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Isolation Endophytic Fungi from Ficus carica L. as Antibiotic Producer against Staphylococcus aureus

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Abstract. Endophytic fungi are microbial fungi which live inside of plant tissue and are also known as producers of secondary metabolites to be be utilized as antioxidant, anticancer, and antibacterial compounds. The aim of this research is to isolate the endophytic fungi from *Ficus carica* L. of Indonesian origin which has antibacterial properties against *Staphylococcus aureus*. A total of five different endophytic fungi were isolated from this plant and only two of them exhibited antibacterial activity against tested bacteria. The antibacterial activity test using MTT assay method showed that the isolated endophytic fungi from the root were able to inhibit the growth of *Staphylococcus aureus* with MIC value of 31.25 μg/ml with 62.29% of cell death. Meanwhile, the isolated endophytic fungi from stem showed antibacterial activity at 500 μg/ml as its MIC with 79.28% of cell death. The results indicated endophytic fungi that live in root and stem of *Ficus carica* L. and have the potential as an antibacterial against *Staphylococcus aureus*.

1. Introduction

To date, the problem of antibacterial resistance and availability in the healthcare world is increasing [1,2]. Another fact mentioned that the number of new antibacterials that have successfully passed the complete development process and finally get approval by the FDA has declined over the past 25 years [3]. Therefore, the discovery of antibacterial compounds is highly essential to overcome the shortage of antibacterials in the medical world.

In recent years, a discovery of new antibacterial agent by utilizing plants as their main source of production still become a promising alternative solution in dealing with antibiotic resistance. However, in practice, high cost and a large number of plants are highly required thus constraining the development on an industrial scale. In addition, the existence of government regulations and international regulations that protect the natural conservation challenging the taking of plants and the development of cultivars of plants out of their origin areas. Therefore, it is necessary to find alternative of bioactive materials that are more efficient and are environmentally-friendly. One of them is by utilizing microorganisms that live in plants which are called endophytic fungi [4,5]. The ability of endophytic fungi in producing secondary metabolite compounds is related to its interaction with its plant host and environment factor surrounding the plant [6,7]. The endophytic fungi are capable of producing secondary metabolite compounds that have antiviral, antimicrobial, antimalarial, anticancer, and antioxidant activity [8]. One plant that has the potential to produce endophytic fungi that have antibacterial activity is *Ficus carica* L. [9-13]. This study aims to evaluate the antibacterial activity produced by endophytic fungi on the roots, stems, leaves, and fruits of *Ficus carica* L. originating from Indonesia.

2. Material and Method

2.1 Plant Collection and Determination

The plant used in this study is a 4-5 month-aged plant that grows in Yogyakarta, Indonesia, which was free of disease. The sample of the plant was then carried out to be determined in Plant Systematic Laboratory, Faculty of Biology, UGM, Yogyakarta, Indonesia.

2.2 Isolation and Purification of Endophytic Fungi

Many parts of the plant such as roots, stems, leaves, and fruit were cut into several sections of 2-3 cm in size. This sample was then subjected to surface sterilization steps by soaking in 5.25% sodium hypochlorite (NaOCl) solution for 2 minutes, then washed with sterile aquadest, immersed in 70% ethanol for 2 minutes, and washed again using a sterile aquadest. The sample then cut using a sterile blade of ± 1x1 cm2 and placed on a petri dish containing 20 ml of Potato Dextrose Agar with 100 µl/ml Chloramphenicol to avoid any bacterial growth. This cultured was then incubated in a dark room at 25-30ºC for 5-14 days depending on fungi growth rate. The fungi colony that successfully grows was then purified by cutting some of the mycelium and was transferred in other PDA medium then incubated with the same condition as previously mentioned. This purification process was repeated to result in a single isolate of endophytic fungi.

2.3 Characterization of Single Isolate Endophytic fungi

The single isolate of the fungi resulted from the purification process was then characterized based on its colony color, colony texture, zonation, growth zone, radial line, and colony diameter at Micology Laboratory, Faculty of Agriculture, UGM, Indonesia, Yogyakarta. Identification of 16S rDNA gene sequencing of fungi was being performed in this study.

2.4 Secondary Metabolite Production and Extraction

Every single isolate of endophytic fungi was partially removed from its mycelium using a sterile knife, then inserted into Potato Dextrose Broth media with the amount of 125 mL in 250 mL volume of Erlenmeyer. This culture was incubated at 28 ± 2ºC at 60-65 rpm for 21 days. Endophytic fungi biomass was separated from its media using filter paper. Each endophytic fungi biomass was crushed then extracted using ethyl acetate in a 1:1 ratio. This step was performed in 3 repetitions with occasional homogenization using an ultrasonicator. Each extract was then dried using a rotary evaporator at 50ºC.

2.5 Antibacterial Activity Test In Vitro

The antibacterial activity test was performed by microdilution method in 96-well micropate based on CLSI protocol guide with little modification. The extract of endophytic biomass fungi was dissolved using 10% of DMSO. Extract with amount of 20 µl was dispense into the first well of a microplate containing 180 µl MBH with a suspension of 10^5 CFU *S.aureus* bacteria. The 2 fold dilution was performed at the second to the ninth well containing 100 µl of bacteria culture to obtain a concentration of 2000 µg/ml-7.8125 µg/ml of extract. This culture was then incubated at 37ºC for 24 hours. Controls used in this experiment were a solvent control which contained 90 µl MHB and 10 µl DMSO, a bacterial control containing 100 µl of bacterial culture and Vancomycin as an antibiotic control.

To determine MIC value of the extract, MTT 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (5 mg/ml) was used as a reagent to assess cell viability. The lowest concentration of the extract which did not exhibit a violet change reaction was defined as the MIC value. In order to

confirm the percentage of the bacterial cell death, the measurement of sample absorbance was conducted using a microplate reader at 570 nm then its calculated using the equation below:

$$\%\ deaths\ \ of\ \ S.aureus = \frac{(Abs.bacterial\ \ control - Abs.treatment)}{(Abs.bacterial\ \ control)} x100\% \quad (1)$$

3. Result and Discussion

3.1 Isolation and Characterization of Single Isolate Endophytic fungi

Based on the endophytic insulation fungi process at the root, stem, leaf, and fruits of *Ficus carica* L. originating from Indonesia, there were 5 isolates, namely isolates A2, Ba2, Bu2, D1, and D2. Table. 1 shows that based on a macroscopic characterization of colony color, colony texture, zonation, growth zone, radial line, and colony diameter from each isolate, there were some differences in each colony. Differences in the characteristics of the isolates in each part of the plant are influenced by several factors such as the environmental conditions in which the host plant grows, the geographical location, temperature, pH, and nutrient levels contained in the soil. In addition, the age factor of the plant affects the distribution of endophytic fungi colonies within the plant tissue. Completely developed plant tissue is more inhabited by endophytic fungi colonies because it enables better nutrient transfer so that fungi colonies can grow and develop properly [14].

Table 1. Characteristic of single isolate endophytic fungi

Observation	**Isolate**				
Macroscopic	**A2**	**Ba2**	**Bu2**	**D1**	**D2**
colony color	white	white	white	blacked dark green	white
zonation	-	-	-	yes	-
colony texture	smooth	smooth	smooth	rough and granulated	smooth
growth zone	-	-	-	yes	-
radial line	-	-	-	-	-
colony diameter	7.5 cm	8 cm	8 cm	5.5 cm	9 cm

Code: A2= isolate from the 2nd root replication, Ba2= isolate from the 2nd root replication, Bu2= isolate from the 2nd fruit replication, D1= isolate from the 1st leaf replication, D2= isolate from 2nd leaf replication.
The minus sign [-] indicates that the isolates do not have characteristic.

3.2 Antibacterial Activity Test In Vitro

Table 2. shows the results of antibacterial activity of A2, Ba2, Bu2, D1, and D2 endophytic isolate extracts show that only A2, Ba2, and D1 isolates exhibit antibacterial activity against *S.aureus* with MIC value of 31.25 µg/ml - 1000 µg/ml, respectively. The endophytic fungi biomass A2 and Ba2 extracts have better activity in inhibiting the growth of *S.aureus* bacteria, when compared with isolate D1. The isolates A2 and Ba2 were able to kill ≥50% of total living *S.aureus* bacteria with a concentration of its MIC value. Based on the existing study, it is revealed that an extract could be said to have a high activity as an antibacterial if it has concentrations of <1 mg/ml or <1000 µg/ml [15].

Table 2. Antibacterial activity of extract against *S.aureus*

Sample	Concentration [µg/ml]	Bacteria cell death [%]±SD
A2	31.25	62.29±19.49
Ba2	500	79.28±3.012
D1	1000	41.10±18.53
D2	-	-
Bu2	-	-

The minus sign [-] indicates the sample D2 and Bu2 do not have activity of inhibition *S.aureus* bacteria growth and do not have percentage value of bacteria cell death.

4. Conclusion

These results suggest promising antimicrobial properties of endophytic fungi isolates from root and stems of *Ficus carica* L. Further study is needed to provide information of its active compound responsible for antibacterial activity.

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