Endophytic fungi from *Syzygium cumini* (L.) Skeels leaves and its potential as antimicrobial agents

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Abstract. One of the harbours of endophytic fungi was medicinal plants. In this study, medicinal plant of *Syzygium cumini* (L.) Skeels was used to isolate endophytic fungi, especially from the leaves. The surface sterilization was first step before isolation of endophytic fungi. Isolation of endophytic fungi used potato dextrose agar (PDA) media and PDA containing plant extract. The single spores from endophytic fungi were obtained using water agar method. Thirty-seven endophytic fungi have been isolated from *S. cumini* and the antimicrobial activity was shown by 17 of endophytic fungi isolates. The result shows that endophytic fungi isolated from leaves of *S. cumini* (L.) Skeels display a good source of natural antimicrobial agents.

1. Introduction

Human life will always depend on nature, especially in finding medicines to cure diseases, therefore new and useful compounds still are required. Many medical problems, such as drug resistance in bacteria and incidence of fungal infections have become affliction at the human, hence drug discovery from nature still are needed to continue. Several drugs from microbial, like antibiotics from bacteria and antifungal agents from various fungal taxa have succeeded to develop, so the drug recovery have focused to microorganisms [1]. Therefore, the prospect of finding a novel compound is higher from microorganisms.

Endophytes are microbes that reside in internal plant tissues without inducing symptomatic infection to their host. Endophytic fungi contributed to their host plants by generating bioactive compounds that afford protection to the plant against predator [2][3]. One or more endophytes can reside in every plant, therefore plants provide a rich repository of microorganisms [4][5]. Endophytic fungi produce bioactive compounds such as terpenoids, flavonoids, steroids, peptides, isocoumarin derivatives, phenolic acids and quinones. Endophytic fungi can produce novel antimicrobial agents, antioxidant, anti-cancer compounds, solvent etc. The medicinal plants are harbour of endophytes, especially endophytic fungi. In this study used medicinal plants namely *S. cumini* (L.) Skeels. In Aceh language, this plant is called jamblang. Many investigations state that this medicinal plant possesses...
antimicrobial activity. Gram postive and Gram negative human patogenic bacteria and yeast can be inhibited by leaves extract from S. cumini (L.) Skeels [6]. This is because of this plant contains bioactive compounds such as myrecetin, isoquercetin, ellagic acid, glucoside, kaemferol and anthocyanins [7]. Based on the many investigations about this plant, it is supposed to contain many endophytic fungi. However, information of endophytic fungi from S. cumini (L.) Skeels leaves is still lacking, especially they possess antimicrobial activity. Therefore this study aims to isolate the endophytic fungi from leaves of S. cumini and determine its antimicrobial activity.

2. Materials and methods

2.1. Chemical and Media
The chemicals used are ethanol and sodium hypochlorite (NaOCl), while for the media are water agar (WA), potato dextrose agar (PDA) and mueller hinton agar (MHA) and sabouraud dextrose agar (SDA). Moreover it also uses antibiotics namely chloramphenicol and ketoconazole.

2.2. Plant collection and identification
Healthy (showing no visual disease) leaves of medicinal plant S. cumini (L.) Skeels was collected from Syiah Kuala district, Banda Aceh, Indonesia by hand picking method. The plant were identified and authenticated at the School of Biological Sciences, Universiti Sains Malaysia, Malaysia.

2.3. Surface sterilization of leaves
The collected leaves were sterilized its surface using surface sterilization procedures [8]. Ethanol and sodium hypochlorite were sterilants that used in surface sterilization of leaves. Briefly, plants leaves were first washed under running tap water for a few minutes, and then washed in distilled water. The surface of plant leaves was sterilized by soaking in ethanol (70%, 80%, 90%) for 30, 60, 90 and 120 s, then followed by immersed in sterile distilled water for 1 min. After drying aseptically, these leaves were soaking in sodium hypochlorite (1%, 2%, 3%) for 30, 60, 90 and 120 s and followed by rinsing them in sterile distilled water for 1 min. Then, the leaves were blotted to dry with sterile filter papers under aseptic conditions. The effectiveness of surface sterilization process was confirmed for each leaf piece [9].

2.4. Isolation of endophytic fungi
After sterilization, the plant leaves were further cut (aseptically) to expose the interior surface to the nutrient media. Four leaf segments were placed on PDA and PDA containing host plant extract (PDA+HP) (10 g/L) which was added with 0.2 g/L chloramphenicol. To seal plate used parafilm, then incubated at 30 ± 2°C for 14 d under dark condition and checked everyday.

2.5. Purification and preservation of endophytic fungi
Isolation from the master plates was done by the transfer of hyphal tips to water agar plates, which this is to obtain single spores. After 48 hours of incubation, the pure cultures were transferred to fresh PDA plates without the addition of antibiotics. Nonsterilized plant tissues were cultured as a positive control. Potato dextrose agar slants containing host plant were used to transfer the endophytic fungi isolates and maintained at 4°C till further use.

2.6. Morphological identification
Morphological study was done by plating the endophytic fungi on PDA containing host plant extract and incubating for 7 d. The growth appearance was then noted by observing front views of the plates.

2.7. Screening for antimicrobial activity by agar plug diffusion assay
Fourteen test microorganisms were obtained from Industrial Biotechnology Research Laboratory, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia and used for the
antimicrobial assay. These microorganisms include four Gram-positive bacteria (Methicillin-resistant *Staphylococcus aureus*, *Staphylococcus aureus*, *Bacillus cereus* and *Bacillus subtilis*), Gram-negative bacteria (*Klebsiella pneumoniae*, *Escherichia coli*, *Salmonella typhimurium* and *Shigella boydii*), four yeast (*Candida albicans*, *Candida utilis*, *Cryptococcus* sp. and *Saccharomyces cerevisiae*) and four fungi (*Aspergillus niger*, *Microsporum fulvum*, *Trichophyton rubrum* and *Fusarium solani*).

Agar plug diffusion method was used to test antimicrobial activity of endophytic fungi isolates [13]. Standard used to adjust overnight cultures of test bacteria and yeast was 0.5 McFarland standard, while the inoculum size of test fungi cultures was determined by haemocytometer (under a light microscope), then adjusted to $1 \times 10^7$ spores/mL. The test microorganisms were then seeded on MHA for the bacteria and SDA for yeasts and fungi using swab streaking method.

Potato dextrose agar plates containing host plant extract were used to inoculate endophytic fungal isolates. After incubation for 20 d at 30°C, the fungal agar plug were cut using a sterile cork-borer into 1.0 cm diameter and 0.4 cm thickness. Further, the bioactive compounds secreted by mycelial (stationary phase) agar plugs were aseptically placed on the agar medium which were previously sowed with test microbes. To allow diffusion of bioactive compounds in the agar, the plates were kept at 4°C for 7 d. Furthermore, all the plates were observed for zone of inhibition after incubation at 37°C (bacteria and yeast) for 24 h and at 30°C (fungi) for 96 h. The presence of inhibition zone displayed the antimicrobial activities. The sensitivity levels of antimicrobial activity were determined by measuring zone of inhibition in millimeter. Positive control used for test bacteria was agar plug containing 30 µg/disc of chloramphenicol, while for test yeast and fungi were agar plug containing 30 µg/disc of ketoconazole.

3. Results and discussion

3.1. Surface sterilization of leaves

Before endophytic isolation process, the surface sterilization procedure was a critical precondition. With good and reliable surface sterilization, the actual endophytes can be isolated and differ from contaminants including epiphytes. The optimal immersion time of the samples in a particular sterilants is very important for the isolation of fungal endophytes. In this study, the optimal conditions for endophytic isolation obtained were at 3% NaOCl for 90 s and 70% ethanol for 120 s. Based on the condition of surface sterilization, the endophytic fungal isolates that were isolated in this study can be considered to be true endophytic fungi. The isolation of endophytes is an independent process and the technique used always gave great influence to the isolated endophytes. Different host and plant parts need a different soaking time in a suitable sterilants, where the thicker leaf required longer immersion time than the thinner ones [10].

3.2. Isolation and identification of endophytic fungi

Thirty-seven endophytic fungi isolates connected to *S. cumini* (L.) Skeels leaves can be cultured in the laboratory. Eleven endophytic fungi isolates can grow on PDA medium, while twenty-six endophytic fungi isolates be able grow on PDA containing host plant (Figure 1). Number of endophytic fungi isolates that growing on PDA medium less than PDA medium containing host plant. This is because of the culture medium that containing host plant has the higher nutrient, therefore endophytic fungal isolates will increase as well. Figure 2 shows the macroscopic and microscopic from one of the isolated endophytic fungal, which the fungal growth was initiated mostly within 2 d of inoculation and in 7 d of incubation, the endophytic fungal showed the optimal growth. The colony morphology of endophytic fungi isolates shows a variety of genera.

The endophytic fungal isolates found were culturable fungi on growth media and can grow quickly. This is because many fungi are incapable to grow in cultures since they are finicky type of endophytes and difficult to cultivate on artificial media [10]. Isolation of endophytes using conventional methodology would produce faster growing culturable fungi and some of those endophytes are never been isolated before [11][12].
Based on the morphological identification which included colony colour and textures [13][14] and also microscopic feature, these endophytic isolates were classified in some genera, namely *Collectotrichum*, *Phomopsis*, *Penicillium*, *Pestalotiopsis*, *Fusarium*, *Aspergillus* and *Myrothecium*.

**Figure 1.** Number of endophytic fungi isolates from leaves of *S. cumini* (L.) Skeels.

**Figure 2.** Macroscopic and microscopic features of one of the isolated endophytic fungi from leaves of *S. cumini* (L.) Skeels.

### 3.3. Screening for antimicrobial activity by agar plug diffusion assay

Based on the screening using agar plug assay, some of endophytic fungi isolates demonstrated antimicrobial activity (Figure 3). Every tested microorganism can be inhibited by compounds produced by endophytic fungi isolates from leaves of this medicinal plant. Therefore, the endophytic fungal isolates were suggested to produce antimicrobial agents. In this study, only seventeen endophytic fungi isolates showed antimicrobial activity against tested microorganisms. This is maybe another endophytic fungi isolates did not produce antimicrobial compounds that can kill the tested microorganisms. In addition, these fungi isolates might be potential as another agent such as antitumor, anticancer, antioxidant, antidiabetes or as enzyme producer. This is confirmed by the statement that novel bioactive compounds produced by endohytic fungi were abundant with enormous potential to use as medicine, and can also be utilized in agriculture and industry areas [15].
Figure 3. Number of endophytic fungi isolates from leaves of *S. cumini* (L.) Skeels that show antimicrobial activity.

4. Conclusion
This study has shown that endophytic fungi isolated from *S. cumini* (L.) Skeels leaves may be a potential natural resource as antimicrobial agents. Further investigation can be focused on isolation of bioactive compounds from these endophytic fungi and purification of its pure compounds.

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