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Antibacterial Activity of Endophytic Fungi Isolated from the Leaves of Jambu Biji (*Psidium guajava L.*)

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Abstract. Antibacterial resistance has been a major health issue and remains a threat to the global health care system. Endophytic fungi, particularly those isolated from medicinal plants are potential as a source of new antibiotics. The endophytic fungi were isolated from the surface of sterilized leaves of jambu biji (*Psidium guajava L.*) by potato dextrose agar (PDA) and the isolates were identified through their morphological characteristics. The antibacterial activities from ethyl acetate extracts of endophytic fungi were evaluated against *Escherichia coli* and *Salmonella tymphi* by Kirby Bauer method. Four endophytic fungi were isolated from the leaves of jambu biji and were identified as *Aspergillus niger Van Tieghem*, *Aspergillus flavus*, *Aspergillus fumigatus* Fres, and *Aspergillus sp.*(14). The antibacterial activity of the extracts was classified as moderate and strong category as its inhibition zone was in the range of $\geq 50\%$ compared to positive control. Therefore, this extract has the potential to be developed into an antibiotic drug.

The first section in your paper.

Keywords : Antibacterial activity, Endophytic Fungi, *Psidium guajava L.*

1. Introduction

People from developing countries such as Indonesia are prone to suffer from diseases caused by poor environment and food intake like diarrhea. Due to the poor environment and lack of awareness on healthy lifestyle, the spread of the disease like diarrhea is rife in slum areas. Unhygienic lifestyle with no concern of sanitation could make colon vulnerable to the attack of pathogenic *Escherichia coli* and *Salmonella tymphi* bacteria that cause diarrhea (1,2).

Antibiotic resistance is a globally popular health problem (3,4). The usage of antibiotics that breaches prescribed rules could make pathogenic microbes resistant to the existing antibiotic (5,6,7). The time duration since the usage of an antibiotic being introduced to the event of resistance varies starting from 0 to 36 years (8). Furthermore, the average duration of an antibiotic to become resistant is about 6.6 years since the introduction. This fact shows the existing antibiotics could not forever be used to cure infection diseases and calls for sustainable development of existing antibiotics as well as newly-sourced antibiotics. The utilization of medicinal plants data as the result of ethnobotany research is one of the effective ways in discovering new chemical materials that could be useful for medication such as to gain new antibiotic compound (9,10).

Another way to gain new antibiotic compound is by utilizing endophytic fungi from each plant. Since there are many kinds of plants, there needs to be an approach to narrow down the search of endophytic fungi that give off antibiotic activity such as plants with history of ethnobotany like Jambu Biji (*Psidium guajava L.*), in which use is related to diarrhea medication so the chance to get new antibiotic compound is higher (11).



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The leaves of Jambu Biji are believed to have the effects of astringent, wound healing, anti-allergy, damaged cell repair, and anti-microbe activities against bacteria that generally infect wounds of surgery and other soft tissues such as *Staphylococcus aureus*, *Streptococcus spp*, *Escherichia coli*, *Salmonella typhi*, *Proteus mirabilis*, and *Shigella dysenteria* (12).

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2. Materials and Methods

2.1. Study Time and Location

The study was conducted in June to September 2017. The antibacterial activity in vitro testing was done at the Microbiology Laboratory in the Faculty of Mathematics and Natural Sciences of Sriwijaya University (FMIPA UNSRI), while as for the extraction and evaporation were done at the Organic Chemistry Lab of Chemistry Department of FMIPA UNSRI.

2.2. Materials

Materials required for the study consisted of the host plant jambu biji leaves while as for sample sterilization it took 70% of ethanol, 0.5% of NaOCl 0.5%, and aquadest sterile. The study used potato Dextrose Agar (PDA) and Potato Dextrose Broth (PDB) as fungal growth media as well as Nutrient Agar (NA) and Mueller Hinton Agar (MHA) as the media in antibacterial activity testing. As for the testing bacteria, the study used *E.Coli* and *S. Tphi* with the antibiotic standard of Ciprofloxacin, and ethyl acetate technical as the organic solvent in sample extraction.

2.3. Methods

2.3.1. Sample Sterilization and Isolation of Endophytic

Samples of jambu biji leaves were washed with flowing water for approx 5 minutes. Then the surface of the leaves were to be sterilized with 70% of alcohol solution poured into a cotton ball for approx. 3 minutes before being washed with aquadest sterile for approx. 1 minute. Next, the leaves were slathered with 5% (b/v) of HgCl₂ (mercury chloride) for approx. 1 minute to later be washed with aquadest sterile for approx 1 minute. The sterilized samples were cut vertically with aseptic technique then were planted in petri dishes containing PDA media and were incubated at 37°C for 2 x 24 hours. Next, the observation was done every day for 3 to 7 days until growing fungi appeared (13).

2.3.2. Purification of Endophytic Fungi

The fungal colony grown in PDA medium that showed different morphological characteristics in shapes, colors, and sizes were later purified. The purification was done by relocating the colony into a new PDA medium with streak plate method, then being incubated at 37°C for 2x24 hours. Next, the purified fungal colony was made into work and stock cultures by growing it in a slant PDA (14).

2.3.3. Identification of Endophytic Fungi

The endophytic fungal strain was identified based on the colony morphology and microscopic observation of mycelia and sexual spores according to the method described in the literature (15).

2.3.4. Cultivation of Endophytic Fungi

Medium (PDB) of 300 mL was used for the cultivation of endophytic fungi and was placed into 3 flasks. Fungal suspension containing 10⁶ spores/mL was inoculated under sterile condition to each of 300 mL PDB medium (ratio 1:1). The cultures were incubated for six weeks in a static condition at room temperature. At the end of incubation period, each culture was filtered to separate the mycelium to later be extracted with ethyl acetate solvent as well as to be evaporated to get concentrated extract.

2.3.5. Extraction of Jambu Biji Leaves

The leaves of jambu biji used as the comparison in the study amounted to approx. 100 grams of fresh leaves, chopped, and extracted by maceration in methanol for 2 x 24 hours. Next, the extract was filtered and evaporated so it would produce concentrated extract towards which later be tested for its antibacterial activity using *E. Coli* and *S. Tphi*, with the same method.

2.3.6. Antibacterial Activity Testing

The making of suspension with 0.5 McFarland standard and the making of testing bacteria suspension (16).

2.3.7. Preparation of Antibacterial Compound Concentration

The antibacterial sample concentration was made with 1,000 µg/disk or 1,000 µg/10 µL and the concentration of ciprofloxacin antibiotic as the standard of 500 µg/disk atau 500 µg/10 µL.

2.3.8. Antibacterial Activity Test by Kirby Bauer Method

Each of the samples dissolved in methanol. The MHA (15 mL) was poured into petri dishes and inoculated with 100 µL of the suspension containing 1×10^8 CFU of bacteria. Ciprofloxacin was used as the positive control and discs treated with methanol were used as the negative control. Sterile paper discs (6 mm) loaded with 10 µL of the samples were placed onto the surface of the agar. The plates were then placed in an incubator at 37°C for 24 h, after which the diameter of the zone of inhibition around each of the discs was measured and recorded. Each experiment was performed in triplicate (17).

2.4. Data Analysis and Management

The determination of antibacterial activity in the test sample (concentrated extracts from endophytic fungi) was done by comparing the inhibition zone in each concentration to the inhibition zone in the positive controls that are standard antibiotic and methanol extracts from the host plant, therefore the relative activity of antibacterial sample tested with positive controls could be determined. The criteria of each antibacterial compound concentration tested against positive controls were determined with the following formula (18).

Weak: $\frac{A}{B} \times 100\% < 50\%$; Moderate: $50 < \frac{A}{B} \times 100\% < 70\%$;

Strong: $\frac{A}{B} \times 100\% > 70\%$

A: zone of inhibition (mm) of tested antibacterial compound

B: zone of inhibition (mm) of positive controls (standard antibiotic and host plant extracts).

3. Results and Discussions

Out of jambu biji leaves, four endophytic fungi were isolated and were characterized in macroscopic and microscopic ways. The four fungi were identified as J1 to J4. The result of such characterization showed the four fungi were *Aspergillus niger* Van Tieghem, *Aspergillus flavus*, *Aspergillus fumigatus* Fres, and *Aspergillus sp.*(J4) (as shown on Figure 1).

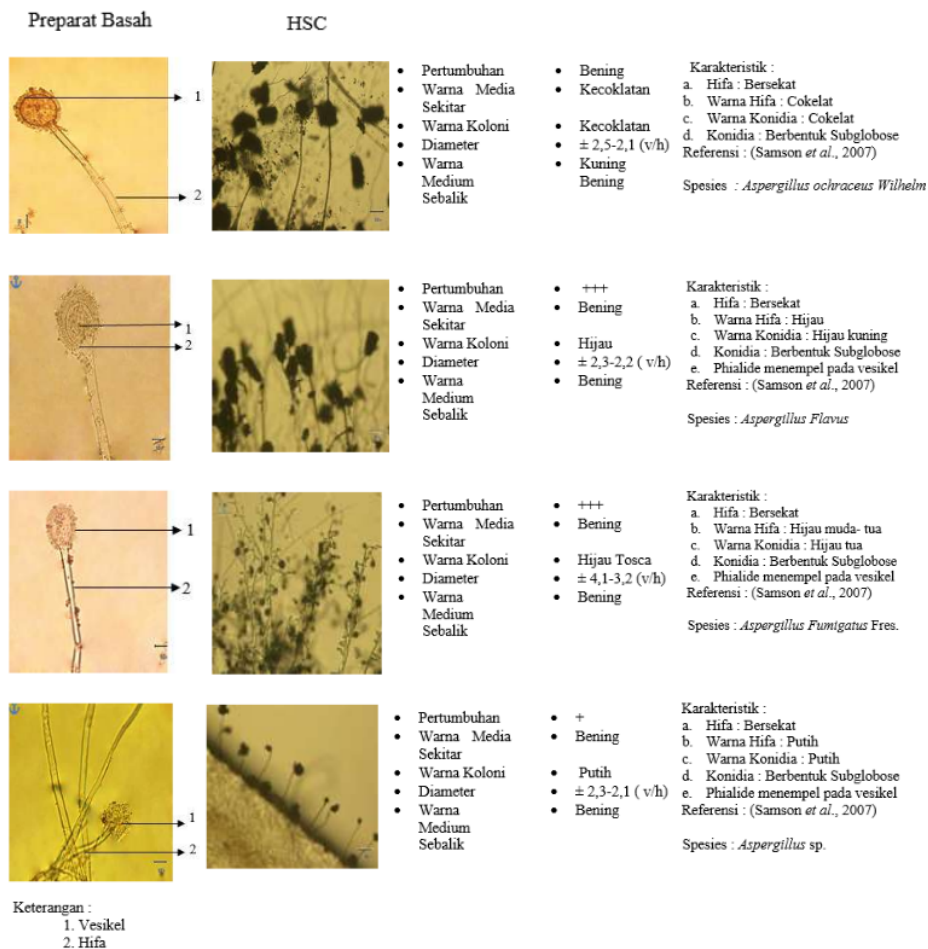


Figure 1. Macroscopic and microscopic observations of endophytic fungi of jambu biji plant

Each fungal isolates obtained was cultivated in 3x300 mL of PDB liquid medium for six weeks to later be filtered and extracted in organic solvent ethyl acetate. The ethyl acetate fraction was then separated and evaporated. Meanwhile, the antibacterial activity testing was conducted on condensed extracts using test bacteria *E. Coli* and *S. Tphi* with Kirby-Bauer method in single concentration that was 1,000 µg/disc. The results of antibacterial testing on the ethyl acetate extracts of endophytic fungi against two test bacteria that have been compared with antibacterial activity of positive controls comprising ciprofloxacin antibiotic (500 µg/disk) and the methanol extract of host plant jambu biji leaves (1,000 µg/disk) are shown on Table 1 and 2.

Table 1. Antibacterial activity percentages of ethyl acetate extracts of endophytic fungi from jambu biji leaves against test bacteria *E.Coli*, compared to the antibacterial activity of positive controls (ciprofloxacin antibiotic and methanol extract of jambu biji leaves)

No	Extracts of endophytic fungi (1,000 µg/disk)	Diameter of zone of inhibition on testing bacteria <i>E. Coli</i>	Percentages of antibacterial activity of methanol extracts on positive controls (%)	
			ciprofloxacin antibiotic(500 µg/disk) 3.53 cm	Extracts of jambubiji leaves(1,000 µg/disk) 1.31 cm
1	Fungal extract 1 (J1)	1.24	35.1 ⁺	94.6 ⁺⁺⁺
2	Fungal extract2 (J2)	1.91	54.1 ⁺⁺	146.0 ⁺⁺⁺
3	Fungal extract3 (J3)	1.38	39.1 ⁺	105.3 ⁺⁺⁺
4	Fungal extract4 (J4)	0.89	25.2 ⁺	68.0 ⁺⁺

Information: + = weak inhibition <50%
 ++ = moderate inhibition 50-70%
 +++ = strong inhibition >70%

Table 2. Antibacterial activity percentages of ethyl acetate extracts of endophytic fungi from jambu biji leaves against test bacteria *S. Tphi*, compared to the antibacterial activity of positive controls (ciprofloxacin antibiotic and methanol extract of jambu biji leaves)

No	Extracts of endophytic fungi (1,000 µg/disk)	Diameter of zone of inhibition on testing bacteria <i>S. tphi</i>	Percentages of antibacterial activity of methanol extracts on positive controls(%)	
			ciprofloxacin antibiotic(500 µg/disk) 3.82 cm	Extracts of jambubiji leaves(1,000 µg/disk) 1.72 cm
1	Fungal extract1 (J1)	1.41	36.9 ⁺	82.0 ⁺⁺⁺
2	Fungal extract2 (J2)	2.15	56.3 ⁺⁺	125 ⁺⁺⁺
3	Fungal extract3 (J3)	0.94	24.6 ⁺	54.6 ⁺⁺
4	Fungal extract4 (J4)	0.83	21.7 ⁺	48.2 ⁺

Information: + = weak inhibition < 50%
 ++ = moderate inhibition 50-70%
 +++ = strong inhibition >70%

The study used ciprofloxacin (500 µg/disk) as a comparator antibiotic standard in the antibacterial test. The zone of inhibition of an antibiotic standard is classified as strong, moderate, and resistant categories with certain levels of antibiotic per disk (19). An antibiotic could be set as a standard if it produces inhibition zones in moderate and weak categories when tested at an inoculum equals to McFarlan 0.5. However, if an antibiotic's zone of inhibition belongs to the resistant category, it could not be used as a standard.

The zones of inhibition listed on Table 1 and 2 have shown that ciprofloxacin is classified as strong category for all testing bacteria so it can be used as a standard in the reference of antibacterial activity test extracts. The zone of inhibition of jambu biji leaves (1,000 µg/disk) worked as a comparator of antibacterial activity power of endophytic fungi extracts against the host plant.

The results on Table 1 and 2 showed that ethyl acetate extracts of four endophytic fungi in which antibacterial activities were tested against 2 test bacteria in single concentration of 1,000 µg/disk produced varied zones of inhibition depending on the strength of the activities.

After the zone of inhibition of each fungal extract against both test bacteria being compared to the zone of inhibition of ciprofloxacin antibiotic standard, the study acquired fungal extract J2, the extract with the strongest level of activity that was at moderate category with the inhibition zone ranging from 50% to 70% (Table 1 and 2). Meanwhile, the other fungal extracts comprising J1, J3, and J4, were grouped into weak category against the two test bacteria. This showed that J2 fungal extracts contained active compound mixed with several other inactive compounds which limited the active compound ability so it would not work optimally to inhibit the test bacteria, as compared to such ability of antibiotic as a synthetic compound.

Next, the zones of inhibition of the four endophytic fungi extracts were compared to those of methanol extracts of the host jambu biji leaves. Jambu biji extract has been known for its healing power in diarrhea medication and has been used in industrial scale. The results on Table 1 and 2 show that fungal extract J2 possesses the strongest activity against test bacteria *E. Coli*, with it being in the strong category with the zone of inhibition range of >70% against the comparator jambu biji leaves extracts. Furthermore, fungal extracts J1 and J3 also showed strong activities. Mean while, the same cannot be said for fungal extract J4 as it being the only extract with activities in moderate category that is 68%. The similar result is shown by fungal extracts J1 to J4 against test bacteria *S. Tphi* as fungal extracts J1 and J2 gave off strong activities, while J3 and J4 each showed moderate and weak activities. The work stages and potentials of endophytic fungi of jambu biji leaves as new antibacterial compound producer are shown on Figure 2.

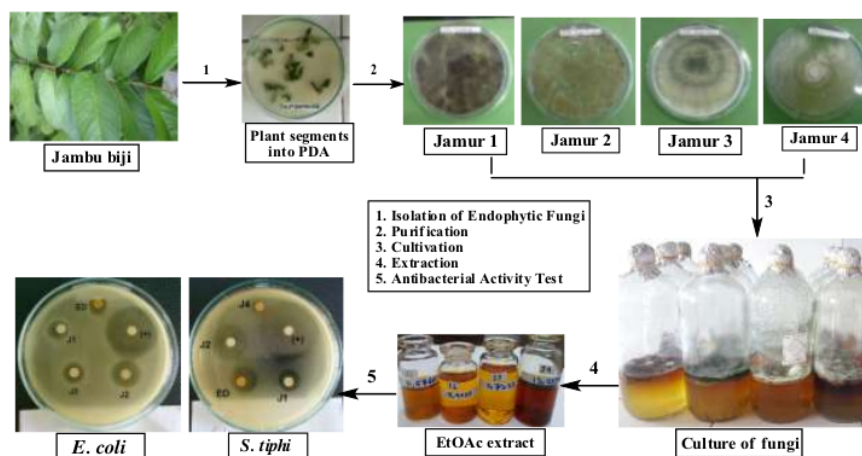


Figure 2. Brief isolation procedures of endophytic fungi from the leaves of jambu biji and antibacterial activity test from their ethyl acetate extracts

The study result shows that fungal extract J2 possesses the strongest antibacterial activity compared to the other three fungal extracts. This indicates the presence of antibacterial active compound in ethyl acetate extracts of J2 which was mixed with several other inactive compounds. If the active compound in J2 is purified, then there will be more potential for antibacterial activity.

The antibacterial activity of J2 extract is stronger than the extracts of the host plant that is jambu biji. This is a breakthrough in the field of medication and is worth to be researched further and in a more structured way to later be developed as a main ingredient of antibiotics in the future. Possible for endophytic fungi to obtain active compound of medicine as the number could be multiplied when necessary in a short time in line with the growth curve (approx. 4 to 8 weeks) and it does not take a wide space and expensive treatment to make it happen (20).

20 Conclusion

From the leaves of a jambu biji plant, there had been the isolation and characterization of four endophytic fungi namely *Aspergillus niger Van Tieghem* (J1), *Aspergillus flavus* (J2), *Aspergillus fumigatus Fres* (J3), and *Aspergillus sp.*(J4). Of all the four fungi, the ethyl acetate extract of *Aspergillus flavus* (J2) possessed the strongest activity, making it belong to the moderate category against ciprofloxacin antibiotic standard and stronger than the activity of methanol extracts of jambu biji leaves. The result shows that with further studies, the active compound in the fungi extract of *Aspergillus flavus* (J2) is potential to be developed as an active ingredient of antibiotics.

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