere with the passage of proteins in the inner layer of cells (Cowan, 1999; Akiyama et al., 2001).

SL4 isolate had a yellow spot with Rf value of 0.225 as phenolic compound and dark yellow which was considered to be an alkaloid with Rf value of 0.575. Previous study conducted by Osman et al., 2009 extracted the leaves and stems of Sembukan (*P. foetida L.*) and discovered that it contained phenolic compounds which were assumed to be the same as secondary metabolites of SL4 isolate in form of yellow spot on the TLC plate. Furthermore, Cowan, 1999 and Guimarães et al., 2019 stated that phenolic compounds are able to form hydrogen bonds with cell proteins, thereby damaging the cell structure and decreasing the cell wall permeability which causes disrupted cell ion and leads to cell lysis.

The TLC result of SL6 isolate in Figure 2 showed that there are two visible spots, namely, a dark yellow spot with Rf value of 0.475 as alkaloid and a purple spot which was terpenoids with Rf value of 0.750. Meanwhile, terpenoids had antibacterial activity and were discovered in Sembukan (*P. foetida L.*) in accordance with the study of Karmakar et al., 2020. Furthermore, Cowan, 1999 and Guimarães et al., 2019, stated that terpenoids have an antibacterial mechanism by interfering with cell permeability and bacterial cell growth, causing cell death.

Meanwhile, the three isolates had dark yellow stains which

Table 1. Antibacterial Activity of Secondary Metabolites Extract of Endophytic Fungi from Sembukan (*Paederia foetida L.*) Against *E. coli* ATCC8739 and *S. aureus* ATCC6538

Isolate	Concentration (µg/ml)	Inhibitory Zone (mm)		Antibacterial Activity (%)	
		E. coli	*S. aureus*	*E. coli*	*S. aureus*
SL1	1000	4	10	36.36	71.43
SL2		2	4	18.18	28.57
SL3		4	7	36.36	50.00
SL4		9	11	81.81	78.57
SL5		4	5	36.36	35.71
SL6		8	9	72.72	64.28
SL7		4	7	36.36	50.00
Tetracyclines		11	14		

Antibacterial activity criteria: <50 % (weak), 50-74 % (moderate), 75 % (strong) (Chan et al., 2007).

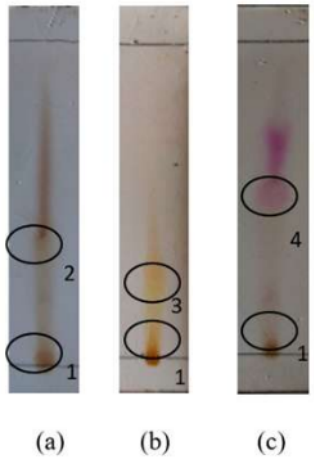

Figure 2. TLC Analysis of Secondary Metabolites Extract of Endophytic Fungi from Sembukan (*Paederia foetida L.*). (a) SL1,(b) SL4, (c) SL6, (1) Alkaloid, (2) Tannin, (3) Phenolic, (4) Terpenoid.

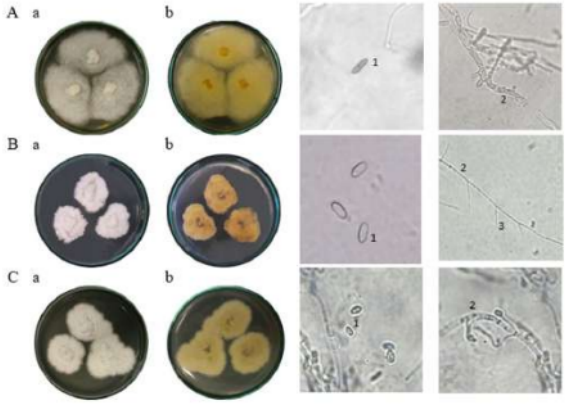

Figure 3. Macroscopic and Microscopic Characters of Endophytic Fungi from Sembukan (*Paederia foetida L.*). (A) SL1. (B) SL4. (C) SL6. (a-b) Colonies on PDA Incubated at room Temperature for 7 Days. (a) Top. (b) Reversed. (1) Conidia. (2) Conidiophore. (3) Branch

was similar to the content of secondary metabolites compounds such as alkaloid compounds. Cowan, 1999 and Cushnie et al., 2014, stated that alkaloid has an antibacterial mechanism by destroying the peptidoglycan synthesis of bacterial cells which causes cell wall layer imperfection and death.

3.5 Characterization and Identification of Endophytic Fungi

Based on the macroscopic characterization, SL1 had a white colony on CDA, MEA and PDA while the fungal growth on PDA medium was the fastest growth compared to other media with more mycellium. The SL1 on PDA medium filled the plate from center to the edge with colony diameter of ± 4.7 cm from the fifth day of cultivation (Figure 3A). Furthermore, the colony texture of SL1 isolates on PDA and CDA media was velvety textured with the aerial mysellium which was short, while the colony texture of SL1 isolates on MEA medium was glabrous. The SL1 has conidia that are fusiform with one septum and hyaline, the hyphae had septate and hyaline while the macroconidia of SL1 had two septa (Figure 3A).

Meanwhile, based on macroscopic and microscopic observations, SL1 isolate was identified as *Fusarium sp.* Moreover, Samson et al., 2004, stated that the properties of *Fusarium sp.* colonies grown on PDA or PSA medium were white, cream, yellowish, brown and reddish. The *Fusarium sp.* growth in both medial was rapid with aerial mycellium having a felty or cottony texture. Meanwhile, the aerial mycellium in *Fusarium sp.* is usually in abundance while it is reduced or absent in *Fusarium sp.* strains which have abundant sporulation characteristics. Pitt et al., 2009 stated that Fusarium have microscopic properties with septate hyphae and the conidia is fusiform to crescent

Table 2. MIC of Secondary Metabolite Endophytic Fungi from Sembukan (*Paederia foetida L.*) Against *E.coli* ATCC8739 and *S. aureus* ATCC6538

Isolate	Concentration (μg/ml)	Inhibitory Zone (mm)	
		E.coli ATCC8739	*S. aureus* ATCC6538
SL1	1000	na	5.0
	500	na	2.0
	250	na	1.0
	125	na	0.0
	62.5	na	0.0
	31.25	na	0.0
	control	na	0.0
SL4	1000	4.0	3.0
	500	1.5	2.0
	250	0.5	1.5
	125	0.0	1.0
	62.5	0.0	0.0
	31.25	0.0	0.0
	control	0.0	0.0
SL6	1000	6.0	na
	500	5.0	na
	250	3.0	na
	125	1.0	na
	62.5	0.0	na
	31.25	0.0	na
	control	0.0	na

*na=not available

which have various forms of microconidia. The white color of SL4 colony on CDA, MEA and PDA media formed a zonation pattern behind the colony. Meanwhile, the reverse zonation pattern of colony isolates on CDA and MEA was brownish while on PDA was yellowish. Furthermore, the reverse colony color of SL4 isolate on PDA turn blacker over time (Figure 3B), and the SL4 on CDA had the slowest growth rate with a colony diameter of ± 3.8 cm after 7 days of incubation.

Based on macroscopic and microscopic characters of the SL4 isolate, it was discovered that the isolates had similarities with *Dematophora sp.* and a white colony which turned blackish brown from the center to the edge, as it was cultivated on PDA medium. Meanwhile, Watanabe, 1992, stated that *Dematophora sp.* on PDA has a mycellium which is initially white and begin to darken partially. The SL4 isolate had long ellipsoidal conidia with hyaline color, one-branched conidiophores, septate and hyaline hypae, and also had fertile portions of conidiophores (Figure 3B). Furthermore, Watanabe, 2002, stated that the conidia *Dematophora sp.* is hyaline which consists of one cell is 3.5-5.5x1.6-2.3 μm in size and has conidiophores with one or two branches. However, *Dematophora sp.* usually carries two rows of spores at the ends of the fertile conidiophores while sporulation occurs in the colony culture when getting old and brown.

The result of macroscopic characterization showed that the colony of SL6 isolate color was white on all media culture and yellow color on the reverse plate with the exception of the colony isolate on CDA medium which was white on the reverse (Figure 3C). Furthermore, the growth of SL6 isolate on CDA was the fastest among the media, with a colony diameter of ± 4.2 cm after 7 days of cultivation.

Microscopic observations of SL6 isolate showed that isolate had septate and hyaline hypae (Figure 3C) while the conidia was long ellipsoidal in form of one cell. Watanabe (2002), stated that the conidiophores of SL6 isolate was simple without branches and became tapered from the base to the top.

Based on the macroscopic and microscopic observations, the SL6 isolate was identified as *Acremonium sp.* Meanwhile, previous study by Peberdy, 1987, stated that *Acremonium sp.* genus have hyaline conidia with globose, ovoid, ellipsoidal and cylindrical forms which consists of single cells and the conidia are rarely encountered with two cells that are fused.

4. CONCLUSIONS

The results showed that the antibacterial activity of secondary metabolites of SL1, SL4 and SL6 against *S. aureus* ATCC6538 and *E. coli* ATCC8739 were moderate to strong activities. Meanwhile, the MIC values of secondary metabolites extract of SL1 and SL4 isolates against *S. aureus* ATCC6538 were 250 μg/ml and 125 μg/ml respectively. Furthermore, the MIC of secondary metabolites extract of SL4 and SL6 isolates against *E. coli* ATCC8739 were 250 μg/ml and 125 μg/ml respectively. The secondary metabolites

extract of SL1 isolate were alkaloid and tannin, SL4 were phenolic and alkaloid, and SL6 isolate were alkaloid and terpenoid. Hence, endophytic fungi SL1 isolate was identified as *Fusarium sp.*, SL4 as *Dematophora sp.*, and SL6 isolate as *Acremonium sp.*

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