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ISOLATION AND IDENTIFICATION OF ENDOPHYTIC BACTERIA FROM ROOTS AND STEMS OF GREEN BETEL PLANT (PIPER BETLE L.) AND EVALUATION OF THEIR ANTIBACTERIAL ACTIVITY

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ABSTRACT

The green betel plant (Piper betle L.) is known to possess antibacterial activity due to its secondary metabolite content, such as tannins, saponins, flavonoids, terpenoids, polyphenols, and steroids. This study aims to determine the characteristics and antibacterial activity of endophytic bacteria isolated from the roots of the green betel plant (Piper betle L.) against Staphylococcus aureus and Escherichia coli. The research employed an experimental method involving bacterial isolation, macroscopic and microscopic identification, as well as antibacterial activity testing of the endophytic bacteria against S. aureus and E. coli using the Kirby-Bauer disk diffusion method. Furthermore, molecular identification of the endophytic bacteria was conducted using the 16S rRNA gene through the Polymerase Chain Reaction (PCR) method. The macroscopic morphological characteristics of the isolated endophytic bacteria showed round, flat-raised colonies with curved edges and a clear white appearance. Microscopic observation through Gram staining revealed that the bacteria were rod-shaped (bacilli) and Gram-positive. The antibacterial activity test showed that the isolate produced an inhibition zone of 11.45 mm against Staphylococcus aureus, which is categorized as weak and indicates that the bacteria have a narrow-spectrum antibacterial effect. Meanwhile, no antibacterial activity was observed against Escherichia coli. It can be concluded that the pure isolates of endophytic bacteria from the roots and stems of the green betel plant belong to the Gram-positive group and exhibit antibacterial activity only against Staphylococcus aureus with a weak inhibitoryeffect

Keywords : Piper betle L; Antibiotic; Gen 16S rRNA

INTRODUCTION

The green betel plant (*Piper betle L.*) is a medicinal plant with potential as a source of endophytic bacteria. It has long been used in traditional medicine due to its content of secondary metabolites such as tannins, saponins, flavonoids, terpenoids, polyphenols, and steroids, which are known to have antibacterial properties. Compounds such as tannins exhibit antimicrobial and antifungal effects by inhibiting bacterial growth. The suggested antibacterial mechanism includes damaging and penetrating the bacterial cell wall and precipitating bacterial proteins, ultimately leading to cell death (Windari et al., 2025).

The continuous use of medicinal plants may lead to overexploitation, thereby requiring the discovery and development of new antibacterial agents by utilizing endophytic bacteria from medicinal plants (Safira et al., 2017). The use of endophytic bacteria is an innovative approach to obtaining antibacterial compounds without having to directly extract them from the plants (Kusumawati et al., 2014). The presence of endophytic bacteria within plant tissues is known to promote plant growth and act as biological control agents. Their ability to penetrate plant tissues is

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facilitated by extracellular enzymes, such as cellulase, produced by the bacteria. After penetration, endophytic bacteria colonize plant tissues and inhibit the growth of pathogenic bacteria through mechanisms such as space and nutrient competition (Pal A, Chattopadhyay A & Paul, 2012).

Endophytic bacteria are microorganisms that live within plant tissues and establish mutualistic symbiosis with their hosts. They are capable of producing secondary metabolites in the form of bioactive compounds similar to those produced by the host plant (Yandila et al., 2018). The advantages of producing bioactive compounds from endophytic microorganisms include ease of cultivation, shorter life cycles compared to plants, and the ability to produce large quantities of bioactive substances (Zulkifli, L., Jekti, DSD., Lestari, N., and Rasmi, DAC., 2016). Therefore, using endophytic bacteria can reduce the need for large-scale harvesting of plant materials, which often takes years to mature. Obtaining active compounds from plants requires more time and complex processes than extracting them from associated endophytic bacteria (Kusumawati et al., 2014).

According to research by (Safira et al., 2017),isolation of endophytic bacteria from green betel plants (P. betle L.) was successful, resulting in 14 pure isolates. Antibacterial screening of these isolates against four pathogenic bacteria showed that three isolates (AS1, BS1, and BS2) had potential as sources of new antibacterial agents, particularly against S. aureus. Additionally, research by Soni Muhsinin et al. (2019) confirmed that endophytic bacteria can be isolated from green betel plants. Antibacterial testing using the microdilution method revealed a Minimum Inhibitory Concentration (MIC) of 1024 ppm for isolates ID1 and ID2, and 16384 ppm for ID7 (Muhsinin et al., 2019).

This study was conducted to characterize and evaluate the antibacterial activity of endophytic bacterial isolates from the roots and stems of green betel plants (Piper betle L.). To determine the species of endophytic bacteria with antibacterial potential, molecular analysis of the 16S rRNA gene was conducted using Polymerase Chain Reaction (PCR), enabling species-level identification.

RESEARCH METHODS

This research was conducted from June to September 2024 at the Microbiology Laboratory, Faculty of Pharmacy, Universitas Perintis Indonesia, and the Microbiology Laboratory, Faculty of Medicine, Universitas Andalas, Padang.

Tools and Materials

Tools

Mask, gloves, Erlenmeyer flask, measuring cylinder, test tubes, test tube rack, dropper pipette, analytical balance, knife, Petri dishes, inoculation loop, tweezers, Bunsen burner, refrigerator, laminar air flow, autoclave, microtubes, micropipette, vernier caliper, label paper, plastic wrap, aluminum foil, tissue, cotton, gauze, light microscope, glass slides, newspaper, spirit lamp, Eppendorf tubes, spin column tubes, thermoblock, PCR machine, and electrophoresis apparatus.

Materials

Root and stem samples of green betel plant (*Piper betle L.*), test bacteria Escherichia coli and Staphylococcus aureus, sterile distilled water, 96% ethanol, 0.9% and 1% NaCl, 1% H_2SO_4, 1.175% $BaCl_2 \cdot 2H_2O$, iodine, fuchsin,

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Nutrient Agar (NA) medium, crystal violet solution, 5.25% Na-hypochlorite, chloramphenicol disc, Lugol's solution, safranin solution, 16S rRNA primers Forward (5' CCAGCAGCCGCGGTAATACG 3') and Reverse (3'-ATCGG(C/T)TACCTTGTTACGACT TC 5'), GelRed, 1 Kbp DNA ladder, loading dye, agarose powder, and TBE buffer.

Research Procedures

Sample Collection and Identification

The sample used in this study was the root of the green betel plant (*Piper betle L.*). The sample was collected from Lubuk Minturun, Koto Tangah District, Padang City, West Sumatra. Botanical identification of the sample was carried out at the Herbarium of the Faculty of Mathematics and Natural Sciences, Universitas Andalas, Padang.

Sterilization of Equipment and Preparation of Bacterial Culture Media

a. Equipment Sterilization

All equipment was first washed and dried. Petri dishes were wrapped in newspaper, test tubes and droppers were sealed with cotton and wrapped individually in newspaper. They were sterilized in a hot air oven at 160°C for 1 hour. The mouths of Erlenmeyer flasks and measuring cylinders were sealed with cotton and wrapped in newspaper, then sterilized in an autoclave at 121°C for 15 minutes at 15 psi. Tweezers, inoculation loops, and slides were flame-sterilized using a spirit lamp.

b. Preparation of Nutrient Agar (NA)

Nutrient Agar was prepared by dissolving 20 g of NA in 1000 mL of sterile distilled water in an Erlenmeyer flask. The solution was heated over a stove while stirring for 10–15 minutes and then sterilized in an autoclave at 121°C for 15 minutes (Retnowati et al., 2011).

Isolation and Purification of Endophytic Bacteria from Roots and Stems of Green Betel

The root samples were washed under running water and cut into 1–3 cm segments. Surface sterilization was carried out in stages: soaked in 96% ethanol for 30 seconds, then in 5.25% Na-hypochlorite for 30 seconds, followed by three rinses in 96% ethanol. The sterilized samples were then plated on Nutrient Agar and incubated until bacterial colonies appeared. Pure cultures were obtained by streaking individual colonies onto new Nutrient Agar plates and incubating at 37°C for 24 hours. Once purified, the endophytic bacteria were transferred to slant NA medium (Kusumawati et al., 2014).

Identification of Endophytic Bacteria

a. Macroscopic Observation

Colony shape (top view): round, oval. Colony elevation (side view): flat, raised. Colony margin (top view): undulate or spreading. Pigmentation: some bacteria produce pigments such as white, yellowish-white, or milky (Faradiska, 2012).

b. Microscopic Observation

The morphology of bacterial cells was observed using Gram staining. A drop of sterile distilled water was placed on a glass slide, and a bacterial colony was picked with a loop and spread evenly. After air-drying, the slide was heat-fixed. Staining was conducted as follows: crystal violet (Gram A) for 1 minute, rinse; iodine solution (Gram B) for 2 minutes, rinse; 96% ethanol (Gram

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C) for 30 seconds, rinse; safranin (Gram D) for 30 seconds, rinse. The slide was then observed under a microscope at high magnification (Waluyo, 2010).

Antibacterial Activity Assay

a. Preparation of 0.5 McFarland Standard

A mixture of 10 mL of 1% H_2SO_4 and 0.05 mL of 1.175% $BaCl_2 \cdot 2H_2O$ was prepared in an Erlenmeyer flask and shaken until a turbid standard solution was formed, to be used as a reference for bacterial suspension turbidity (Sihombing et al., 2018).

b. Preparation of Test Bacterial Suspension

Test bacteria (E. coli – Gram-negative, S. aureus – Gram-positive) were inoculated and then transferred (~1 loopful) into 5 mL of 0.9% NaCl solution to match the turbidity of the McFarland 0.5 standard. A 200 µL aliquot of this suspension was spread onto the surface of the agar medium.

c. Preparation of Endophytic Bacterial Suspension

Isolates were picked using an inoculation loop and suspended in 9 mL of 0.9% NaCl, then vortexed until homogeneous.

d. Antibacterial Activity Testing

A total of 200 µL of the test bacterial suspension (S. aureus and E. coli) was spread on Mueller Hinton Agar. Three sterile paper discs were placed on each plate, each disc treated with the endophytic bacterial suspension, with chloramphenicol disc used as a positive control. The plates were incubated at 37°C for 24 hours. Each test was performed in triplicate.

e. Observation and Measurement of Inhibition Zones

After 24 hours of incubation, clear zones around the paper discs were observed. The diameter of the inhibition zones was measured using a vernier caliper. The zone sizes were classified according to CLSI standards.

Table 1. Classification of Microbial Inhibition Response

Diameter of Inhibition Zone (mm)	Growth Inhibition Response
≤14	Weak
15-18	Moderate
≥19	Strong

Source :(Clinical Laboratory Standart Institute, n.d.), 2013

6. Molecular Identification of Endophytic Bacteria

a. DNA Isolation

The endophytic bacterial isolate exhibiting the strongest antibacterial activity was taken from slant medium using a micropipette and transferred into a microtube containing 1 mL of Phosphate Buffer Saline (PBS). The sample was then centrifuged at 10,000 rpm for 5 minutes. After centrifugation, the supernatant was discarded, and 1 mL of TE buffer was added. The mixture was vortexed and then incubated in a heating block at 95°C for 10 minutes. Following incubation, the bacterial suspension was centrifuged again at 10,000 rpm for 5 minutes. The resulting supernatant, containing the extracted DNA, was transferred into a 1.5 mL microtube and stored in a freezer (Sihombing et al., 2018).

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b. Amplification of 16S rRNA Gene Using PCR

The isolated DNA was then subjected to PCR amplification, which involved three main steps: denaturation, annealing, and extension (Table 4), using specific 16S rRNA primers (Rahmani et al., 2006). The DNA amplification was carried out using Taq DNA Polymerase with the Go Taq Mastermix reagent.

Table 2. Components and mixture for 16S rRNA primer and sample DNA

Reagent	Volume (µL)
Template DNA	3 µL
Go Taq mastermix	25 µL
Forward Primer	1 µL
Reverse Primer	1 µL
Nuclease free water	20 µL
Total Volume	50 µL

Table 3. PCR Machine Cycle

Cycle Number	Temperature (°C)	Time
1	95	3 minutes
35	95 (denaturation) 55(annealing) 72(extension)	30 seconds 30 seconds 30 seconds
1	F extension 72	5 minutes
1	Cooling 12	Until completion

7. Agarose Gel Electrophoresis and Visualization

A 0.2% agarose gel was prepared by dissolving 0.2 grams of agarose powder in 20 mL of TE buffer. The mixture was then heated in a microwave for 1–2 minutes until completely dissolved and transparent. Afterward, 3 µL of GelRed was added to the solution, and the mixture was poured into a casting tray equipped with a comb. Once the gel solidified (±30 minutes), 5 µL of the PCR amplicon sample was loaded into the wells. To determine the size of the PCR amplification products, 3 µL of 1 Kbp DNA ladder and 3 µL of loading dye were loaded into the first well, followed by the amplified DNA samples in subsequent wells. The electrodes were connected to a power supply and the electrophoresis was run for 40 minutes. After the run, the power supply was turned off, and the gel was transferred to a UV transilluminator for visualization and documentation of the results (Sihombing et al., 2018).

8. Data Analysis

The data analysis employed both experimental and descriptive

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approaches. It included the results of isolation and identification of endophytic bacteria using molecular techniques based on rRNA and PCR, as well as the antibacterial activity test of the obtained endophytic bacterial isolates. All data were derived from direct observations, sample collection, and documentation throughout the research process, which served as the evidentiary basis for analysis.

RESULTS AND DISCUSSION

Sample Identification

The samples used in this study were roots (AK) and stems (BT) of the green betel plant (*Piper betle* L.), collected from Lubuk Minturun, Koto Tangah District, Padang, West Sumatra. The plant specimens were identified at the ANDA Herbarium to confirm that the samples used were indeed *Piper betle* L. Based on the identification results, the samples were confirmed to be green betel with the species name *Piper betle* L.

Macroscopic and Microscopic Identification of Endophytic Bacteria

Surface sterilization was carried out on both plant samples, followed by incubation on Nutrient Agar (NA) medium in petri dishes. Pure colonies of bacteria grown on NA media were then isolated and observed for morphological identification. The macroscopic characteristics of the isolated endophytic bacteria are presented below.

Table 4. Macroscopic Morphology Observation of Endophytic Bacteria

Macroscopic Characteristic	Results of Observation	
	Root (AK)	Stem (BT)
Shape	Circular	Circular
Surface	Raised, flat	Raised, flat
Margin	Curved	Curved
Color	Translucent white	Translucent white

Note: AK = Root, BT = Stem

Based on the table above, it can be seen that the morphological observations of endophytic bacteria showed circular shape, raised-flat surface, curved margin, translucent white, and yellowish-white colors. This is consistent with Faradiska (2012), who stated that endophytic bacterial colonies generally have a circular or oval shape. The colony color can be white, yellowish-white, or milky, with wavy and spreading margins (Faradiska, 2012).

Subsequently, the isolates were microscopically identified using Gram staining. The observations showed that all endophytic bacterial isolates were Gram-positive, indicated by purple-stained colonies in rod (bacillus) shape. The following table presents the Gram staining observations of the endophytic bacteria:

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Gram Staining of Endophytic Bacteria

The isolates were microscopically identified using Gram staining. The observations showed that all endophytic bacterial isolates were Gram-positive, indicated by purple-stained colonies in rod (bacillus) shape. The following table presents the Gram staining observations of the endophytic bacteria:

Table 5. Gram Staining Observations of Endophytic Bacteria

Isolate Code	Gram Staining	Shape
AK	Positive	Bacillus
BT	Positive	Bacillus

Note: AK = Root, BT = Stem

Based on Table 5, it can be observed that both AK and BT isolates are Gram-positive bacilli. Gram-positive bacteria are characterized by their ability to retain the primary stain, crystal violet, during Gram staining, resulting in a purple appearance under microscopic observation. This is due to the thick peptidoglycan layer in their cell walls, which binds the dye and is not disrupted during the alcohol decolorization step.

This structural composition allows Gram-positive bacteria to provide more effective protection against physical and pathogenic threats within the host tissues. In contrast, Gram-negative bacteria have a thinner peptidoglycan layer and a higher lipid content in their outer membranes, which makes them more susceptible to decolorization by alcohol. As a result, they lose the crystal violet stain and appear red after being counterstained with safranin (Imawati, 2015).

Endophytic bacteria are more frequently found among Gram-positive groups, potentially due to their lower lipid content (1–4%) compared to Gram-negative bacteria, which contain about 11–12% lipids in their cell walls (Pelczar, M. J. dan Chan, 1986).

Antibacterial Activity of Endophytic Bacterial Isolates

The antibacterial activity of endophytic bacterial isolates was tested to evaluate their inhibitory effects against two pathogenic bacteria: the Gram-positive *Staphylococcus aureus* and the Gram-negative *Escherichia coli*. These bacteria are among the most common causative agents of infection. *Staphylococcus aureus* is part of the normal human skin flora, while *Escherichia coli* is normally found in the intestinal tract but can cause intestinal infections under certain conditions, including diarrhea due to its ability to damage mucosal cells (Brooks, G. F., S. J. Butel, 2001).

The antibacterial activity assay was performed using the agar diffusion method, with endophytic bacteria isolated from the roots and stems of green betel (*Piper betle* L.) tested against *S. aureus* and *E. coli*. The results are presented in the following table.

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Table 6. Antibacterial Activity of Endophytic Bacterial Isolates from Roots and Stems of Green Betel (Piper betle L.)

Isolate Code / Control	Inhibition Zone Diameter (mm)							
	Staphylococcus aureus			Average	*Escherichia coli*			Average
	Replicate				Replicate			
	1	2	3		1	2	3	
AK	15,46	9,08	9,82	11,45	0	0	0	0
BT	13,58	12,76	13,7	13,34	0	0	0	0
Positive Control (Chloramphenicol)	31,6	32	31,02	31,54	30,02	28,3	28,36	28,89

Note: AK = Root, BT = Stem

Based on the table above, it can be seen from the average measurement results of the clear zone diameter that the antibacterial activity against the test bacterium *Staphylococcus aureus* from isolate AK was 11.45 mm and from isolate BT was 13.34 mm. Meanwhile, the antibacterial activity against *Escherichia coli* from both AK and BT isolates was 0.00 mm, indicating that the endophytic bacterial isolates fall into the weak inhibition category. According to the Clinical and Laboratory Standards Institute (CLSI), an inhibition zone diameter ≤14 mm is classified as weak, 15–18 mm as moderate, and ≥19 mm as strong inhibition. Therefore, the endophytic bacterial isolates were able to inhibit the growth of *S. aureus* but not *E. coli*, indicating that these isolates are narrow-spectrum bacteria.

This result suggests that the roots and stems of green betel plant (*Piper betle* L.) contain antibacterial compounds that can inhibit the growth of *Staphylococcus aureus*, although the inhibition strength is categorized as weak. According to Purwanto (2014), the formation of clear zones in the root and stem sections of green betel is due to the fact that roots are the primary entry point for endophytic bacteria into plant tissues, while no clear zones were observed in the leaves. The presence of these clear zones indicates that the endophytic bacteria produce bioactive compounds such as saponins, tannins, flavonoids, terpenoids, polyphenols, and steroids that function as antibacterial agents (Tan & Zou, 2001).

The mechanism of bacterial growth inhibition by secondary metabolites may involve disrupting the synthesis of bacterial cell wall components, increasing cell membrane permeability, which causes the loss of essential cellular components (Sepriana et al., 2017). Based on the average measurements, the inhibition zone diameter produced by the endophytic bacterial isolates was greater against the Gram-positive *Staphylococcus aureus*, indicating that the antibacterial activity of isolates from the roots and stems of *Piper betle* L. was more effective. Gram-positive bacteria have a cell wall structure with more peptidoglycan, fewer lipids, and the cell wall contains teichoic acids, which are water-soluble polymers that function as ion

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transporters. Because of their polar nature, Gram-positive cell walls are more polar than Gram-negative ones. Flavonoids and tannins are polar compounds, which allows them to more easily penetrate the polar peptidoglycan layers compared to the nonpolar lipid layers of Gram-negative bacteria. This explains why the inhibitory activity is higher against Gram-positive bacteria than against Gram-negative bacteria. The structural differences in the bacterial cell walls lead to different responses, with the BT isolate showing a larger average inhibition zone diameter compared to the AK isolate.

Molecular Identification of Endophytic Bacteria

Endophytic bacterial isolates from the roots (AK) and stems (BT) of the green betel plant (*Piper betle* L.) were identified using molecular analysis targeting the 16S rRNA gene to determine their bacterial species. Molecular identification of microorganisms involves four main stages: DNA isolation, PCR amplification, electrophoresis, and sequencing. DNA isolation was conducted using the boiling method, a simple technique involving physical disruption of bacterial cells through high-temperature heating (95–100°C). High-temperature heating increases cell wall permeability, allowing surrounding fluids and molecules to enter the cell and internal materials to exit. DNA is then separated and used as a template for the PCR process (Harvianti, 2017).

The extracted DNA was amplified using the PCR method with universal 16S rRNA gene primers. The PCR amplification process consists of the denaturation phase (separating DNA strands), annealing, and extension. The materials used include H_2O, Taq Master Mix, forward primer (338F), and reverse primer (1525R). The Master Mix acts as a ready-to-use solution containing DNA polymerase and other components for DNA amplification. The forward primer initiates synthesis from the 5’ to 3’ end, while the reverse primer initiates from the 3’ to 5’ end. The DNA template serves as a blueprint for the synthesis of a new DNA strand (Harvianti, 2017).

The next step is DNA electrophoresis, a technique used to measure the migration rate of charged particles in an electric field. This method takes advantage of the negative charge on molecules such as DNA. DNA, RNA, or proteins can be separated using this technique. When negatively charged molecules are placed in a medium such as agarose gel and an electric current is applied, the molecules will migrate from the negative pole to the positive pole (Gaffar, 2007).

Electrophoresis results for the root (AK) and stem (B T) isolates

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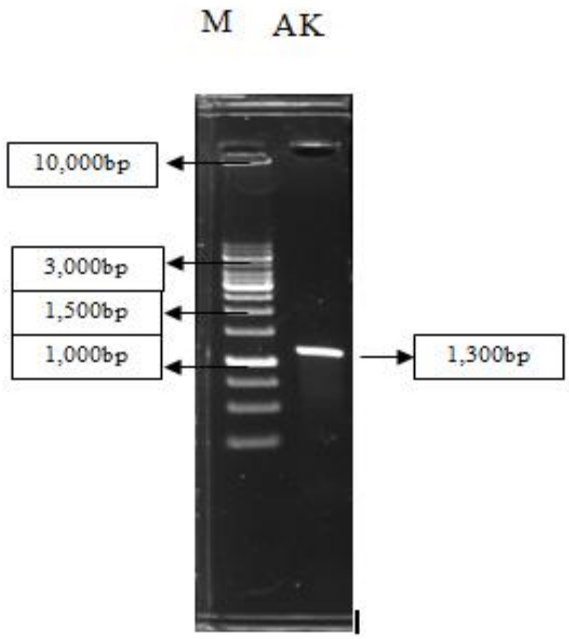

Figure 1. Electrophoresis result of the 16S rRNA gene amplification product of the endophytic bacterial isolate from root (AK), showing a DNA band size of approximately ±1300 bp. M: DNA marker (1 kb DNA ladder).

Figure 2. Electrophoresis result of the 16S rRNA gene amplification product of the endophytic bacterial isolate from stem (BT), showing a DNA band size of approximately ±1300 bp. M: DNA marker (1 kb DNA ladder).

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Based on the figures above, it can be observed that after the amplification process was visualized through electrophoresis, the result of the electrophoresis for the isolate with code AK in Figure 1 showed a clear band that was well separated and aligned with the 1300 bp marker. It is evident from the image that the DNA marker (M) used was a 1 kb DNA ladder, marked clearly at 1000 bp and 3000 bp. This indicates that the amplified gene fragment was approximately ±1300 bp in length, which corresponds to the expected size of the 16S rRNA gene (~1500 bp), suggesting that the bacterial amplification process was successful. The success of the PCR technique depends on the compatibility of the primers with the targeted isolate as well as the optimization during the PCR process (Rinanda, 2011).

Meanwhile, the electrophoresis result of the isolate with code BT in Figure 2 showed no visible band, indicating that no gene fragment of the expected size (~1500 bp) was amplified. This suggests that the amplification process for the bacterial DNA was unsuccessful. This result highlights the need for further optimization in both the DNA extraction process and the PCR reaction conditions in order to effectively amplify the 16S rRNA gene from the endophytic bacterial isolate obtained from the stem (BT) of Piper betle L.

CONCLUSION

1. Two endophytic bacterial isolates were successfully obtained from the root (AK) and stem (BT) of green betel plant (Piper betle L.). The morphological identification revealed that the colonies were round, raised-flat, with lobate margins, and were clear white to yellowish-white in color. Microscopic identification through Gram staining showed that the isolates were rod-shaped and Gram-positive.
2. The endophytic bacterial isolates from the root and stem of Piper betle L. demonstrated antibacterial activity against *Staphylococcus aureus*, with average inhibition zone diameters of 11.45 mm and 13.34 mm, respectively, which are classified as weak. These isolates are considered narrow-spectrum antibacterial agents.

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