Isolation, antibacterial activity, and molecular identification of endophytic fungi from *Pogostemon cablin*

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Abstract. *Pogostemon cablin* is the medicinal plants that produces patchouli essential oils as secondary metabolites which has multiple functions including antibacterial ability. The secondary metabolites in plants mostly associated with their endophytic fungi. In this study we isolated endophytic fungi from *Pogostemon cablin*’s leaves and examined antibacterial activity of the endophytic fungi against *Escherichia coli* and *Staphylococcus aureus*, as well as find out the identity of most potential isolate based on Internal Transcribe Spacer (ITS) region. The isolation of endophytic fungi was performed using surface sterilization method on Malt Extract Agar (MEA) medium. The antibacterial activity was tested using paper disc on Muller Hinton Agar (MHA) medium and molecular identification was amplified using ITS 4 and ITS 5 primers. The isolation process resulted in 5 isolates of endophytic fungi. The antibacterial assay indicated one potential isolate with the highest antibacterial activity when tested against *E. coli* and *S. Aureus*, exhibited 20.9 mm and 19 mm clear zone respectively. Molecular identification from ITS region database depicted that the potential isolate has high homology with *Nigrospora* sp. by 99% similarity. This result suggested that the antibacterial ability of essential oils from the *Pogostemon cablin*’s leaves might has high correlation with the occurrence of endophytic fungi.

1. Introduction

*Pogostemon cablin* [Blanco] Benth, known as patchouli, is a perennial fragrant herb belongs to family Lamiaceae. This plant is Southeast Asia origin and has been widely used as traditional medicine. *P. cablin*’s leaves contain an essential oil called patchouli oil yielded from dried leaves maceration [1]. Patchouli oil consists of a major component called patchoulol (patchouli alcohol) and several sesquiterpenes such as α- and β-guaiene, pogostol, α- and β-bulnesense, α-, β-, γ-, δ-patchouline and norpatchoulenol [2,3,4]. Patchouli oil also has a strong fixative property; hence it becomes one of paramount base ingredients in fragrance products such as perfumes, soaps, detergents, and cosmetic products.

Patchouli oil extracted from leaves was reported having various properties such as antimicrobial [5, 6], antioxidant [7], anti-inflammatory [8], analgesic [9], antiemetic [10], antimitogenic [11], antithrombotic [12], and cytotoxic activities [13, 14]. Previous research showed that the pharmacological ability of plants secondary metabolites might has correlation with their endophytic organisms through several mechanisms
[15]. However, there is no report about correlation of the pharmacological features of the patchouli oil with endophytic fungi that reside within *Pogostemon cablin*.

Endophytic fungi are organisms which live inside plant’s tissues, both intra and intercellular, without causing any sign of diseases or morphological changes. Plant endophytic fungi could create communities that differ in species composition, host and tissue preference. Endophytic fungi produce bioactive compounds mainly to provide protection for the host plant to resist external abiotic and biotic stress by enhancing the immune response against invading pathogens [16]. Besides, endophytic fungi are also capable of producing biochemical compounds similar to those produced by their host plants [17]. Further analysis in bioprospecting of secondary metabolite compounds from microbes requires pure culture. Therefore, isolation step is needed as initial and crucial steps of bioprospection [18, 19]. Endophytic fungi are promising source for the discovery of new secondary metabolites with various bioactivity features [20]. Recently, several findings proved that several endophytic isolates from leaves have antibacterial activity against pathogenic bacteria [21, 22]. However, the antibacterial activity of endophytic fungi from *Pogostemon cablin* has not been reported yet.

The objectives of this study were to isolate and investigate the ability of endophytic fungi from *Pogostemon cablin* to produce secondary metabolite compounds with antibacterial activity against pathogenic bacteria. The potential isolates were then identified based on internal transcribe spacer (ITS) region as the one of DNA barcoding in Fungi [22]. Recent study by Liu et al. [23] found two novel metabolites from endophytic fungi from *P. cablin*. However, these compounds showed zero activity against pathogenic bacteria. Hence, the finding in this study would give the novel perspective about the potential isolate of endophytic fungi from *P. cablin* with antibacterial activity.

2. Material and methods

2.1 Isolation of endophytic fungi

The isolation of endophytic fungi from Nilam (*Pogostemon cablin*) leaves was performed using surface sterilization method according to Okane [24]. Endophytic fungi isolated using *Malt Extract Agar* (MEA) medium and then incubated at 25° C for 3 weeks. The growing colonies were selected based on morphotype and purified further into *Potato Dextrose Agar* (PDA) medium.

2.2 Fermentation, extraction, and determination of antibacterial activity

The endophytic fungi were inoculated into PDB for 7 days for fermentation According to Santos et al. [21]. The culture broth (filtrate) was extracted using ethyl acetate (EtOAc) by comparison of the filtrate and solvent were 1:3. The filtrate was evaporated using rotary evaporator to produce a crude extract. The antibacterial activity test was carried out using the crude extract of endophytic fungi with paper disc diffusion method based on the National Committee for Clinical Laboratory Standards. The crude extract was dissolved with dimethyl sulfoxide (DMSO) on 30 µg/disc and 50 µg/disc concentration respectively. Finally, 10 µl of mixture was dropped on sterile paper disc (6mm in diameter) to tested antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* and it was incubated on 37°C for 24 hours. Positive control used chloramphenicol disc on 30 µg/disc concentration and the negative control used sterile disc contained DMSO.

2.3 DNA extraction and polymerase chain reaction

The DNA of endophytic fungi was extracted based on *Chelating Ion Exchange* (Chelex) method according to Lamballerie et al [25]. The genomic DNA was amplified by *Promega Kit* with total volume 25 µl *master mix*, consisting of 15 µl nuclease free water, 5 µl *Bioneer PCR Master Mix* (Promega), 2 µl primer ITS 4 (10 pmol), 2 µl primer ITS 5 (10 pmol) and 1 µl (cons. 30-200 ng/µl) DNA genome as
template. The PCR cycle was performed with 35 cycles of 3 minutes pre-denaturation at 95°C, 30 seconds denaturation at 95°C, 30 seconds annealing at 55°C, and 1 minutes extension at 72°C.

2.4 Homology and phylogenetic analysis
The raw sequence data was analyzed automatically by Chromas Pro version 1.5 software. The final sequence data were submitted to National Center for Biotechnology Information (NCBI) database to obtain the the most closely related species. Phylogenetic analysis was performed using MegaX software based on Neighbor-Joining [26], with Kimura 2 parameter method [27].

3. Results and discussion

Table 1. The result of morphotype selection and screening of antibacterial activity of endophytic fungi

| No | Isolates morphotype | Isolates code and characters | Diameter of inhibition zone (mm) |
|----|---------------------|------------------------------|----------------------------------|
| 1. | DN 1.1.2 (Velvety)  | -                            | 13,3                             |
| 2. | DN 3.1 (Granular)   | -                            | 14,9                             |
| 3. | DN 4.1 (Cottony)    | 14,4                         | 24,2                             |
| 4. | DN 6.1 (Cottony)    | -                            | 14,9                             |
| 5. | DN 6.2 (Cottony)    | -                            | 14,7                             |

The results of screening from 5 isolates of endophytic fungi showed that the average size of inhibition zone ranging 13.3-24.2 mm (Table 1). Isolate DN 4.1 had the best inhibition zone against two testing bacteria with the diameter were 14.4 mm against Escherichia coli and 24.2 mm against Staphylococcus aureus. Other isolates mostly only had activity against Gram negative bacteria. Therefore, isolate DN 4.1 was used for further antibacterial test.
The antibacterial assay of the crude extract of isolate DN 4.1 were done using the disc diffusion method and the results were presented on Table 2. Both of tested pathogens are highly susceptible to the crude extracts. The results of antibacterial activity showed that isolate DN 4.1 against Gram negative bacteria produced larger inhibition zone rather than against Gram positive bacteria. According to Cappucino and Sherman [30], the factors that influence the size of inhibition zone are the interaction of the antibacterial substances with the tested microbes and its ability to diffuse into the media. Other factors involve the number and growth rate of the tested microbes, as well as the microbial sensitivity level towards antibacterial substances.

| Isolate       | Concentration | Average of inhibition zone (mm) |
|---------------|---------------|---------------------------------|
|               | **Escherichia coli** | **Staphylococcus aureus**       |
| DN 4.1        | 30 µg/disc    | 19,3                            |
|               | 50 µg/disc    | 25,1                            |
| K+ (kloramfenikol) | 30 µg/disc | 24,5                            |
| K- (DMSO)     |               |                                 |

The highest inhibition zone was showed on concentration 50 µg/disc (25,1 mm against *Escherichia coli* and 18,4 mm against *Staphylococcus aureus*). These results indicated that antibacterial activity of Patchouli’s leaves which reported by previous research [31] might have the correlation with the occurrence of endophytic fungi inside the plant tissues. Based on their activity, antibiotics are divided into several groups. Major part of antibiotic compounds have narrow spectrum ability, which are only able to inhibit gram positive bacteria growth (30%). Some antibiotics (15%) are called as broad antibacterial spectrum compounds that are active against gram-negative, gram-positive and mycobacteria. Besides, there are also broadest spectrum compounds (12%) which has antifungal activity as its additional antibiotic feature. [32]. The higher concentration of extract would give the higher inhibition zone because higher number of antibacterial compounds which had been released. Thus, the penetration of compounds into cell are faster [33]. The DN 4.1 as the potential isolate then identify using molecular method based on Internal Transcribed Spacer (ITS) region.

The molecular identification showed that endophytic fungi isolate with the highest antibacterial activity (DN 4.1) was closely related with *Nigrospora aurantiaca* with 99% similarity, indicating the similar species. Previous study by Suwannarach et al. [34] showed that *Nigrospora aurantiaca* was the endophytic fungi isolated from *Cinnamomum zeylanicum*. This species uniquely produced the red pigment which can also be observed in DN 4.1 isolate (Table 1). This red pigment, namely bostrycin, has high stability at temperature ranging from 20—50 °C and showed usefulness for for textile dyeing process. However, there are still no reports about the antibacterial ability of *Nigrospora aurantiaca* which isolated from plant tissues, especially from Patchouli’s leaves. Thus, this research is the initial report of antibacterial activity of *Nigrospora aurantiaca* which is isolated from the leaves of *Pogostemon cablin*. 
Figure 1. The result of phylogenetic analysis using Neighbor Joining method using Kimura-2 parameters with 1000 bootstrap replications showed that isolate DN 4.1 formed monophyletic group with *Nigrospora aurantiaca*. The outgroup is *Pseudopestalotiopsis cocos*.

Phylogenetic analysis from Neighbor Joining tree indicated that the DN 4.1 isolate formed monophyletic group with *Nigrospora aurantiaca* as type strain with bootstrap value 61%. Although with moderate bootstrap value, the phylogenetic tree also confirmed that isolate DN 4.1 is the member of genus *Nigrospora*. In many previous studies, the member of genus *Nigrospora* known as endophytic fungi with cosmopolitan distribution and wide host range [35,36]. In addition, the member of genus *Nigrospora* also has been regarded as the alternative of source natural product as it has the ability of produce secondary metabolites which potential for industrial application [37]. However, there are still no reports about the finding of genus *Nigrospora* from *Pogostemon cablin* with antibacterial ability against two pathogenic bacteria.

4. Conclusion

Most endophytic fungi from Patchouli (*Pogostemon cablin*) leaves have antibacterial activity against Gram-positive bacteria. However, there is one isolate (DN 4.1) which has activity against both Gram positive and negative bacteria. Molecular identification as well as phylogenetic analysis indicated this isolate is identified as *Nigrospora aurantiaca*, the member of genus *Nigrospora* in Ascomycota group.

Acknowledgements

This research was funded by Hibah Mahasiswa Undip 2017 from Diponegoro University Indonesia which granted to all authors. Thanks to Muhammad Ilyas, M.Si from InaCC LIPI for providing materials for preservation and Norma Sainstika Pangestu for helping during molecular identification.
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