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Screening of Antibacterial Activities of the Endophytic Fungi Isolated from the Leaves of *Medinilla speciosa* Blume

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Abstract
Endophytes or endophytic fungi have been investigated as a storehouse of bioactive compounds. This study was aimed to evaluate the potential antibacterial activity of endophytic fungi isolated from the leaves of Medinilla speciosa Blume. The antibacterial test was determined by measuring the inhibition zone with disk-diffusion method. Twenty endophytes were isolated from the leaves of Medinilla speciosa Blume and identified morphologically. The results demonstrated that ten isolates showed variation in their antibacterial activity against Escherichia coli ATCC 25922, Staphylococcus aureus ATCC 25923, Bacillus subtilis ATCC 6633, and Shigella dysenteriae ATCC 13313. Further investigation will be needed to explore and identify the bioactive molecules of the isolated endophytic fungi.

I. Introduction

The discoveries of natural bioactive compounds and their therapeutic properties have been investigated since the emergence of new diseases and drug-resistant problems. In recent years, the research to develop a new and safe drugs from natural resources is explored extensively. The bioactive compounds can be lead from many sources; plants, animals, marine organisms, and endophytic microbes (Christina, Christapher and Bhore, 2013).

Plants are known as a reservoir of endophytes, including fungi, bacteria, and actinomycetes (Strobel *et al.*, 2004). The ability of endophytic microbes to produce bioactive compounds has been widely investigated since the discovery of penicillin from *Penicillium notatum* by Alexander Fleming in 1928 (Akanksha, Bhardwaj and Agrawal, 2014). Endophytic are microorganism which grown in living tissues of higher plants, establishing mutual relationship without causing any apparent symptoms and negative effect on their host.

It has been known that endophytic fungi are an important source of bioactive compounds. Up to now, the production of endophytes metabolites as antibacterial, antifungal, antidiabetic, anticancer, antiviral activities, and other pharmacological activities has been increasingly investigated (Gouda *et al.*, 2016). Using endophytic microbes as a source of raw material for developing new drugs from the herbal plant was very beneficial advantageous because it can be obtained without killing the host plant, hence environmental sustainability can be preserved (Kumala, DwiYuliani and Simanjuntak, 2015).

One of Indonesian plant species which has not been fully explored in terms of pharmacology is Medinilla. *Medinilla speciosa* Blume is distributed in Indonesia, Malaysia, and the Philippines. Traditionally, the fruit of this plant has been consumed by pregnant women and also used as medicine for diarrhea, mouth sores, inflammatory, cancer, and bacterial infection (Hanum, Prihastanti and Jumari, 2017). In order to searching for more bioactive endophytic fungi of this plant, the isolation and antibacterial screening from the leaves was carried out. Herein are reported the endophytic isolates and their antibacterial activities against *Escherichia coli, Staphylococcus aureus, Bacillus subtilis,* and *Shigella dysenteriae.*

II. Research Method

II.1 Sample Preparation

The leaves of *Medinilla speciosa* Blume were collected from Mount Muria in Kudus Regency, Central Java, Indonesia in May 2015. Authentication and identification of plant were carried out at Centre for Plant Conservation Botanic Gardens, Bogor, Indonesia.

II.2 Isolation and Purification of Endophytic Fungi

Fresh leaves of *M. speciosa* Blume were washed in running tap water for 10 min to remove the dust and transferred to aseptic condition. The

selected leaves then immersed 95% ethanol for 30 sec, 4% sodium hypochlorite solution for 3 min, and 95% ethanol for 30 sec followed by rinsing with sterile distilled water three times. After drying, they were cut into small approximately 0.5 cm and placed on potato dextrose agar medium (PDA) supplemented with streptomycin (100 µg/ml) to suppress bacterial growth. The agar plates were incubated at 25 ± 2 ºC for a maximum of 21 days for fungal growth (Bhardwaj *et al.*, 2015).

II.3 Screening of Antibacterial Activity of Isolated Endophytic fungi

The screening of antibacterial activity of the isolated endophytic fungi was carried out by using the agar well diffusion method (Balouiri, Sadiki and Ibnsouda, 2016) with four pathogenic bacterial strains: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, and *Shigella dysenteriae* ATCC 13313. A sterile cork borer of 6 mm was used to cut the portion of mycelia mat of the isolates and transferred to pre-sterile nutrient agar medium plates contains bacterial test suspension. The clear zone around the isolates demonstrated microbe inhibition zone area and was measured in millimeter (mm) scale.

II.4 Morphological Characteristic of Isolated Endophytic Fungi

The morphological identification of isolated endophytic fungi was performed by microscopic identification and macroscopic morphological characterization, including the color of the colonies, the exudate drops, zonation, also the types of hyphae, and mycelium (Alsohaili and Bani-Hasan, 2018).

II.5 Fermentation of Isolated Endophytic Fungi

Pure endophytic isolates were transferred to freshly prepared PDA to establish stock cultures and incubated 25 ± 2 ºC for 6-7 days. Three pieces of inoculum were selected with a sterile cork borer of diameter 6 mm and transferred into 200 ml pre-sterile liquid fermentation media potato dextrose yeast (PDY). This was further fermented for the next 14 days at 25 ± 2 ºC. The supernatant was separated from biomass via centrifugation at 3000 rpm for 15 min and refiltered for the estimation of antibacterial activity (Aharwal *et al.*, 2018).

II.6 Antibacterial Activity Test

All the bacterial tests were inoculated into the nutrient broth (NB) media and incubated at 35 °C -37 °C for 18-24 hours. Each of these bacterial suspensions was compared to McFarland standard 0.5 (1.5 x 108 CFU/ml) (Techaoei *et al.*, 2020) for density uniformity and then 100 µl of the bacterial suspension was spread onto nutrient agar (NA) using a sterile cotton bud in a Petri dish until all surface was covered. Then, a filter paper discs (about 6 mm in diameter), containing 20 µl of the test compound are placed on the agar surface. The discs were incubated at 35 °C -37 °C for 18-24 hours. The sensitivities of the microorganism against the fungal isolates were determined by measuring the diameter of inhibitory zones compared to chloramphenicol as a positive control and aquadest as a negative control (Balouiri, Sadiki and Ibnsouda, 2016).

III. Results and Discussion

Totally, 20 endophytic fungi isolates were successfully obtained from the leaves of *M. speciosa* Blume collected from Central Java, Indonesia. Fermentation of endophytic fungi was done by using PDY medium because it contains rich carbon sources from potato extracts and dextrose, also nitrogen source from its yeast extract. The fermentation process was performed at room temperature under static conditions (Kumala, DwiYuliani and Simanjuntak, 2015).

All fungal isolates were screened for their antibacterial activity against bacterial test Gram-positive bacteria (*S. aureus*, *B. subtilis*) and Gram-negative bacteria (*E. coli*, *S. dysenteriae*). Chloramphenicol was chosen as a positive control because it has a wide spectrum against Gram-positive bacteria and Gram-negative bacteria and it is also known as a bacteriostatic agent (Jayatilake and Munasinghe, 2020). Out of twenty, ten endophytic isolates were selected based on their ability to inhibit the growth of bacterial tests as shown in Table 1.

Table 1. Screening of isolated endophytic fungi against four pathogenic bacteria

Fungal Isolates	Zone of inhibition (mm)			
	S. aureus	***B. subtilis***	***E. coli***	***S.dysenteriae***
DPU1 (a)	9.71	-	-	-
DPU3 (b)	12.4	7.00	-	-
DPU4 (c)	-	8.70	20.13	-
DTE1 (d)	9.65	-	10.66	-
DTE3 (e)	11.67	-	11.06	-
DTU1 (f)	9.10	-	16.32	-
DTU4 (g)	-	7.22	10.55	6.90

DTU6 (h)	-	6.42	-	7.05
DTU7 (i)	-	6.75	12.35	6.92
DTU9 (j)	-	6.72	9.75	-
Positive Control	20.21	15.32	13.63	15.52
Negative Control				

After screening the antibacterial activity of the endophytic fungi isolates, ten selected isolates were further identified based on their macroscopic and microscopic characteristic (Figure 1). The morphological identification of endophytic fungal strains is based on the morphology of the fungal culture colony or hyphae (Ma et al., 2018), the characteristics of the spores (Sudirga, 2016), and reproductive structures (Mbilu et al., 2018). Based on their cultural and microscopic properties (Navi et al., 1999), these fungi show different characteristics and successfully identified as described in Table 2.

Table 2. Phenotypic characteristics of isolated endophytic fungi from the leaves of *M. speciosa* Blume

Fungal Isolate	Macroscopic Characteristic	Microscopic Characteristic	Probable Genera/Species
DPU1 (a)	Colonies were powder in texture and black with conidial production	Hyphae septate hyaline dichotomously branched vesicle, round, radiate head, black conidia.	*Alternaria* sp.
DPU3 (b)	The surface of the colony is white orange; the color is white orange in reverse.	Multi-celled spores, conidia are oval-shaped and attached to conidiophores arise from a septate mycelium.	*Colletotrichum* sp.
DPU4 (c)	Colony surface greenish-white, greenish-yellow inverted color, thick hyphal texture.	Conidiophores arising from a septate mycelium.	*Penicillium* sp.
DTE1 (d)	The surface of the colony is brownish-white, the reverse color is brownish-green white.	Hyphae septate and branched, conidia which form a brownish-yellow chain	*Aspergillus* sp.
DTE3 (e)	The surface of the colony is white brownish-yellow, the color is a brownish-yellow reversal, forming concentric circles	Hyphae septate and branched, having conidia and chlamydospores that appear on the septate mycelium.	*Humicola* sp.
DTU1 (f)	The surface of the colony is brownish-white, the color is brownish-yellow in reverse	Hyphae septate and branched, conidiophores arise from a septate mycelium.	*Corynespora* sp.
DTU4 (g)	Colony like cotton is white and flat	Hyphae septate and branched and the colony has spores that are oval rod-like in shape.	*Trichophyton* sp.
DTU6 (h)	Upper colonies are hyaline to dark then turn brownish-gray, in contrast to brownish color.	Hyphae septate and branched, conidia obovoid.	*Botrytis* sp.
DTU7 (i)	Colony like cotton is white and flat	Hyphae septate and branched and the colony has spores that are oval rod-like in shape.	*Trichophyton* sp.
DTU9 (j)	The surface of the colony is uneven and stringy like cotton, the color of the surface is grayish-white and the color is creamy with black circles.	Hyphae septate and branched, conidiophores arise from a septate mycelium.	*Phomopsis* sp.

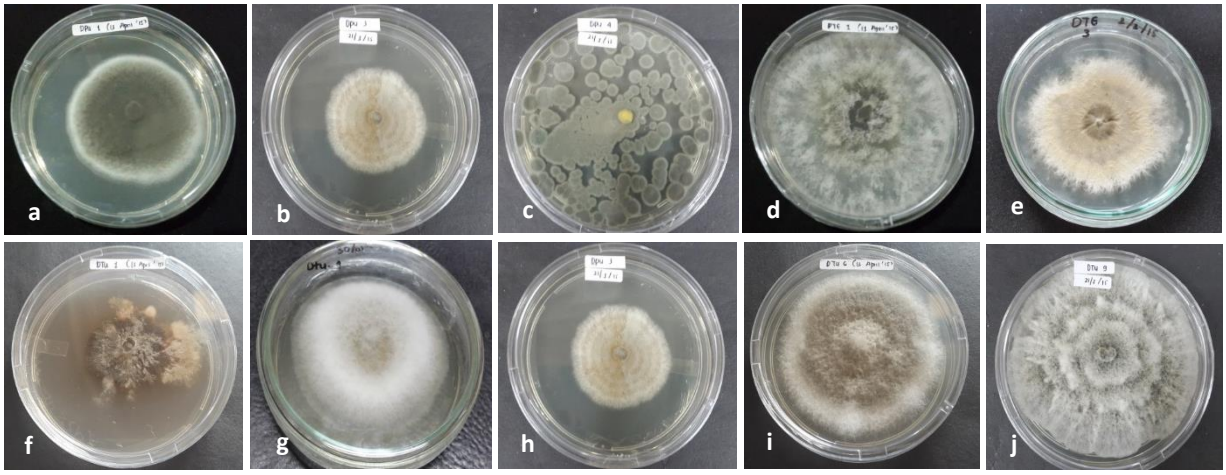

Figure 1. Endophytic Fungi Isolates
Note; (a)=DPU1; (b)=DPU3; (c)=DPU4; (d)=DTE1; (e)=DTE3; (f)=DTU1; (g)=DTU4; (h)=DTU6; (i)=DTU7; (j)=DTU9

The antimicrobial activities of the endophytic microbes, primary was determined by measuring the inhibition zone with the disk-diffusion method. The test microorganisms were chosen due to their pathogenicity. The bacterial growth curve was studied to determine the growth rate of the bacteria. The chloramphenicol was used as a positive control and aquadest was used as a negative control.

The result of antibacterial activity of the endophytic fungi fermentation showed that DPU1, DPU3, DPU4, DTE1, DTU1, DTU7, and DTU9 were active against *S. aureus* ATCC 25923; DPU3, DPU4, DTE1, DTU1, DTU4, and DTU6 were active against *B. subtilis* ATCC 6633; all isolates were actives against *E. coli* ATCC 25922, and all isolates were active against *S. dysenteriae* ATCC 13313 except for DTU7 (Table. 3).

Table. 3. Zone of inhibition of potent endophytic fungi isolates

Fungal Isolates	**Zone of inhibition (mm)**			
	S.aureus	***B.subtilis***	***E.coli***	***S.dysenteriae***
DPU1 (a)	7.85	-	6.42	6.68
DPU3 (b)	7.53	6.21	6.38	6.46
DPU4 (c)	7.78	6.11	6.26	6.68
DTE1 (d)	6.96	7.05	6.91	7.31
DTE3 (e)	-	-	7.03	6.11
DTU1 (f)	6.95	7.21	7.28	6.71
DTU4 (g)	-	5.76	6.86	6.11
DTU6 (h)	-	7.03	6.35	7.68
DTU7 (i)	6.55	-	6.91	-
DTU9 (j)	6.61	-	6.91	5.92
Positive Control	19.46	14.52	10.94	16.91
Negative Control	-	-	-	-

IV. Conclusions

Twenty endophytic fungi isolates were successfully obtained from the leaves of *M. speciosa* Blume. Ten of the isolates have shown the variation of inhibiting zone against the bacterial test. The study revealed the presence of good antibacterial activity for the isolates DPU3, DPU4, DTE1, and DTU1. These isolates could be a good source for bioactive compounds and the secondary metabolites compounds may be further isolated.

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