

Antibacterial Activity of Endophytic Bacteria from Butterfly Pea (Clitoria ternatea L.) Root against Staphylococcus aureus and Escherichia coli

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Abstrak - Indonesia is rich in biodiversity, with many medicinal plants such as Clitoria ternatea (butterfly pea) known for antibacterial properties. This study aimed to evaluate the antibacterial activity of ethyl acetate extracts from endophytic bacteria isolated from C. ternatea roots, with potential application in developing natural antibacterial agents. The bacteria were identified as belonging to the genera Bacillus, Pseudomonas, and two strains of Azomonas (A and B). Extracts were tested against Staphylococcus aureus and Escherichia coli using Disk Diffusion Assay (DDA), Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), and Time-Kill assays. Results showed Pseudomonas extract at 40 mg/ml had the largest inhibition zone against S. aureus (6.75 ± 0.353 mm), while Azomonas A was most effective against E. coli (3.187 ± 0.618 mm). The lowest MIC against S. aureus was 2.5 mg/ml from Azomonas B, with MBCs of 20 mg/ml for Pseudomonas and Azomonas B. Against E. coli, Azomonas A showed MIC and MBC of 10 mg/ml and 40 mg/ml, respectively. Time-Kill assays confirmed bacterial reductions at MIC and higher concentrations. These findings suggest C. ternatea's endophytic bacteria extracts have promising antibacterial potential for sustainable medicinal use.

Kata kunci: Antibacterial, Bacillus, Pseudomonas, Azomonas, Clitoria ternatea L., Staphylococcus aureus, Escherichia coli.

INTRODUCTION

Indonesia is known as a country with abundant flora and biodiversity, which provides great potential in the utilization of plants as traditional medicine (Safitri et al., 2024). One plant that has attracted attention is Bunga Telang (Clitoria ternatea L.), which has long been used in traditional medicine and has various properties, including antibacterial potential (Yurisna et al., 2022). Endophytic bacteria, which live inside plant tissues without causing disease, can contribute to plant health and produce secondary metabolite compounds that have biological activities, including antibacterial properties. The utilization of these compounds can not only help in the development of new drugs, but also play a role in maintaining plant biodiversity in Indonesia (Tobing et al., 2024).

Several studies have explored the utilization of bacterial extracts endophytic bacterial extracts from medicinal plants along with their activity tests against various bacterial pathogens. pathogens. Research by Sepriana et al. (2020) found that 4 isolates from 5 bacterial isolates of endophytes of clove plants had strong inhibition (diameter of inhibition zone ≥ 11 mm) against S. aureus bacteria. (Hamtini et al., 2024) obtained isolates of endophytic bacteria from namnam leaves as many as 11 isolates inhibited S. aureus and 2 isolates of E. coli with a strong inhibition zone. strong inhibition zone. Another study showed that telang flower leaf extract gave zone of inhibition against E. coli of 26 mm, while methanol extract of telang flower seeds gave a zone of inhibition against *E. coli* (Chakraborty et al., 2017).

This study aimed to explore the antibacterial activity of supernatant extracts of endophytic bacteria isolated from C. ternatea roots, specifically from the genus Bacillus, Pseudomonas, and Azomonas A and Azomonas B. The antibacterial activity was tested against two pathogens. The



antibacterial activity was tested against two important pathogens, *Staphylococcus aureus* and *Escherichia coli*, which are common causes of human infections. Through a series of antibacterial activity tests, including Disc Diffusion Assay, Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), and Time-Kill testing, this study is expected to provide scientific evidence regarding the antibacterial potential of the supernatant extract of *C. ternatea* endophytic bacteria. The results of this study are expected to contribute to the development of alternative sustainable natural resource-based medicine and support efforts to preserve biodiversity in Indonesia.

MATERIALS AND METHODS

Research Design

The research conducted is included in the type of descriptive and experimental research. Experimental research includes the treatment of potential endophytic bacterial supernatant extracts of *C. ternatea* roots made into several concentration series for antibacterial activity testing. While descriptive research was conducted to describe the characteristics and number of endophytic bacteria from *C. ternatea* roots that were successfully isolated and grew on the media.

Research Sampling

Root samples of *C. ternatea* were taken from one of the house yards in the Gegerkalong, KPAD, Bandung. The main root 10 cm long was cut and brought to the laboratory for isolation. The roots were cleaned with running water for 15 minutes, then immersed in 70% alcohol for 1 minute, 5.25% sodium hypochlorite solution for 5 minutes, and again immersed in 70% alcohol for 1 minute. After the sterilization process, the roots were cut into small pieces and planted sterile in Nutrient Agar (NA) media containing nystatin to prevent fungal contamination. The medium was incubated at 37°C for 48 hours.

Endophytic Bacteria Selection

Furthermore, the antagonist test is carried out, this test is carried out for the selection of endophytic bacterial isolates that have the ability to produce antibacterial compounds against pathogenic bacteria. The test was carried out by point inoculation method of each endophytic bacterial isolate on the surface of MHA media that had been homogenized with test bacteria (Widowati et al., 2019).

Growth Curve

One ose of potential bacterial colonies was inoculated on 10 ml of NB medium and incubated for 24 hours on an incubator shaker. After 24 hours, 1 ml of potential bacterial culture was transferred into a new erlenmeyer containing 100 ml of NB medium and then incubated for 24 hours at 37°C with a speed of 121 rpm to measure turbidity (OD) using a UV-VIS spectrophotometer with a wavelength of 600 nm every 1 hour interval for 24 hours until the initial stationary phase was found (A'yun & Nugraheni, 2023b).

Extraction

After knowing the initial stationary phase for harvesting time, one ose of potential bacterial isolates was inoculated on sterile NB medium and then incubated on a shaker incubator until it reached the stationary phase according to the growth curve of the bacterial isolate. Then the bacterial culture was centrifuged at 5000 rpm for 15 minutes. The centrifuged supernatant was



transferred into a separating funnel and then extracted using ethyl acetate (Romadhonsyah et al., 2024) with a ratio of 1: 1 and homogenized by shaking for 5-10 minutes until an organic layer (ethyl acetate) was formed. The organic layer was separated from other layers that were insoluble by ethyl acetate, then concentrated using a rotary evaporator at 50 °C (Uche-Okereafor et al., 2019). The crude extract was then dissolved in 10% DMSO as stock and diluted in concentration series of 2.5 mg/ml, 10 mg/ml and 40 mg/ml.

Antibacterial Activity Test

Each concentration series was subjected to the DDA test, where sterile paper disks were placed on Mueller Hinton Agar (MHA) media that had been inoculated with test bacteria (S. aureus and E. coli). Then 10 μ l of extracts of various concentrations, positive control (Chloramphenicol 30 mg/ml) and negative control (DMSO 10%) (Nugraha et al., 2023) were dripped onto the disc. After incubation for 24 hours at 37°C, the diameter of the inhibition zone was measured to determine the inhibition category.

Zone of inhibition
$$=\frac{(DV - DC) + (DH - DC)}{2}$$

Description:

DV: Vertical diameter of clear zone

DC: Disc diameter

DH: Horizontal diameter of clear zone

In addition to the DDA test, MIC and MBC tests were also conducted. The MIC test was carried out with the two-fold serial dilution method on a 96-well microtiter plate. The 1st well was filled with positive control which is 100 µl of test bacteria plus 100 µl of chloramphenicol for each test bacteria (S. aureus and E. coli). The 2nd well is filled with negative control, namely 100 µl of test bacteria plus 100 µl of 10% DMSO. The 3rd to 12th wells were filled with treatments. The concentration of supernatant extract used ranged from 0.078 mg/ml to 40 mg/ml (Nxumalo et al., 2020). In addition to observations based on the clarity of the visible solution, OD values were also observed using a UV-VIS spectrophotometer based on the results of the MIC test (Warokka et al., 2016). The MBC test was carried out using the agar plate method by inoculating 100 µl from the four closest wells of the lowest concentration that showed positive results of the MIC test into the MHA medium and then each medium with different concentrations was incubated for 24 hours at 37°C. Finally, antibacterial activity was tested with the Time Kill Assay method used which is a modification of the research of Israyilova et al. (2022). The tested extract levels include 0xMIC, 1xMIC, 2xMIC, and 4xMIC. Test bacterial inoculum with a concentration of about 1 x 10⁸ CFU/ml was put into tubes containing extracts at 1xMIC and 2xMIC levels, as well as negative control tubes without extracts. Samples were taken at 0, 4, 8, 12, and 24 hours for serial dilution. Colony counting was done by the drop plate method, by dripping 10 µl of sample 10 times on MHA medium. Petri dishes were incubated at 37°C for 24 hours, then the growing colonies were counted to determine the number of bacteria (CFU/ml). A curve was created based on the number of colonies (CFU/ml) on the Y axis and sampling time (hours) on the X axis.



RESULT AND DISCUSSION

Based on the identification results, two isolates of the genus *Azomonas*, one isolate of the genus *Bacillus* and one isolate of the genus *Pseudomonas* were obtained. In the DDA test, no supernatant extract was found that did not produce an inhibition zone, or in other words, all supernatant extracts of potential endophytic bacteria from *C. ternatea* roots showed antibacterial activity against both test bacteria.

Table 1. DDA Test Results against S. aureus

No	Potential Isolate	Concentration	Inhibition Zone Diameter (mm) ± SD
1	Bacillus	40 mg/ml	4.375 ± 0.176
		10 mg/ml	2.375 ± 0.176
		2.5 mg/ml	0.5 ± 0.707
2	Pseudomonas	40 mg/ml	6.75 ± 0.353
		10 mg/ml	3.625 ± 1.237
		2.5 mg/ml	0.875 ± 1.237
3	Azomonas B	40 mg.ml	4.125 ± 0.176
		10 mg/ml	2.75 ± 0
		2,5 mg/ml	2 ± 0.353
4	Chloramphenicol	30 mg/ml	12.24 ± 0.12

Table 2. DDA Test Results Against E. coli

No	Potential Isolate	Concentration	Inhibition Zone Diameter (mm) ± SD
1	Azomonas A	40 mg/ml	3.187 ± 0.618
		10 mg/ml	2.687 ± 0.088
		2.5 mg/ml	0.187 ± 0.265
2	Azomonas B	40 mg/ml	1.25 ± 0.707
		10 mg/ml	1 ± 0.883
		2.5 mg/ml	0.75 ± 1.06
4	Chloramphenicol	30 mg/ml	11.42 ± 0.45

Seen in Table 1. The highest inhibition zone diameter against the test bacteria *S. aureus* was produced by the supernatant extract of the genus *Pseudomonas* with a concentration of 40 mg/ml which was able to form an inhibition zone with a diameter of 6.75 mm. While in Table 2. shows the DDA test against *E. coli* bacteria, the largest concentration of inhibition is in the supernatant extract of the genus *Azomonas* A with a concentration of 40 mg/ml which is 3.187 mm. The inhibition zone formed is caused by the presence of antibacterial compounds, these compounds are thought to be flavonoids contained in *C. ternatea* root endophytic bacteria. Flavonoid compounds are known to have the ability to inhibit bacterial growth by disrupting the permeability of bacterial cell walls, namely by denaturing bacterial cell proteins and damaging the cytoplasmic membrane in bacteria which causes the leakage of important metabolites and activates the bacterial enzyme system. This damage to the cytoplasmic membrane causes nucleotides and amino acids to seep out and the entry of active ingredients causes the death of the test bacteria. In cytoplasmic membrane damage, H⁺ ions from phenol compounds and their derivatives such as flavonoids will attack polar compounds or phosphate groups so that phospholipid molecules will break down into glycerol, carboxylic acid and phosphoric acid. This causes phospholipids to be

unable to maintain the shape of the cytoplasmic membrane so that bacteria experience growth resistance and even death. Beta rings and -OH groups on flavonoids are thought to be the structures responsible for inhibiting the growth of test bacteria (Handayani, 2021).

The difference in diameter results in each supernatant extract is caused by the level of strength of each endophytic bacteria in inhibiting the growth of test bacteria. The structure of the test bacteria is also one of the factors why the resulting diameter is different. This is because the performance of antibacterial compounds is influenced by the structure of the bacterial cell wall. Gram-negative bacteria have cell walls that contain many lipids and little peptidoglycan, and have an outer membrane that is bilayer (selective defense) (Oktasila et al., 2020). The outer membrane of Gram-negative bacteria itself consists of phospholipids composed of lipid A which is non-polar. Antibacterial compounds present in *C. ternatea* root endophytic bacterial extracts such as flavonoids and saponins are polar compounds that are difficult to penetrate the non-polar lipid layer in Gram-negative bacterial cell walls. In this study, there was no extract concentration that had an inhibition zone diameter equal to or greater than 30 mg/ml chloramphenicol (positive control), but the results of the inhibition zone formed from the crude extract of the supernatant of *C. ternatea* root endophytic bacteria increased with each additional concentration.

After obtaining the inhibition zone from the DDA test, further tests were carried out, namely the Minimum Inhibitory Concentration (MIC). The MIC test was conducted to determine the minimum concentration of supernatant extract of endophytic bacterial isolates to inhibit the growth of test bacteria.

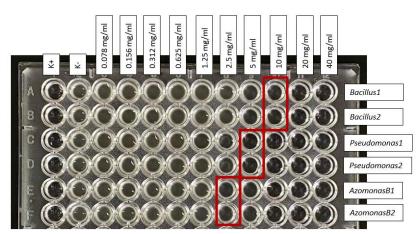


Figure 1. MIC test results against *S. aureus* characterized by the appearance of the medium in the test wells that is the same or close to the medium in the positive control (clear)

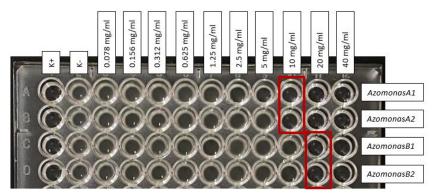


Figure 2. MIC test results against *E. coli* is characterized by the appearance of the medium in the test wells that is the same or close to the medium in the positive control (clear)



The MIC test begins with the comparison of bacterial suspensions in Mueller Hinton Broth (MHB) medium with a turbidity standard of 0.5 McFarland. MIC test results can be observed from changes in media turbidity in each well that has been solvent, supernatant extract and test bacterial inoculum after 24 hours of incubation.

MIC results against the test bacteria *S. aureus* showed MIC values in isolates of the genus *Bacillus*, *Pseudomonas*, and *Azomonas* B were 10 mg/ml, 5 mg/ml, and 2.5 mg/ml, respectively (Figure 1). While the MIC values against *E. coli* in *Azomonas* A and *Azomonas* B were 10 mg/ml and 20 mg/ml, respectively (Figure 2). The results of the MIC test using a 96-well microtiter proved the presence of inhibition of bacterial growth in each treatment of bacterial supernatant extract of endophyte extract of *C. ternatea* root. The MIC value also shows that each bacterium has different growth. However, further tests are needed in order to However, further tests are needed to show clearer inhibition of bacterial growth. Therefore, it is necessary Minimum Bactericidal Concentration (MBC) test is also needed to determine the value of the concentration of the supernatant extract of endophytic bacteria in killing 99% of the test bacteria.

The MBC test was conducted by growing the inoculum in the 96-well microtiter wells onto the MHA medium. The inoculum was then incubated for 24 hours at 37°C. The MBC test results are indicated by the absence of growth of test bacterial colonies on MHA medium (**Figure 3**). This shows that the supernatant extract of endophytic bacteria from *C. ternatea* roots has been able to cause the death of 99% of the test bacteria as a whole.

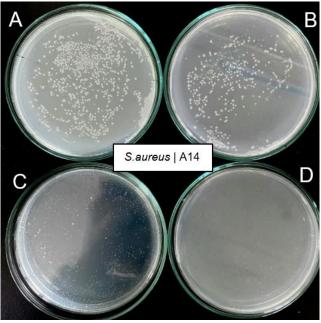


Figure 3. MBC test results of isolate *Azomonas* B against *S. aureus* that characterized by the absence of growth of test bacterial colonies on media.

(A) concentration 5 mg/ml, (B) concentration 10 mg/ml, (C) concentration 20 mg/ml, (D) concentration 40 mg/ml

MBC results against the test bacteria *S. aureus* showed MBC values in isolates of the genus *Bacillus*, *Pseudomonas* and *Azomonas* B were 40 mg/ml, 20 mg/ml, and 20 mg/ml, respectively (Figure 2). While the MBC value against *E. coli* in *Azomonas* A is 40 mg/ml and *Azomonas* B is above 40 mg/ml. The MIC and MBC values obtained in this study are used as the basic value to

determine the concentration in the next antibacterial activity test, namely the Time-Kill Assay. The values used for Time-Kill are 1 x MIC, 2 x MIC and 4 x MIC.

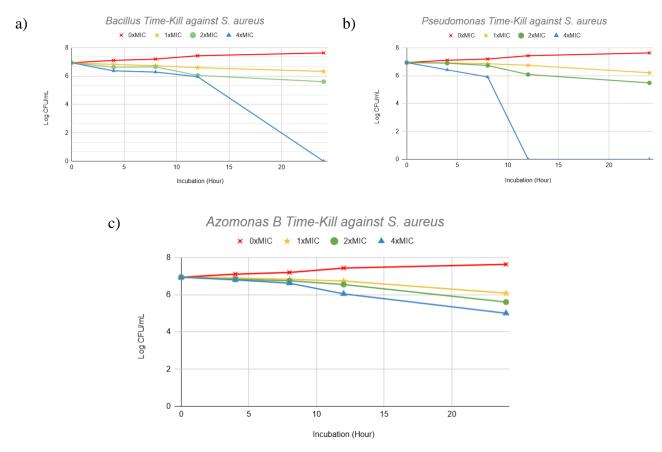


Figure 4. Time and Concentration for Endophytic Bacteria to Kill S. aureus Formed by The Time Kill Curve.
a) Bacillus, b) Pseudomonas, c) Azomonas B

Based on the data in Figure 3a. it is clear that the growth of S. aureus bacteria at a concentration of 0xMIC (without the provision of Bacillus endophytic bacteria supernatant extract continues to increase over time from hour 0 to hour 24. Whereas in the administration of Bacillus endophytic bacteria extract concentrations with 1xMIC and 2xMIC concentrations, there was a decrease in the number of bacteria from time to time, but not yet to kill the test bacteria completely. At 4xMIC concentration, there was a zero point at the 24th hour. Based on the data in Figure 3b, it can be seen that the growth of S. aureus bacteria at a concentration of 0xMIC (without the provision of supernatant extract of *Pseudomonas* endophytic bacteria) continues to increase over time from hour 0 to hour 24. Whereas in the administration of *Pseudomonas* endophytic bacterial extract concentrations with 1xMIC and 2xMIC concentrations, there was a decrease in the number of bacteria from time to time, but not yet to kill the test bacteria completely. At 4xMIC concentration, there was a zero point at the 12th hour. Based on the data in Figure 3c, it can be seen that the growth of S. aureus bacteria at a concentration of 0xMIC (without the provision of supernatant extract of Azomonas B endophytic bacteria) continues to increase over time from hour 0 to hour 24. Meanwhile, the concentration of Azomonas B endophytic bacteria extract with 1xMIC, 2xMIC and 4xMIC concentrations showed a decrease in the number of bacteria from time to time, but not yet to kill the test bacteria completely. This is also in accordance with the results obtained in the previous MBC test, where Azomonas B extract can kill all S. aureus bacteria at a concentration of 20 mg/ml or 8 times the MIC value.



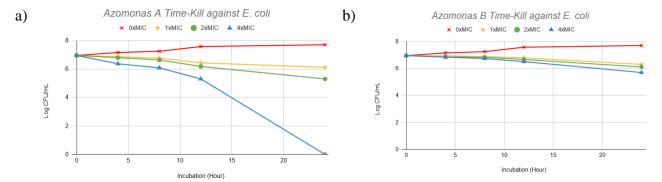


Figure 5. Time and Concentration for Endophytic Bacteria to Kill *E.coli* Formed by The Time Kill Curve.

Azomonas B

Azomonas B**

Based on the data in Figure 4a, it can be seen that the growth of *E. coli* bacteria at a concentration of 0xMIC (without the provision of supernatant extract of *Azomonas* A endophytic bacteria) continues to increase over time from hour 0 to hour 24. Meanwhile, the concentration of *Azomonas* A endophytic bacteria extract with 1xMIC and 2xMIC concentrations showed a decrease in the number of bacteria from time to time, but not yet to kill the test bacteria completely. At a concentration of 4xMIC, there was a zero point at hour 24. Based on the data in Figure 4b, it is clear that the growth of *E. coli* bacteria at a concentration of 0xMIC (without the provision of supernatant extract of *Azomonas* B endophytic bacteria) continues to increase over time from hour 0 to hour 24. Whereas in the administration of *Azomonas* B endophytic bacterial extract concentrations with 1xMIC, 2xMIC, and 4xMIC concentrations, there was a decrease in the number of bacteria from time to time, but not yet to kill the test bacteria completely. This is in accordance with the results obtained in the previous MBC test, where the extract of *Azomonas* B isolate could not kill all bacteria at a concentration of 40 mg/ml (4xMIC).

CONCLUSIONS

The largest inhibition zone in the supernatant extract of Bacillus, Pseudomonas and Azomonas B isolates against S. aureus was 4.375 mm, 6.75 mm, and 4.125 mm, respectively. While the diameter of the largest inhibition zone formed in the supernatant extract of Azomonas A and Azomonas B isolates against E. coli were 3.187 mm and 1.25 mm, respectively. The minimum concentration of supernatant extract of C. ternatea root endophytic bacteria that can inhibit the growth of S. aureus by supernatant extract of Bacillus, Pseudomonas and Azomonas B isolates are 10 mg/ml, 5 mg/ml, and 2.5 mg/ml, respectively. While against E. coli by supernatant extracts of Azomonas A and Azomonas B isolates were 10 mg/ml and 20 mg/ml, respectively. The minimum concentration of supernatant extract of *C. ternatea* root endophytic bacteria capable of killing 99% of S. aureus bacteria by Bacillus, Pseudomonas and Azomonas B supernatant extracts were 40 mg/ml, 20 mg/ml and 20 mg/ml, respectively. The lowest concentration killing E. coli by Azomonas A was 40 mg/ml. While Azomonas B isolates have not been able to kill E. coli bacteria at a concentration of 40 mg/ml. The value of concentration and incubation time that is effective in killing the growth of S. aureus bacteria is 4xMIC with 24 hours incubation time by Bacillus isolate extract, and 4xMIC with 12 hours incubation time by *Pseudomonas* isolate extract. While to reduce the growth of *E.* coli is 4xMIC with an incubation time of 24 hours by the bacterial extract of Azomonas A isolate.

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