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Research Article

Diversity and potential of local *Actinobacteria* from Bedengan Forest for sustainable antibiotic solutions

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ABSTRACT

Natural antibiotics may offer a solution to the growing issue of pathogenic bacterial resistance caused by the widespread use of synthetic antibiotics. This study investigates the diversity and potential of *Actinobacteria* from the rhizosphere of Pinus in the Selorejo Bedengan Forest, Malang City, which has been exposed to synthetic agricultural chemicals for an extended period. Soil samples were collected and analyzed for various environmental factors, including pH, moisture, organic matter, and light intensity. A total of seven *Actinobacteria* isolates were identified, belonging to the genera *Streptomyces* and *Nocardia*. The diversity of these isolates was assessed using the Shannon-Wiener index, yielding a moderate diversity value (H' = 2.74). Antibacterial activity was tested against *Escherichia coli* and *Shigella dysenteriae* using the pitting method. The results indicated that isolates AC5 and AC7 exhibited inhibition zones of ≥11 mm and ≥12 mm, respectively, categorizing them as having strong antibacterial activity. These findings highlight the significant potential of local *Actinobacteria* as candidates for the production of natural antibacterial compounds to combat pathogenic bacteria. This research not only supports the development of more environmentally friendly antibiotic alternatives but also encourages the sustainable use of local microbial resources.

Keywords: *Actinobacteria*, Bedengan Forest, natural antibiotics, bacterial resistance, sustainability

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INTRODUCTION

Pathogenic bacteria are among the main agents causing infections that threaten public health worldwide, especially in Indonesia (Okparasta et al. 2020). Efforts to prevent and treat these infections have long involved the use of synthetic antibiotics. However, the widespread and prolonged use of these antibiotics in both humans and animals has had serious consequences, leading to antibiotic resistance in pathogenic bacteria (Pisestyani et al. 2023).

The problem of antibiotic resistance is becoming increasingly urgent, highlighting the need to explore natural antibiotic sources. Research by Manilal et al. (2020) shows that one of the most promising approaches to overcome the problem of bacterial resistance is the use of natural compounds as an alternative to synthetic antibiotics. Not only do they have lower side effects, but the use of natural materials is also considered more environmentally friendly. In the study, researchers used plant extracts to obtain antibacterial bioactive compounds.

However, a new challenge arises next is that the biomass will be quickly depleted if produced on a large scale, and ultimately leading to a decline to a decline in plant abundance.

As an innovative approach, research has shifted its focus to specific microbes, such as *Actinobacteria*, which are known to be natural producers of antibiotics. Several studies have been conducted to isolate and characterise antibiotic-producing microbes from plant rhizospheres. Research by Janatiningrum et al. (2024) showed that 50% of *Actinobacteria* isolated from the rhizosphere of *Pometia pinnata* exhibited antimicrobial activity for *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Escherichia coli*, and 20.8% inhibited fungal growth. Another study conducted by Andini et al. (2016) successfully isolated *Actinobacteria* from the rhizosphere soil of the Suranadi Forest in West Lombok, demonstrating potential to inhibit the growth of the pathogenic bacterium Methicillin-Resistant *Staphylococcus aureus* (MRSA).

The presence of *Actinobacteria* can contribute to the production of natural compounds such as antibiotics with a biomass that is easily and rapidly propagated (Pratiwi, 2018). The plant rhizosphere has been recognized as an environment rich in organic compounds that can be utilized by soil microbes, such as *Actinobacteria*, in producing bioactive compounds (Michael et al. 2017). The symbiotic relationship between plant roots and rhizosphere soil microbes provides a strong conceptual foundation for this study.

Most research related to the exploration of *Actinobacteria* as potential candidates for producing antibacterial compounds has been conducted in lush tropical forests, far from agricultural fields, to avoid contamination by synthetic agricultural chemicals. Andini et al. (2016) reported that forest soil located far from agricultural land is rich in potential *Actinobacteria*. However, no studies have been conducted to isolate *Actinobacteria* from forests adjacent to agricultural land that have been exposed to synthetic chemicals from fertilizers, pesticides, herbicides, fungicides, and other agrochemicals for years. Therefore, it is intriguing to isolate *Actinobacteria* with antibacterial potential that also exhibit resistance to synthetic agricultural chemicals. Research by Sriragavi et al. (2023) demonstrated that *Streptomyces* sp. strain BS-16, isolated from bamboo rhizosphere soil, exhibits remarkable antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis*, and *Streptococcus pyogenes*.

Pine (*Pinus sylvestris*) contains phenolic compounds like stilbenes and flavonoids, which have demonstrated antibacterial activity. *Actinobacteria*, particularly those from the genus *Streptomyces*, are prolific producers of antibiotics and other bioactive compounds. These bacteria are often found in symbiotic relationships with plants, including pines, where they contribute to the plant's defense mechanisms by producing antimicrobial substances (van der Meij et al. 2017 & Świecimska et al. 2023). Actinobacteria isolated from Scots pine pollen have shown significant antibacterial and antifungal activities. These strains can inhibit pathogens like *Candida albicans* and various bacteria, suggesting their role in protecting the plant from pathogenic microbial threats (Axenov-Gribanov et al. 2016).

Based on this background, this research aims to explore pine rhizosphere *Actinobacteria* from Selorejo Bedengan Forest, Malang City, East Java, as a potential source of antibacterial agent's pathogens. The forest is adjacent to agricultural land, making it an interesting site to study the diversity and potential of these *Actinobacteria*, including their varying abilities to inhibit pathogenic bacteria. Through this approach, it is expected that superior isolates will be identified to help combat pathogenic bacteria and, at the same time, promote the sustainable use of natural resources in the field of microbiology.

METHODS

Location and Object of Research

This research focused on *Actinobacteria* isolated from the rhizosphere soil of Pinus in Bedengan Selorejo Forest, Malang City (coordinates: 7°56'15"S, 112°32'00"E; Figure 1). The study aimed to explore the diversity and potential of *Actinobacteria*. The research site was selected through a purposive sampling method due to its proximity to agricultural land that has been cultivated by local residents for decades. Microbial enumeration and observation were conducted at the Microbiology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya.

Actinobacteria Sampling

Samples were taken from the soil around the rhizosphere near the root system of plants as a composite at a depth of 5-10 cm using a 5 cm diameter soil drill and stored in plastic bottles. Each soil sample was a composite with three replicates (Andini et al., 2016; Mijic et al., 2021). In this study, soil physicochemical environmental parameters were measured, including 1) soil pH, 2) light intensity, 3) soil moisture, 5) total organic content in the soil, and 6) temperature.

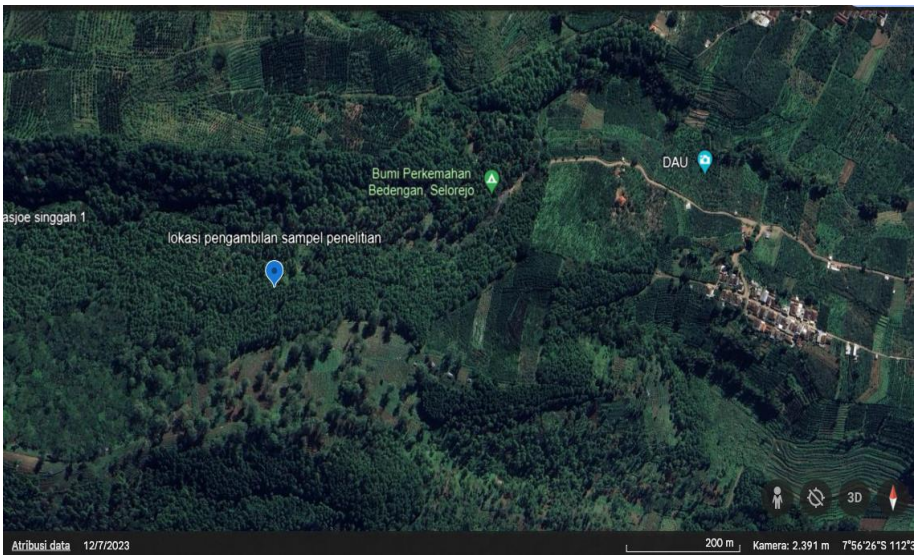

Figure 1. Location research

Sample Measurement and Observation

Soil pH was measured using an electrometric method with pH meter (Jenway) in the laboratory. Light intensity was measured using a luxmeter, while soil moisture was measured using the gravimetric method. Soil moisture and total organic matter were calculated by drying in an oven and then weighing, the values were then calculated using the appropriate formula (Cherlinka, 2024).

$$Dry\ weight = \frac{(\mathrm{Wt}-\mathrm{wt})}{(\mathrm{Wt}-\mathrm{w})} \text{ x } 100$$

Humidity (%) – 100 % - % dry weight
Description:
Wt : soil weight and cup weight before drying (g)
wt : soil weight and cup weight after drying (g)
w : cup weight (g)

Organic material (%) = $(a-b).(a)^{-1}$ x 100%
Description:
a : soil weight before burning (g)
b : soil weight after burning (g)

Isolation and Observation of *Actinobacteria*

A total of 1 g of soil sample was dissolved in 9 mL of physiological saline (NaCl 0.85%) followed by serial dilutions from 10^{-1} to 10^{-5} were made. The soil sample suspension was taken a 0.1 mL aliquot and inoculated on the surface of the Starch Casein Agar (SCA) medium in a Petri dish that supplemented with the antifungal agent nystatin using the pour plate method. The inoculated medium was incubated at 28°C for 7-8 days. The number of *Actinobacteria* colonies was determined using the Total Plate Count (TPC) method and subsequently transferred to a new SCA medium to obtain a single isolate (purification). The isolation of single colonies was performed using the dilution method (spread plate) (Pratiwi, 2018).

Actinobacteria isolates obtained from soil were characterized visually characterized through macroscopic and microscopic observations. Macroscopic observations included the shape, colour, size, edges, elevation, and mycellium of the colonies. Microscopic observations were made through gram staining and cell morphology analysis under a microscope include type of gram, cell shape, type of filament. Diversity of *Actinobacteria* analyzed using the Shannon-Wienner index (H') (Sunarto et al. 2015).

$$H' = -\sum_{i=1}^{n} pi \ln pi$$

H'= Shannon-Wienner index, S= number of types, Pi= proportion of the number of individuals of the i-th species to the total number of individuals. If H' < 1,0 then the diversity is low, 1,0 < H' < 3 then the diversity is moderate, H' > 3 then the diversity is high.

Screening and Testing the Potential of *Actinobacteria* in Producing Antibiotics

This study aims to isolate *Actinobacteria* with the ability to inhibit the growth of pathogenic bacteria through antagonistic tests against *Escherichia coli* and *Shigella dysenteriae*. However, the antagonistic tests have not been conducted against gram-positive bacteria due to the unavailability of pathogenic isolates at the Microbiology Laboratory of Brawijaya University. The antagonistic tests were conducted using the Completely Randomized Design (CRD) method, with three replications in each treatment. *Actinobacteria* isolates were prepared by subculturing on Starch Casein Broth (SCB) media with a volume of 50 mL and incubating at 28°C for 7 days. *Escherichia coli* and *Shigella dysenteriae* were grown in Nutrient Broth (NB) media until they reached a concentration of 10^7 cells/mL, then spread on petri dishes containing Nutrient Agar (NA) media and left in the refrigerator for 1 hour to solidify. The culture suspension of *Actinobacteria* isolates with a density of 10^7 cfu/mL was inoculated with 20 µL into wells with a diameter of ± 5 mm on Petri dishes that had been inoculated with pathogenic bacteria, then incubated at 28°C for 24 hours. The zone of inhibition (clear zone) formed around the wells was measured and was considered positive if the inhibition zone was ≥ 3 mm, indicating antimicrobial activity and the ability to produce antibiotics. Observational data were analyzed using statistical tests at a specific significance level (e.g., $p < 0.05$).

RESULTS AND DISCUSSION

Rhizosphere Environmental Factors of the Bedengan Forest

The analysis of composite soil from Selorejo Bedengan Forest, including pH value, moisture, light intensity, soil organic content, temperature, and total plate count (TPC), was presented in Table 1. The rhizosphere soil of Bedengan Forest in Malang has a pH value that tends to be acidic. Acidic soil pH conditions are influenced by microbial activity and the decomposition of complex organic matter. The main factor limiting the growth of *Actinobacteria* in forest soil is its low (acidic) pH.

Barka et al. (2016) stated that *Actinobacteria* are acid-intolerant and tend to be unable to survive if the pH is below 6. However, other studies show contradictory results. For example, research by Dorchenkova et al. (2022) reported that *Actinobacteria* can still adapt to low pH, and Golińska & Dahm (2011) successfully isolated *Actinobacteria* from forest soils with an acidic pH ranging from 4.0 to 5.5. Additionally, researchers have successfully obtained *Actinobacteria* isolates from the rhizosphere of pine trees at pH 5.4.

Table 1. Measurement of environmental factors

Number	Environmental Factors	Value
1	Soil pH	5.4
2	Soil humidity	59.27%
3	Light Intensity	525 x 10 lux
4	Soil Organic Matter	5,8%
5	Soil Temperature	19°C
6	TPC (*Total Plate Count*) cfu/g	2.4 x 10^4

The average light intensity in Bedengan Forest, Malang, was moderate at 525 × 10 lux. This value aligns with the low soil temperature of approximately 19°C and sufficient soil moisture of 59.27%. Light intensity in the soil category tends to be low, indicating that sunlight exposure is largely blocked by the forest's vegetation cover, preventing much light from directly reaching the ground surface.

The sampling location's position in the highlands of Malang City contributes to the relatively low temperatures. Bhatti et al. (2017) stated that *Actinobacteria* grow optimally under moderate soil moisture and soil temperatures ranging from 25–30°C. The soil temperature conditions in Bedengan Forest, Malang, were below this optimal range, which may limit the growth and development of *Actinobacteria*. The data show that the microbial population obtained from the rhizosphere of Bedengan Forest was 2.4 × 10^4 cfu/g. This value was lower compared to the findings of Alwi et al. (2020), who reported an *Actinobacteria* density of 1.53 × 10^7 cfu/g in the rhizosphere of Leda plants.

Soil organic matter in Bedengan Forest, Malang, was found to be 5.8%, which is related to the soil pH measured at that location. The high organic matter content enhances the soil's water-holding capacity, allowing a large number of microorganisms to thrive. According to research by Bhadha et al. (2017), increasing soil organic matter by just 1% can significantly improve water retention, enabling the soil to hold moisture for longer periods. These

factors facilitate the rapid decomposition of organic matter by microorganisms and contribute to a decrease in soil pH, leading to acidic conditions.

Soil pH and organic matter content play a crucial role in soil function and nutrient availability for plants in this area. Neina, (2019) confirmed that soil pH influences plant growth and biomass yield by affecting biogeochemical processes, nutrient availability, mobility, and soil biological activity. Hoffland et al., (2020) added that soil organic matter serves multiple functions, including acting as a nutrient source, enhancing water-holding capacity, promoting soil aggregation, and serving as a key indicator of soil quality.

Rengel (2015) explained that acidic soils contain higher levels of micronutrients such as Fe, Mn, B, Cu, and Zn, while macronutrients such as K, S, Ca, and Mg tend to be less available. Kumar et al., (2016) noted that this occurs because micronutrients readily adhere to and bind strongly to soil surfaces with high concentrations of H^+ ions. The abundance of H^+ ions is what characterizes acidic soils.

Actinobacteria Diversity of Rhizosphere Bedengan Forest Selorejo Malang City

The diversity index is the result of a combination of species richness and evenness (Chen & Grinfeld, 2024). Based on the results of calculations using the Shannon-Wiener diversity index in Table 2, the *Actinobacteria* diversity index at the Selorejo Bedengan Forest location in Malang City was classified as medium (2.74). This diversity was lower than that reported by Laila (2018), who isolated rhizosphere bacteria in UB Forest, Malang Regency, and obtained a high diversity value (0.92) as measured by Simpson's diversity index.

According to Omayio & Mzungu (2019), the diversity index has not reached a high category due to the dominance of certain species over others. Isolate AC2 appears to be more abundant than other isolates, though not by a significant margin. If certain isolates are significantly more abundant than others, the diversity index may decrease, leading to lower productivity and indicating high ecological pressure and an unstable ecosystem. According to Singh & Gupta (2018), the loss of microbial diversity and abundance is directly correlated with the decline of soil microbial biomass, which is considered an indicator of soil fertility and ecosystem productivity.

Table 2. Diversity analysis of *Actinobacteria*

Isolate	Total	F	RD	RF	IVI	H'
AC1	14	3	11.86	11.11	22.98	0.36
AC2	28	5	23.73	18.52	42.25	0.49
AC3	18	3	15.25	11.11	26.37	0.41
AC4	15	3	12.71	11.11	23.82	0.38
AC5	10	5	8.47	18.52	26.99	0.30
AC6	17	4	14.41	14.81	29.22	0.40
AC7	16	4	13.56	14.81	28.37	0.39
Total	118	27	100	100	200	2.74

Note: F = Frequency; RF = Relative frequency; RD = Relative density; IVI = Importance value index; H' = Diversity index

Actinobacteria diversity is influenced by various environmental factors, one of which is soil organic content. A high organic content is generally directly proportional to the abundance of *Actinobacteria*. Bedengan Forest is known to have a medium to high soil organic content (Table 1). High organic content, combined with suitable soil moisture, creates ideal conditions for microbial growth. However, this also tends to make the soil pH more acidic. The diverse and abundant presence of *Actinobacteria* in Bedengan Forest soil indicates that the site still maintains a high level of soil fertility. de Menezes et al. (2015) explained that the soil C/N ratio and humic organic carbon are important drivers of *Actinobacteria* GH48 community compositions, highlighting their connection to soil organic carbon dynamics.

Morfologi Isolat *Actinobacteria*

A total of seven isolates were obtained from soil samples collected from the Selorejo Bedengan Forest, Malang City. These isolates were designated with the codes AC1 to AC7. *Actinobacteria* are gram-positive, rod-shaped (bacillus), and can form branched filaments or substrate mycelia. They are typically small, thin, and have non-concentrated hyphae. They are non-motile, and some species produce pigment. *Actinobacteria* are generally not acid-resistant, thrive in aerobic environments, although most are facultative anaerobes, and their networks can branch before transforming into a rod shape (Barka et al., 2016). The results of macroscopic and microscopic observations are presented in Table 3 and Figure 2.

Table 3. Morphological characteristics of *Actinobacteria* isolates

Macroscopic Characteristics	Isolat (AC)						
	1	2	3	4	5	6	7
Colony Shape	irregular	irregular	rooted	Round	Rooted	irregular	irregular
Edges	Not flat	Not flat	Serrated	Serrated	Fillamentous	Entire	Curly
Colony colour	White	White	White	Light Yellow	White	White	White
Flat	+	+	+	+	-	+	-
Convex	-	-	-	-	+	-	+
White Substrate Mycelium	-	-	+	-	+	-	+
Greyish-White Aerial Mycelium	-	-	-	-	+	-	+
Chip	+	-	-	-	-	+	-
Slimy chip	-	-	-	+	+	-	+
Smooth	-	+	+	-	-	-	-
Microscopic							
Bacil	-	+	+	-	-	+	-
Gram +	+	+	+	+	+	+	+
Filamentous	+	+	+	+	+	+	+
Branched Filaments	+	+	+	+	+	+	+
Condensed Hyphae	-	-	-	+	-	-	-

Note: (+) have the mentioned characters, dan (-) does not have the mentioned characters

Seven isolates were identified into two genera: *Nocardia* (isolates AC1, AC2, AC4, and AC6) and *Streptomyces* (isolates AC3, AC5, and AC7). Isolates belonging to the genus *Nocardia* exhibit a mushroom-like cell shape due to the presence of branching mycelia, which form monopodial branches (Henderson & Sutherland, 2017). *Streptomyces* exhibits a diverse range of cell shapes, varying by species. Its unique cell structure is characterized by filamentous growth and complex differentiation, transitioning from filaments to spores. The cell wall of Streptomyces is composed of peptidoglycan and glycopolymers, which are essential for maintaining cell shape and integrity (Bhowmick et al. 2024). Additionally, Streptomyces follows a mycelial lifestyle, setting it apart from many other bacteria. Its mechanism of cell division is also distinctive, involving septum formation and unique nucleoid separation, unlike bacteria such as *Escherichia coli* (Jakimowicz & Van Wezel, 2012). The mycelium consists of vegetative hyphae that grow on the surface, penetrate the medium, and fragment when cultured on SCA media.

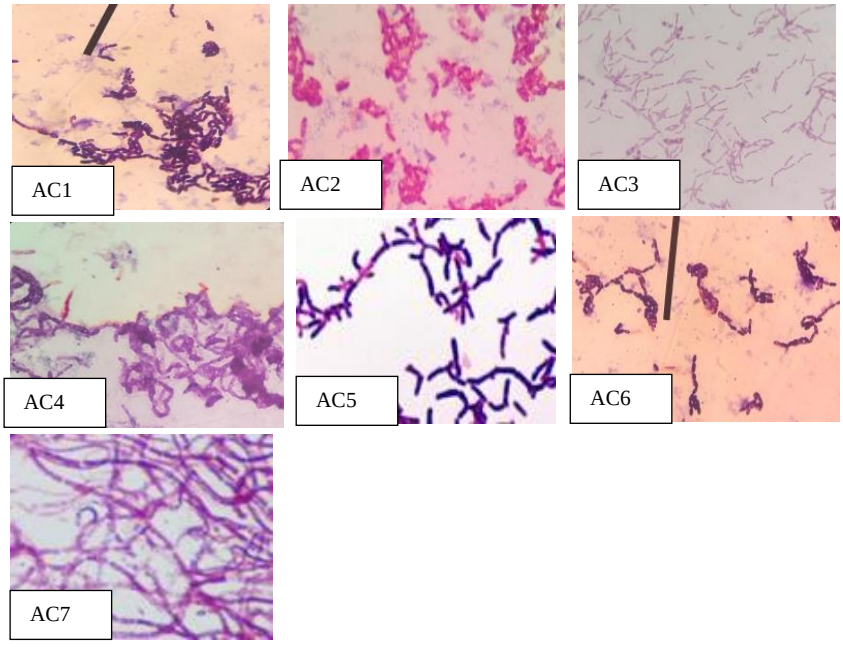

Figure 2. Microscopic of *Actinobacteria* isolates with 100 x 10 magnification

According to Takashima et al. (2017) the genus *Nocardia* is Gram-positive and grows optimally under aerobic conditions. In addition to being found in rhizosphere soil, *Nocardia* can also thrive in various environments, including plant tissues (Liu et al. 2017), and animal or human hosts. Research by Nammali et al. (2022) reported that *Nocardia coffeae* sp. nov. is a novel endophytic actinobacterium strain isolated from the roots of *Coffea arabica*, exhibiting typical chemotaxonomic properties. Additionally, a study by Zhuang et al. (2021) found that strain WCH-YHL-001T is a novel *Nocardia* species, isolated from a human skin biopsy specimen in China.

Isolates AC3, AC5, and AC7 exhibit morphological characteristics similar to those of the *Actinobacteria* genus *Streptomyces*. Macroscopically, the colonies of these isolates are root-shaped and irregular. The substrate mycelium is white, while the aerial mycelium is slightly yellowish-ash and greyish-white (Table 3). According to Donald et al. (2022), *Streptomyces* colonies are known for their filamentous and chalky appearance. These colonies often have a hairy, rough, or mealy texture and vary in color from white to grey. This distinctive filamentous growth pattern results from the formation of a branched network of multinucleated hyphae, which distinguishes *Streptomyces* from other bacteria.

The morphology of *Streptomyces* species is characterised by filamentous growth from a single spore, leading to the formation of a multinucleated and branched hyphal network. The filamentous nature of *Streptomyces* is a distinctive characteristic that contributes to its ability to produce a wide range of bioactive metabolites (Wang et al. 2017). The potential of these metabolites is of interest in inhibiting the growth of pathogenic bacteria.

Potential of *Actinobacteria* as Antibacterial Pathogens

Most *Actinobacteria* in soil produce bioactive compounds, including antifungal and antibacterial agents. This group is commonly found in the rhizosphere of plants, where it aids in defense against pathogenic microbes (Tistechok et al. 2021). Notable secondary metabolites produced by *Actinobacteria* include antibiotics such as streptomycin (Alwali & Parkinson, 2023), erythromycin, tetracycline (Jakubiec-Krzesniak et al. 2018), and chloramphenicol (Som et al. 2017). Additionally, compounds like rifampicin, actinomycin, and vancomycin are significant products of *Actinobacteria*, particularly from the genus *Streptomyces*, which play an essential role in treating bacterial infections (Sharma & Manhas, 2019). The presence of *Actinobacteria* in the plant rhizosphere not only benefits plants by combating pathogenic microbes but also offers substantial potential for discovering new antibiotics. The results of the antibacterial activity test against pathogens are shown in Figure 3.

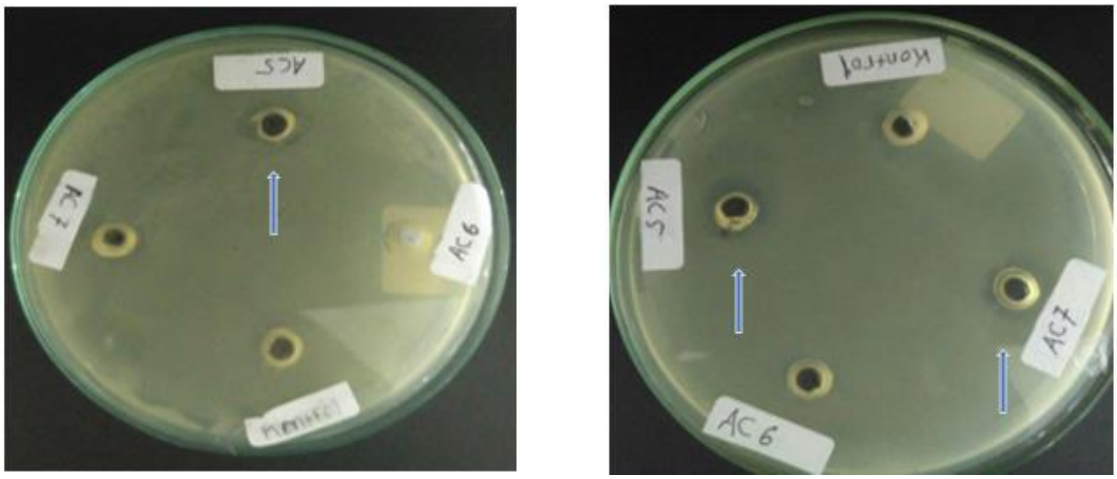

Figure 3. Antibacterial activity of pathogenic *Actinobacteria* isolates

In this study, the antibacterial activity test showed that isolate AC5 effectively inhibited the growth of *Escherichia coli*, with an inhibition zone of 9.67 mm. Isolates AC5 and AC7 also demonstrated antibacterial activity against *Shigella dysenteriae*, with inhibition zone diameters of 8.24 mm and 9.17 mm, respectively. Based on the antibacterial strength criteria defined by Kaur et al. (2018) and Zhang et al. (2017), an inhibition zone diameter of ≤5 mm is categorized as weak, 5–10 mm as moderate, 10–20 mm as strong, and ≥20 mm as very strong. These findings indicate that isolates AC5 and AC7 have significant potential as sources of natural antibiotics.

The antibacterial properties of this isolate are likely attributed to its production of secondary metabolites, such as daptomycin, which is known for its broad-spectrum activity against both Gram-positive and Gram-negative bacteria (Luo et al. 2018; Desouky et al. 2022). Furthermore, the successful isolation of these *Actinobacteria* provides strong evidence that rhizosphere soil, particularly in forest ecosystems like Bedengan Forest, harbors unique metabolites that could serve as alternative sources for antibiotic development. Additionally, the ability of *Actinobacteria* to thrive in soils exposed to synthetic chemical pesticides for extended periods demonstrates their

ecological resilience. This resilience offers opportunities to investigate the molecular mechanisms underlying these adaptations, including their capacity to produce antimicrobial secondary metabolites. Further research is needed to identify the specific compounds produced by isolates AC5 and AC7 and to evaluate their effectiveness against a broader range of pathogens.

CONCLUSION

The *Actinobacteria* diversity index in Bedengan Forest is classified as medium, with a value of 2.74. This indicates a moderate level of *Actinobacteria* diversity at the site, although it has not reached a high level. In this study, seven *Actinobacteria* isolates with distinct characteristics were successfully obtained. Among these, isolates AC5 and AC7 exhibited significant potential as antibacterial agents, particularly against the pathogenic bacteria *s*

However, further research is highly recommended to evaluate their potential antibacterial activity against gram-positive pathogenic bacteria. These findings suggest that *Actinobacteria* from Bedengan Forest could serve as a promising source of bioactive compounds for antimicrobial development. Additionally, more in-depth characterization is necessary to identify the bioactive compounds produced by these isolates, elucidate their mechanisms of action, and assess their potential applications in the health sector and pharmaceutical industry.

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AUTHOR CONTRIBUTIONS

All authors have read and approved the manuscript to be published.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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