Bioactivity of Antibacterial Compounds Produced by Endophytic Actinomycetes from *Neesia altissima*

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ABSTRACT

Identification of alternate bioactive compounds isolated from microbial endophytes from plants may lead to better solution against antibiotic resistance. Main objective of this research is to determine the bioactivity of antibacterial compounds produced by endophytic actinomycetes, which was isolated from stem bark of *Neesia altissima* Bl. at Halimun Salak Mount. Detection of bioactive compounds was assayed using Thin Layer Chromatography (TLC) by employing bioautography. Bioactivity was assayed using crude extract against five microorganisms using agar diffusion methods. Tetracycline was used as a positive control. The result of antibacterial activity from endophytic actinomycetes isolate revealed that the bioactive compounds were effective in inhibiting the growth of bacteria *Bacillus cereus* (1.25 mm), *Salmonella typhimurium* (5.75 mm), and *Shigella flexneri* (4.37 mm). Bioactivity of ethyl acetate extract from bioactive compounds of endophytic actinomycetes has an antibacterial activity against bacteria *Bacillus cereus* (13.00 mm) and *Staphylococcus aureus* (15.90 mm), at a concentration of 250,000 ppm. Each of TLC fraction was able to inhibit growth of *B. cereus* at a minimum concentration of 10,000 ppm. In conclusion, the bioactive compounds produced by endophytic actinomycetes can have inhibitory effect towards *B. cereus*. The category of that compounds are narrow spectrum. The bioactive compound isolated from endophytic actinomycetes may have other implications such as anticancer, antifungal, and antioxidant.

Keywords: Bioactivity, bioactive compounds, effectivity, *Neesia altissima* Bl., Streptomyces sp.

INTRODUCTION

The long-term and extensive use of antibiotics has led to the emergence of a large number of drug resistant strains. According to the report incidences of *Mycobacterium tuberculosis* infection have reached up to 10.2%. Pathogenic bacteria such as *Mycobacterium tuberculosis* and *Staphylococcus aureus* are susceptible to mutations and are more likely to cause drug resistance [1]. The increasing incidence of drug resistance in pathogenic bacterial, fungal infections and certain cancers has prompted a search for more and better agents [2]. Actinomycetes is the main source of antibiotics and endophytic actinomycetes isolated from medicinal plants has considerable development potential. *Neesia altissima* Bl. (Bengang) is one of medicinal plants that have been used for centuries as remedies for diarrhea, disuria, and gonorrhea diseases at around west Java, Indonesia. Kudo et al. (1998) [3]; Chen et al. (2009) [4]; Qin et al. (2008 [5], 2010 [6]) have found a few actinomycetes from tissue of medicinal plants constantly from the present findings. Moreover, most endophytic actinomycetes of medicinal plants can produce important compounds and some of them have new chemical structure [7, 8, 9]. Igarashi et al. (2002) [10] isolated 398 actinomycete strains from leaves, stems and roots of cultivated and wild plants that showed antagonistic activity against phytopathogenic fungi and bacteria at 10 – 20% of the n-butanol extracts of their fermentation broths. Igarashi et al. (2000) [11] reported that a wide range of endo-
phytic actinomycetes have a potential to produce anti-
microbial compounds. Sasaki et al. (2001a, 2001b) [12, 13] identified several new bioactive compounds pro-
duced by actinomycetes isolated from live plants. Two new novobiocin analogs produced by Streptomyces sp. collected of Aucuba japonica [12] and cedarmycins by Streptomyces sp. collected from Cryptomeria japonica [13] were determined to be antimicrobial metabolites. A new naphthoquinone antibiotic, alnumycin, was also re-
ported in Streptomyces sp. isolated from root nodules of Alnus glutinosa collected in Germany [14]. Shimizu et al. (2004) [15] proved that Streptomyces galbus strain R-5 produced actinomycin X2 and fungichromin that could account for the in vitro anti-bacterial and anti-fun-
gal activities.

The development and improvement of accurate and efficient methods for rapid antibiotic susceptibility test-
ing is important for public health. Antimicrobial suscepti-
bility information about pathogens may significantly reduce morbidity and mortality, cost of treatment, and duration of hospitalization if this information can be provided to clinicians in a rapid and timely fashion [16]. The aim of this study was to investigate the bioactivity of antibacterial bioactive compounds isolated from endophytic actinomycetes N. altissima.

MATERIALS AND METHODS

Isolation of the compounds

For the preparation of crude extract samples of endophytic Actinomycetes were soaked twice with ethyl acetate for 24 hours. The extract was evaporated under reduced pressure and at a temperature of 35°C until total evaporation of the ethyl acetate.

Determination of antimicrobial activities

For the antimicrobial testing, a crude extract of the ethyl acetate was prepared in pure dimethylsulfoxide (DMSO) at 250,000; 50,000; 10,000; 5,000; 1,000; and 100 ppm. Streptomyces sp. strain UICC B-92 was isolated from Neesia altissima and kept on International Streptomycetes Project (ISP2) medium. The isolate have stored at Universitas Indonesia Culture Collection (UICC), UI Depok. Overnight broth cultures, adjusted to yield approximately 10⁸ cfu/mL for bacteria were streaked with a calibrated loop on plates containing appropriate solid medium. Filter paper discs (6 mm diameter; Oxoid) were placed on the inoculated agar surfaces and impregnated with 15 mL of stock solutions. Pure DMSO (15 mL) was used as a negative control while tetracycline discs (100 mg) were used as positive controls. The plates were observed after 18 hours at 37°C. All tests were performed in duplicate and the antibacte-
rial activity was expressed as the mean of inhibition di-
ameters (mm) produced by the crude extracts of endophytic Actinomycetes.

Extraction for production of secondary metabolite

An established slant of isolate was inoculated in a 250 mL flask containing 50 mL of vegetative medium (ISP2 medium) consisting of yeast extract 4 g/L, malt extract 10 g/L, dekstroza 4 g/L, and demineral water 50 ml. The flask was incubated at 30 °C for 2 days in an incubator-shaker. Fifty milliliters of the culture was transferred to 1,000 mL of the fermentation medium. The fermentation medium consisted of glucose 4 g/L, bacto peptone 15 g/L, yeast extract 3 g/L, Fe (III) citrate hydrate 0.3 g/L, and dematerialized water 1000 mL [17]. The fermentation was carried out at 30°C for 5 days in an incubator-shaker.

The residue was suspended in water and extracted successively three times with hexane, chloroform, ethyl acetate and methanol. The n-methanolic extract was then separated on a column chromatography using silica gel 60 G F254 [18].

Detection of antibacterial activity by using bioautography method

The agar overlay method (immersion bioautog-
raphy) was used for detection of antibacterial bioactive compounds. TLC plate (Merck Silica Gel 60 F254) was loaded with 10 μL of crude extract of endophytic Acti-
omycetes. The solvent system used was chloroform : methanol (5 : 1). The chromatogram was kept for evap-
oration of the solvent. Developed chromatogram was placed on sterile nutrient agar plate (15 mL) for detection of antibacterial activity. For detection of antibacte-
rial activity, 5 mL of nutrient agar was seeded with 1 mL of spore suspension of Bacillus cereus (0.1A540 = 10⁷ spores/mL) was poured on chromatogram. After agar got solidified the petri plates were kept at 4°C for diffusion for 3 hours. Plates were then incubated at room temperature for 24 hours for bacterial culture. The anti-
microbial activity was observed by inhibition zone and the hRf value was defined.

RESULTS AND DISCUSSION

The results of the antibacterial activity of endophytic Actinomycetes and ethyl acetate extracts are listed in
Table 1. Antibacterial activity of endophytic actinomycetes

| Isolate | Diameter of inhibition zone (mm) |
|---------|----------------------------------|
|         | B. cereus | E. coli | S. aureus | S. typhimurium | S. flexneri |
| UICC B-92 | 1.25 | - | - | 5.75 | 4.37 |
| K (+) | 9.00 | 9.00 | 9.00 | 9.00 | 9.00 |

Note: (-): no inhibition zone; K (+): positive control

Table 2. Antibacterial activity of ethyl acetate extract of bioactive compounds from endophytic actinomycetes

| Treatment | Concentration (ppm) | Diameter of inhibition zone (mm) |
|-----------|---------------------|----------------------------------|
|           |                     | B. cereus | S. flexneri | S. aureus |
| With shaking | 50,000 | 7.50 ± 7.00 | - | - |
|            | 10,000 | 6.50 ± 7.00 | - | - |
|            | 5,000 | - | - | - |
|            | 1,000 | - | - | - |
|            | 100 | - | - | - |
|            | 250,000 | 13.00 ± 13.00 | 8.00 ± 8.00 | 7.00 ± 6.50 |
| K (+) | 9 | 9 | 9 |

With shaking

| No shaking | 50,000 | 7.00 ± 7.00 | - | - |
|            | 10,000 | 7.00 ± 7.00 | - | - |
|            | 5,000 | - | - | - |
|            | 1,000 | - | - | - |
|            | 100 | - | - | - |
|            | 250,000 | 7.50 ± 6.50 | - | 16.50 ± 15.20 |
| K (+) | 9 | 9 | 9 |

Note: (-): no inhibition zone; K(+): positive control (tetracycline)

Table 1 and 2, respectively. The bioautography revealed clear zones of bacterial growth inhibition for Bacillus cereus while, for Staphylococcus aureus, zones of inhibition were less visible.

The endophytic actinomycetes have been identified at previously research [19]. They are Streptomyces sp. UICC B-92. The genus Streptomyces is an important group of actinomycetes because of its ability to produce many types of secondary metabolites [20] and approximately two-thirds of all known natural antibiotics are produced by these bacteria. Streptomyces is known to produce antitumour drugs such as the anthracycline antibiotic, daunorubicin, or the bleomycin complex; autoimmune active agents such as the macrolide tacrolimus; antimicrobial compounds like phenazines [21]. Antibacterial activity of endophytic actinomycetes isolate, Streptomyces sp. UICC B-92 have inhibited the growth of Bacillus cereus, Salmonella typhimurium and Shigella flexneri (Table 1). That result is different from crude extract of compounds (Table 2). The reason of this difference may be due to the fact that target compounds have not been isolated yet. One or more components could be separated from a mixture by the extraction process.

In the fermentation process, yield more was produced from Streptomyces sp. UICC B-92 isolate during 5 – 7 days incubation. That bioactive compounds of endophytic actinomycetes showed high in vitro antibacterial activity against B. cereus ATCC 10876 and S. aureus ATCC 25923 but not against Gram negative bacteria. The crude extract from the endophytic Actinomycetes isolate showed that the isolate of UICC B-92 have antibacterial activity against B. cereus more than S. aureus and another test bacteria (Table 2). The standard devia-
The antibacterial activity of an isolate has been detected by various methods like dilution methods (agar diffusion and MIC) and bioautography methods. Bioautography is sensitive method for detection of antimicrobial compounds even in small amounts [24]. Hence for detection of antibacterial compounds, bioautography is suitable method in initial stages as compared to agar dilution methods [25]. Bioautography is a sensitive method and can be directly employed for isolation of active constituents [26]. Bioautography screening methods are based on the biological activities, e.g. antibacterial, antifungal, antitumour, and antiprotozoae of the tested substances [27]. The detection method in this research used thin-layer chromatography (TLC) (Figure 2). The methods have been successful due to combination of layer liquid chromatography techniques. Bioautography visualization of antibacterial bioactive compounds from endophytic Actinomycetes (Figure 3) revealed inhibition zone of the spot at 41 – 42 fraction on TLC. The result of bioautography shown antibacteria
activity toward B. cereus has valid value of Rf 4.66, 6.16, and 7.67 while for the size of inhibition zone has sufficiently wide, 7.25 mm averaged (Table 3). From that results shown repeatedly the potential of endophytic Actinomycetes for the discovery of novel bioactive compounds, having antibacterial activity. The data of this result suggested that Streptomyces sp. UICC B-92 from N. altissima provided potential sources for the discovery of new secondary metabolites to combat the diarrhea causing bacteria.

CONCLUSION
Bioautogram showed that Rf value of spot derived from the bioactive compounds isolated from Streptomyces sp. strain UICC B-92 are 4.66, 6.16, and 7.67, which have significant for the average of inhibition zone is 7.25 mm. It was found that the crude extract of bioactive compounds from endophytic actinomycetes has an antibacterial activity for controlling Gram positive bacteria, especially B. cereus ATCC 10876.

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