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Antibacterial activity of actinomycetes associated with clove plant (*Syzygium aromaticum*) against *Staphylococcus aureus* and *Escherichia coli*

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ABSTRACT

Actinomycetes form associations with plants through colonizing plant tissues (endophytes) or by residing in the soil around some plants' roots (rhizosphere). Actinomycetes are known to produce antibacterial compounds. This study aimed to investigate the antibacterial activity of actinomycetes associated with the clove plant (*Syzygium aromaticum*) against *Staphylococcus aureus* and *Escherichia coli*. Actinomycetes were isolated from clove plants and the rhizosphere, and their antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli* was evaluated using the agar plug method, where the presence of transparent zones around 10-day-old actinomycete growth indicated inhibition of bacterial growth. Four isolates showed inhibitory effects against *Staphylococcus aureus*, while only three isolates, B.4, T.3, and T.4, demonstrated inhibitory activity against *Escherichia coli*, as indicated by the presence of inhibition zones. Isolate T.3 exhibited the highest inhibition zone of 8.5 mm against *S. aureus*, whereas B.4 displayed the highest inhibition zone of 7.7 mm against *E. coli*. In conclusion, the actinomycetes found in clove plants (*Syzygium aromaticum*) demonstrate antibacterial properties against *Staphylococcus aureus* and *Escherichia coli*, indicating their potential as antibacterial agents.

Keywords:
Antibacterial, actinomycetes, *Escherichia coli*, *Syzygium aromaticum*, *Staphylococcus aureus*.

Introduction

Antibiotic release into the environment and persistent antimicrobial residues have been widely studied globally, as antibiotics overuse and misuse lead to increased presence and spread in the background. Non-medical antibiotic use has an impact on both the environment and human health. This issue has garnered more attention with the rise of drug-resistant bacteria infections globally.

Numerous studies have shown that bacteria have developed resistance to antibiotics commonly used in clinical treatment (Klein et al., 2019; Saha et al., 2021; Sulis et al., 2022). It has been reported that community-acquired *Escherichia coli* infections are resistant to commonly used oral antibiotics, such as amoxicillin, cefixime, and ciprofloxacin, presenting a challenge for outpatient treatment (Lee et al., 2018; Seok et al., 2020; Mfoutou et al., 2021). Additionally, the global issue of increasing resistance to antimicrobial agents by *Staphylococcus aureus*, particularly in the case of methicillin-resistant strains, presents a significant challenge in managing infections caused by these bacteria (Gajdács et al., 2019; Guo et al., 2020). The presence of resistant bacteria can lead to more extended hospital stays, increased healthcare costs, and, most critically, higher rates of illness and death (Dadgostar et al., 2019). Therefore, exploring natural bioactive compounds as potential new antibiotics is crucial to address this problem.
Actinomycetes, which are frequently discovered in the tissues of plants (endophytes) and around plant roots (rhizosphere), have been explored as a promising source for the production of bioactive compounds (Quach et al., 2021; Natsagdorj et al., 2021; Elshafie et al., 2022).

Actinomycetes are widely recognized for producing various secondary metabolites, including antibiotics, antitumor agents, and plant growth hormones, which are significant for both the pharmaceutical and agricultural industries (Selim et al., 2021; De Simeis et al., 2021). Research has shown that these microorganisms are abundant and associated with medicinal plants, and some produce biologically active substances with distinct chemical compositions (Oberhofer et al., 2019; Aamir et al., 2020). Despite this, the diversity of actinomycetes and their bioactive compounds associated with medicinal plants in unique environments remains limited. Efforts to identify the functional properties of endophytic actinomycetes hold significant promise in addressing the current challenge of drug resistance. Thus, this study examines the antibacterial properties of actinomycetes found in association with the clove plant (*Syzygium aromaticum*) against *Staphylococcus aureus* and *Escherichia coli*.

Methods

Materials

The clove plants were obtained from a plant market in Yogyakarta. The rhizosphere soil was collected around the clove plant roots from a 5 cm radius. The test bacteria, *Staphylococcus aureus*, and *Escherichia coli*, were obtained from the Microbiology Laboratory, Universitas Ahmad Dahlan. The media used were Starch Casein Agar (Himedia®), Nutrient Agar (Oxoid®), and Nutrient Broth (Oxoid®). Other chemical substances utilized were distilled water, 70% alcohol, 0.5% NaClO solution, and gram staining (methylene blue, safranin, iodine, and crystal violet).

Isolation of actinomycetes

The isolation of actinomycetes from clove plants began with sterilizing the surface of the plant organ samples (leaves and stems) based on the method described by (Passari et al., 2015). The pieces were then cleaned with flowing water and gradually treated with 70% (v/v) alcohol for 1 minute, 0.5% (v/v) NaClO solution for 3 minutes, 70% (v/v) alcohol for 30 seconds, and sterilized water twice before being dried. The samples were cut and ground aseptically with a mortar, placed on Starch Casein Agar (SCA) media supplemented with 1% nystatin, and then incubated at room temperature (26°C) for 2 weeks.

Isolation of actinomycetes from the rhizosphere of clove plants involved drying a soil sample at room temperature for 4 days (Jiang et al., 2016). One gram of soil was placed in a tube containing 9 mL of sterilized 0.85% NaCl solution and vortexed for 5 minutes. The sample suspension was placed in hot water at 50°C for 10 minutes and then serially diluted. The 10-3 and 10-4 dilution suspensions were inoculated using the pour plate method on SCA media supplemented with 1% nystatin. The samples were incubated at 26°C for 2 weeks.

Morphological characterization

The successfully isolated actinomycetes were grown on SCA media for 10 days at room temperature. The macroscopic characterization of Actinomycetes involved analyzing colony morphology and color in SCA media (Katili et al., 2017). Color observation includes air mycelium and substrate mycelium colors. Colony morphology was observed to determine characteristics such as colony edge, elevation, and shape (Li et al., 2016). Microscopic characterization was done by following the cell and spore shapes of the actinomycetes under a microscope. A pure isolated culture was aseptically transferred onto a glass slide and gram-stained to examine actinomycetes' cell and spore morphology. Actinomycete isolates that met the characteristic morphology criteria (gram-positive, mycelial growth & spore formation) were screened for antibacterial activity.

Antibacterial Activity

The antimicrobial activities of each actinomycete were tested against pathogenic bacteria (*Staphylococcus aureus* and *Escherichia coli*) using the agar plug method (Balouiri et al., 2016). The actinomycetes were grown on SCA media at 28°C for 10 days while the bacteria were cultured on NB media. Subsequently, a 5 mm agar plug containing 10-day-old actinomycetes growth was aseptically placed in the center of a 9 cm NA culture plate. A 5 mm agar plug containing SCA media

was used as a negative control. Following this, all the dishes were appropriately sealed with parafilm and then incubated at 28 ± 2°C for 24 hours. The development of a transparent zone surrounding the actinomycete colonies indicated an inhibition zone (Ortlieb et al., 2021). This test was conducted three times. The standard for measuring antibacterial activity is based on Davis and Stout's classification, which includes weak (less than 5 mm), moderate (between 5 and 10 mm), strong (between 10 and 19 mm), and very strong (greater than 20 mm).

Data Analysis

The experiments were carried out in triplicate and the results were presented as the mean value ± standard deviation. Shapiro-Wilk test was used to do a prior normality test. One-way analysis of variance (ANOVA) and the Tukey test were used to determine the statistical significance of the data. The statistical evaluations were carried out using GraphPad Prism.

Results and Discussions

A total of 4 actinomycete isolates were successfully isolated from the internal tissues of the plant (endophytes) and the rhizosphere. Initially, the isolation process yielded 1 endophytic actinomycete isolates and 3 actinomycete isolates from the rhizosphere of clove plants. Among the plant organs, the lowest number of isolates was obtained from the leaves. This finding is consistent with the results of a study by Saini et al. (2016) on the isolation of actinomycetes from *Syzygium cumini* plants, where a higher number of isolates were found in the stems (21) compared to the leaves (1). Endophytic actinomycetes are more commonly found in the roots and stems than in the leaves. Nevertheless, investigations into actinomycete colonization in different parts of medicinal plants have yielded inconsistent outcomes (Nalini et al., 2017).

Based on macroscopic observations, the isolated actinomycetes exhibited similar characteristics, with white and whitish-brown colors (Table 1.). The actinomycete isolates displayed varying aerial and substrate mycelium. On SCA media, all four isolates showed densely packed colony consistency, with colonies T.3, T.4, and T.5 exhibiting dry, powdery colony characteristics. The macroscopic features can be seen in Figure 1. Based on the Gram staining results, all isolates showed gram-positive characteristics (purple) and displayed filamentous cell morphology.

Table 1. Colony morphological characteristics.

Isolate	B.4	T.3	T.4	T.5
Shape	Irregular	Circular	Circular	Circular
Elevation	Raised	Flat	Raised	Umbonate
Edge	Undulate	Entire	Entire	Entire
Aerial mycelium	White	White	Gray	White-brown
Substrate mycelium	White	White-brown	Grayish-white	Brown

Several studies have provided evidence of the antimicrobial activity exhibited by Actinomycetes (Chen et al., 2021; Sarika et al., 2021). The antagonist test, a rapid and effective method yielding tangible outcomes, was employed to evaluate the potency of Actinomycetes (Rozirwan et al., 2020). The antibacterial assay was conducted using the agar plug method to quantitatively determine the formation of inhibition zones. Actinomycetes were cultivated on SCA media, and during their growth, microbial cells released various bioactive compounds that diffused into the agar medium. After incubation, agar plugs with a diameter of 6 mm containing Actinomycetes were placed onto NA media that had been inoculated with the test bacteria. Substances diffused from the agar plugs into the NA media. Subsequently, the antimicrobial activity of the secreted molecules was detected by the appearance of inhibition zones surrounding the Actinomycete plugs.

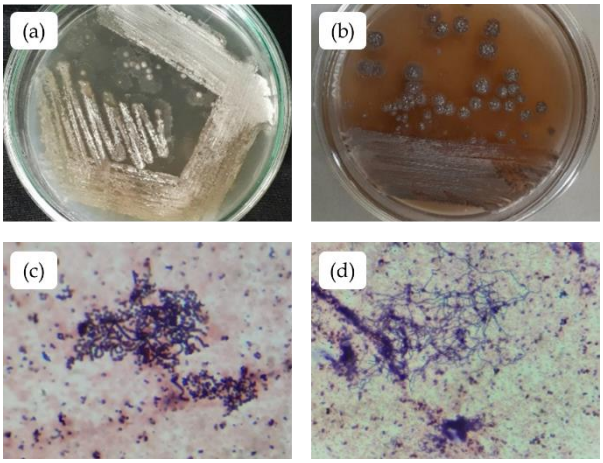

Figure 1. Colony morphology of actinomycete isolates (a) T.3, (b) T.5 and microscopic features of actinomycete isolates (c) T.3, (d) T.5.

The Actinomycete isolates demonstrated antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*, forming clear inhibition zones around the Actinomycete plugs. The agar plug method, as described by Balouiri et al. (2016), was employed to assess antibacterial activity, where microbial plugs inoculated onto the test media diffused into the agar, resulting in the secretion of antibacterial compounds by the microorganisms. Clear inhibition zones surrounding the Actinomycete plugs indicated antibacterial activity. All four Actinomycete isolates tested against *Staphylococcus aureus* exhibited inhibition or clear zones around the Actinomycete plugs (Figure 2.). However, among the four Actinomycete isolates tested against *Escherichia coli*, only three isolates produced inhibition zones or clear zones, as isolate T.5 did not show a clear zone. Isolate T.3 displayed the largest inhibition zone, measuring 8.5 mm against *S. aureus*, while separating B.4 showed the highest inhibition zone of 7.7 mm against *E. coli* (Figure 3.). Considering that all isolates exhibited inhibition zones ranging from 5 to 10 mm, the activity of all isolates can be categorized as moderate.

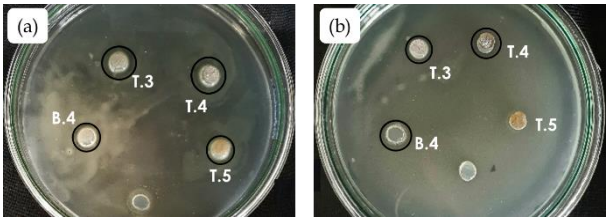

Figure 2. Antibacterial activity of actinomycete isolates against *Staphylococcus aureus* (a) and *Escherichia coli* (b).

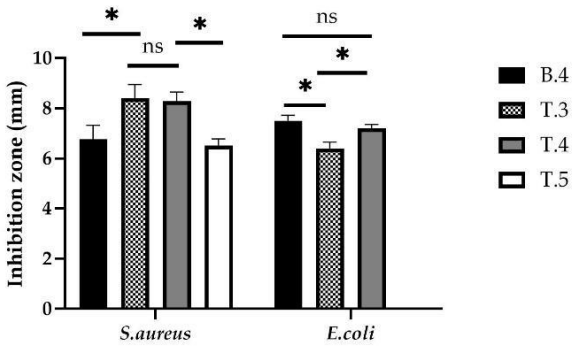

Figure 3. Zone inhibition of the antibacterial activity of actinomycete isolates against *Staphylococcus aureus* and *Escherichia coli*

The inhibition mechanism of bacterial growth by antimicrobial compounds can occur through various processes, including cell damage by inhibiting cell wall formation, disrupting the cytoplasmic membrane's permeability, leading to cellular fluid leakage, and modifying protein and nucleic acid molecules. The ability to inhibit the tested microorganisms obtained from potential isolates may also be influenced by bacterial communication patterns or quorum sensing, which regulate metabolic regulation. This allows bacteria to release specific compounds into the environment to prevent the colonization of other bacteria (Lubis, 2015).

The antibacterial test results indicated that *Staphylococcus aureus* demonstrated a greater susceptibility to the metabolites or antibacterial compounds produced by Actinomycetes than *Escherichia coli*. This finding is consistent with the research conducted by Budhathoki & Shrestha (2020) who reported that *Staphylococcus aureus* displayed the highest susceptibility among 12 Actinomycete isolates obtained from the soil. Furthermore, the inhibition zones formed around *Staphylococcus aureus*, a gram-positive bacterium, were more significant than those observed around gram-negative test bacteria, indicating a higher activity of the Actinomycete isolates against gram-positive bacteria. This difference in antibacterial activity could be attributed to variations in the cell morphology between gram-positive and gram-negative bacteria and the specific mode of action of the antibiotic compounds targeting the components of gram-positive cell walls more effectively than gram-negative cell components.

The disparity in the formation of inhibition zones between gram-positive and gram-negative bacteria is primarily influenced by differences in the composition of their respective cell walls (Aminingsih et al., 2012). Notably, as a gram-positive bacterium, Staphylococcus aureus possesses a structurally simpler cell wall with lower lipid content. In contrast, *Escherichia coli*, a gram-negative bacterium, exhibits a more complex cell wall structure characterized by a higher content of complex lipids. Consequently, the cell wall of gram-negative bacteria presents more excellent resistance to the penetration of antibacterial substances.

Actinobacteria can synthesize diverse secondary metabolites with critical pharmacological properties (Anandan et al., 2016). This has resulted in identifying a wide range of antibiotic compounds, predominantly from the Streptomyces genus. Numerous potent secondary metabolites and antibiotics are among the approximately 7600 compounds discovered to be produced by the Streptomyces genus. Consequently, Streptomycetes have emerged as the predominant microorganisms responsible for the synthesis of antibiotics that are extensively employed by the pharmaceutical sector.

Conclusion

Among the isolates tested, four of them exhibited inhibitory effects against *Staphylococcus aureus*. In contrast, only three isolates (B.4, T.3, and T.4) demonstrated inhibitory activity against *Escherichia coli*, as evidenced by inhibition zones. Notably, isolate T.3 displayed the highest inhibition zone, measuring 8.5 ± 0,56 mm, against *S. aureus*, whereas B.4 exhibited the most significant inhibition zone of 7.7± 0,21 mm against *E. coli*. These findings highlight the antibacterial properties of actinomycetes derived from clove plants (*Syzygium aromaticum*) against both *S. aureus* and *E. coli*, suggesting their potential as effective antibacterial agents.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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