Antimicrobial activity of secondary metabolites of endophytic bacteria F4 of papaya leaf (Carica papaya L)

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Abstract. Papaya leaves have been known to contain secondary metabolite compounds as antimicrobial, so that endophytic bacteria is also suspected of having secondary metabolite compounds as antimicrobial. In previous research, endophytic bacteria have been isolated from papaya leaf and obtained 5 endophytic bacteria which were F1, F2, F3, F4 and F5. Three bacteria that have been known for their antibacterial activity were F1, F3, and F5. This research aims to determine the antimicrobial activity of F4 endophytic bacteria. The results showed that single isolates of endophytic bacteria F4 is a type of Gram-positive bacteria with a basil cell shape (stem). F4 endophytic bacteria has 3 growth phases; they are logarithmic phase occurs at 0-8 hours, stationary phase at 8-34 hours, and phase of death at 34-46 hours. Phytochemical results showed that MS18 contained saponin group compounds, while MS36 contained alkaloids and saponin group compounds. The highest antimicrobial activity was in the death phase, MS36 with minimum inhibitory concentration (KHM) to Escherichia coli, Candida albicans, and Bacillus subtilis were 15.5 mg/mL, respectively; 16.5 mg/mL and 16.5 mg/mL by disc diffusion method, whereas to Aspergillus niger of 0.5 mg/mL by dry weight method.

1. Introduction
Papaya leaf is one of the parts papaya plant that contains antimicrobial compounds [1]. Ekaiko et al. (2015) reported that secondary metabolites in papaya leaves have antimicrobial activity [2]. Antimicrobial activity can also be found in secondary metabolites produced by endophytic bacteria. This statement has been proven by Das et al. (2017) that secondary metabolites produced by endophytic bacteria from Hyptis suaveolens can inhibited growth of Bacillus subtilis, Staphylococcus aureus, Eschericia coli, and Candida albicans [3].

The application of endophytic bacteria as producer of antimicrobial compounds on a large scale is more effective than plants, because life cycle of endophytic bacteria is shorter than plants to save production time and does not require a large area. Another advantage, it can maintain the preservation of medicinal plants, especially rare medicinal plants [4].

In previous research, Ramadhan (2015) discovered five endophytic bacteria, namely F1, F2, F3, F4, and F5 [5], Endophytic bacterial isolate F3 can produce secondary metabolite compounds of alkaloids, flavonoids, saponins, tannins, and triterpenoids which function as antioxidants and antibacterials. Secondary metabolite F3 bacteria able to inhibit DPPH free radical and has scavenging activity of 68.5%. Secondary metabolite F3 shows antibacterial potential to inhibit S. aureus, S. Typhi and E. coli.
[6]. The antioxidant activity of secondary metabolites of F1 using the DPPH method has IC$_{50}$ of 22.472 ppm. Phytochemical screening shows that the production of secondary metabolites of F1 contains alkaloids, flavonoids, tannins, and saponins [7].

2. **Research methods**

2.1. *Morphological identification of endophytic bacteria F4*

Pure colonies of endophytic bacteria F4 were identified morphologically by observing the colony shape, color, elevation, colony edge shape, and Gram-type bacteria. Production of secondary metabolites of endophytic bacteria F4.

Pure colonies of endophytic bacteria F4 were inoculated in an Erlenmeyer flask containing liquid *zobell* and incubated for 8 hours as a starter inoculum. Then starter inoculum was poured again in an Erlenmeyer flask containing liquid *zobell* and incubated for 18 hours. Then they were centrifuged with rate 6000 rpm for 15 minutes. Centrifugation aims to separate filtrate and residues. Then the filtrate was compacted with freeze drying method.

2.2. *Phytochemical screening*

Phytochemical test aims to analyze secondary metabolites qualitatively. The screening includes the determination of saponin, alkaloids, tanin, triterpenoid/steroid and flavonoids. All stages were carried out based on the analysis method described by Sherwani et al. (2013) [8].

2.3. **Assay of Antibacterial Activity**

2.3.1 *Turbidimetry method.* Suspension of test bacteria (*Escherichia coli dan Bacillus subtillis*) has been adjusted to 0.5 Mac Farland. Then 0.35 mL of test bacteria suspension was transferred into 3.15 mL *Nutrient Broth*, added 0.35 mL secondary metabolites, and incubated for 24 hours. Furthermore, optical density measurement was carried out with a UV-Vis spectrophotometer.

2.3.2 *Disc Diffusion Method.* Suspension of test bacteria (*Escherichia coli dan Bacillus subtillis*) has been adjusted to 0.5 Mac Farland. Then 40 μL of test bacteria suspension was poured into a petri plate containing *Nutrient Agar*. Then disc paper is soaked into secondary metabolites. As positive control, disc paper was soaked into 0.3 mg/mL antibiotic. As negative control, disc paper was soaked into aquades. Furthermore, Petri plate was incubated for 24 hours and measured clear zones (zones of inhibition) with a transparent rule.

3. **Results and discussion**

3.1. *Morphological identification of endophytic bacteria F4*

Gram staining method with a microscope was used to morphological identification of endophytic bacteria F4. The result was shown in Figure1.
Figure 1. The observation results of Gram staining of endophytic bacteria F4

Figure 1 shows that endophytic bacteria F4 are gram-positive bacteria, because they are purple. The purple color of endophytic bacteria F4 is caused by compound complex of crystal violet dye which strongly bond by the cell wall of Gram-positive bacteria even though it is given laxative (alcohol) so it cannot be colored with safranin [9].

| Colony Color | White  |
|--------------|--------|
| Colony Shape | Round  |
| Colony Elevation | Convex |
| Shape of a Colony Edge | Smooth |
| Cell Form | Basil  |
| Gram | Positive |

Table 1. Characteristics of endophytic bacteria F4

Table 1 shows characteristics of endophytic bacteria F4 microscopically and macroscopically. Microscopic observations show that endophytic bacteria F4 are a type of gram-positive bacteria with bacillar cell form, while macroscopic observation shows white colonies, rounded colony shape, convex colony elevation shape, and smooth colony edge shape.

3.2. Production of secondary metabolites of endophytic bacteria F4

Production results show that secondary metabolites are brown solution but after concentrated by freeze drying method produces secondary metabolites in the form of brown solids.

3.3. Phytochemical screening

The results of Phytochemical screening shown in Table 2 to show the differences in phytochemical test results between secondary metabolites and ethanol extract of papaya leaves that has been done by Ramadhan (2015). The ethanol extract of papaya leaves contains alkaloids, saponins, flavonoids, tannins, and steroids but in secondary metabolites only contain saponins. The results obtained are not in accordance with the theory which states that endophytic bacteria are capable of producing secondary metabolites in accordance with their hosts [10]. The differences in the results of suspected papaya leaves are various types of endophytic bacteria whereas in this study there is only one type of endophytic bacteria, where each type of bacteria has a different genetic code so that it can produce various kinds of secondary metabolites.

| Phytochemical test | Secondary metabolites | Ethanol Extract of Papaya Leaves [5] |
|--------------------|-----------------------|-------------------------------------|
| Alkaloid           | -                     | +                                   |
| Flavonoid          | -                     | +                                   |
| Tannin             | -                     | +                                   |
| Saponin            | +                     | +                                   |
| Steroid            | -                     | +                                   |
| Triterpenoid       | -                     | -                                   |

3.4. Assay of antibacterial activity
In this study, the antibacterial activity test used turbidimetry and disc diffusion methods. Figure 2 shows the results of antibacterial testing using the turbidimetric method. It can be seen that the percentage of metabolite inhibition secondary to *Escherichia coli* is greater than *Bacillus subtilis*. These results indicate that secondary metabolites are more effective in inhibiting the growth of *Escherichia coli* than *Bacillus subtilis*. This is because the cell wall of *Bacillus subtilis* (15-80 nm) is thicker than the cell wall of *Escherichia coli* (2-3 nm) so that the antimicrobial substances contained in secondary metabolites are difficult to penetrate into *Bacillus subtilis* cells [11].

In this study also determined the Minimum Inhibitory Concentration (MIC) with disc diffusion method. In the disc diffusion method has used secondary metabolites with the same concentration as the concentration of positive control but does not show a clear zone, so the extract concentration is increased again to indicate the existence of a clear zone. The result was shown in Figure 3.

![Figure 2](image1.png)

**Figure 2.** Antibacterial activity with turbidimetry method

![Figure 3](image2.png)

**Figure 3.** Determination of MIC with disc diffusion method

Figure 3 shows that secondary metabolites have different MIC against *Escherichia coli* and *Bacillus subtilis*, which are 16.5 mg/mL and 17.5 mg/mL, respectively. Figure 3 also shows that the increase in the concentration of secondary metabolites results in a greater inhibition zone. This is consistent with the theory that the greater the concentration of the active compounds contained, the greater the percentage of inhibition produced will also be greater [12].
In Figure 3 shows the high concentration (17.5 mg/mL) of inhibition zone diameter produced by metabolites secondary to Escherichia coli and Bacillus subtilis, which are 9 mm and 4 mm, respectively. Greenwood (1995) said that the inhibitory response to microbial growth can be classified as follows, if the inhibition zone diameter of more than 20 mm is categorized as strong, the 16-20 mm inhibition zone is categorized as moderate, 10-15 mm inhibition zone is categorized as weak, and the inhibition zone is less than 10 mm is categorized as less effective [13]. Based on these classifications it can be concluded that the antimicrobial activity of secondary metabolites is categorized as less effective antimicrobials.

Antibacterial activity testing gives different results in Escherichia coli and Bacillus subtilis, even though both are types of bacteria. This is because Escherichia coli and Bacillus subtilis are two different types of gram bacteria. Because of the differences in cell wall structure, so that the mechanism of metabolic antimicrobial activity of the secondary to Escherichia coli and Bacillus subtilis is different. Escherichia coli (gram negative) has a thin layer of peptidoglycan on the cell wall and is surrounded by lipoprotein, lipopolysaccharide (LPS), phospholipids and some proteins. It is described LPS has a lipid structure and polysaccharides. The inhibitory mechanism in gram-negative bacteria is the formation of hydrogen bonds between bioactive compounds and polysaccharides in gram-negative bacteria, then blocking the flow of nutrients so that bacteria will die due to lack of nutrients. In Bacillus subtilis (gram positive), the cell wall is composed of many pore tissue and thick peptidoglycan layer, and surrounded by ketoic acid layer. The mechanism that occurs in gram-positive bacteria, bioactive compounds will form hydrogen bonds with glycan in peptidoglycan which causes damage to the cell wall, so that bacteria cannot survive external influences and die immediately [14].

The presence of antibacterial activity in secondary metabolites due to the presence of active compounds, where phytochemical screening results indicate the presence of saponins. Saponins as antibacterials are by diffusing through the outer membrane and cell walls of susceptible bacteria then binding the cytoplasmic membrane so that it disrupts and reduces the stability of the cell membrane, this causes the cytoplasm to leak out of the cell resulting in cell death [15].

Ampicillin as much as 0.3 mg/mL was used as a positive control in order to inhibit the growth of Escherichia coli and Bacillus subtilis. Bacterial growth can be inhibited using ampicillin through inhibition of bacterial wall synthesis by covalently binding to the transpeptidase bound to peptidoglycan [16]. The use of ampicillin at a smaller concentration compared to the concentration of secondary metabolites so that the results shown are not too different.

4. Conclusion

Endophytic bacteria F4 from papaya leaves are bacteria in the form of bacillus cells and Gram-positive types where the secondary metabolites produced provide high antibacterial activity against gram-negative bacteria compared to gram-positive bacteria.

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