

## 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 reports 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 gonorrhoea 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-

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How to cite:

Pratiwi RH, Hanafi M, Artanti N, Pratiwi RD (2018) Bioactivity of Antibacterial Compounds Produced by Endophytic Actinomycetes from *Neesia altissima*. J. Trop. Life. Science 8 (1): 37 – 42.

phytic actinomycetes have a potential to produce antimicrobial compounds. Sasaki *et al.* (2001a, 2001b) [12, 13] identified several new bioactive compounds produced 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 reported 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-fungal activities.

The development and improvement of accurate and efficient methods for rapid antibiotic susceptibility testing is important for public health. Antimicrobial susceptibility 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 Streptomyces Project (ISP)2 medium. The isolate have stored at Universitas Indonesia Culture Collection (UICC), UI Depok. Overnight broth cultures, adjusted to yield approximately  $10^8$  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 antibacterial activity was expressed as the mean of inhibition diameters (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 bioautography) 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 Actinomycetes. The solvent system used was chloroform : methanol (5 : 1). The chromatogram was kept for evaporation of the solvent. Developed chromatogram was placed on sterile nutrient agar plate (15 mL) for detection of antibacterial activity. For detection of antibacterial activity, 5 mL of nutrient agar was seeded with 1 mL of spore suspension of *Bacillus cereus* (0.1A540 =  $10^7$  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 antimicrobial 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)				
	<i>B. cereus</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>S. typhimurium</i>	<i>S. flexneri</i>
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)		
		<i>B. cereus</i>	<i>S. flexneri</i>	<i>S. aureus</i>
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
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; auto-immune 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-

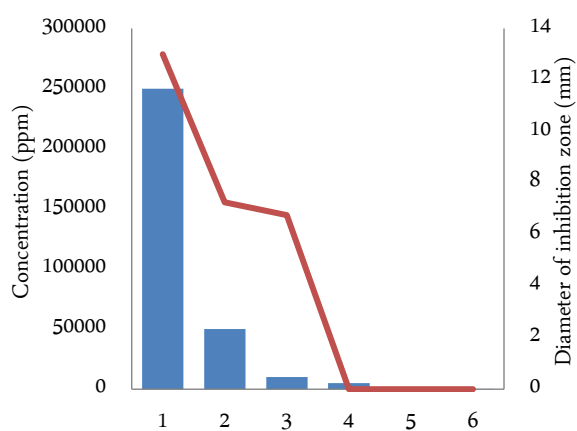
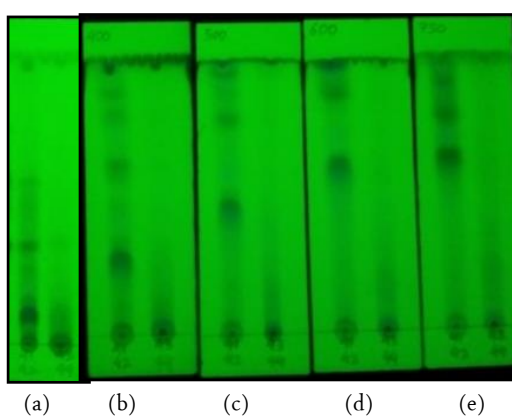
Figure 1. Antibacterial activity for *B. cereus*

Figure 2. TLC of endophytic actinomycetes (fraction 41 – 42) before purification with chloroform ( $\mu\text{L}$ ): methanol ( $\mu\text{L}$ ) = 2000 : 250 (a); 2000 : 400 (b); 2000 : 500 (c); 2000 : 600 (d); 2000 : 750 (e)

tion of antibacterial activity for *B. cereus* is 0.76. The value of standard deviation showed homogeneity for the data sample (Figure 1). The higher sensitivity of the gram positive bacteria than gram negative bacteria is possibly due to their differences in cell wall constituents and their arrangement. The outer membrane of gram negative bacteria carrying the structural lipopolysaccharide components is an effective barrier against hydrophobic substances [22, 23], while gram positive bacteria cell wall contain a peptidoglycan layer, which is an ineffective permeability barrier. Based on the result, concentration of 250 ppm showed 6.25 mm inhibition zone against *B. cereus*. This data suggested that these Actinomycetes endophytes from *N. altissima* provided potential for production of secondary metabolites against the diarrhea-causing bacteria. They indicated promising capability to produce useful bioactive compounds.

The antibacterial activity of an isolate has been de-

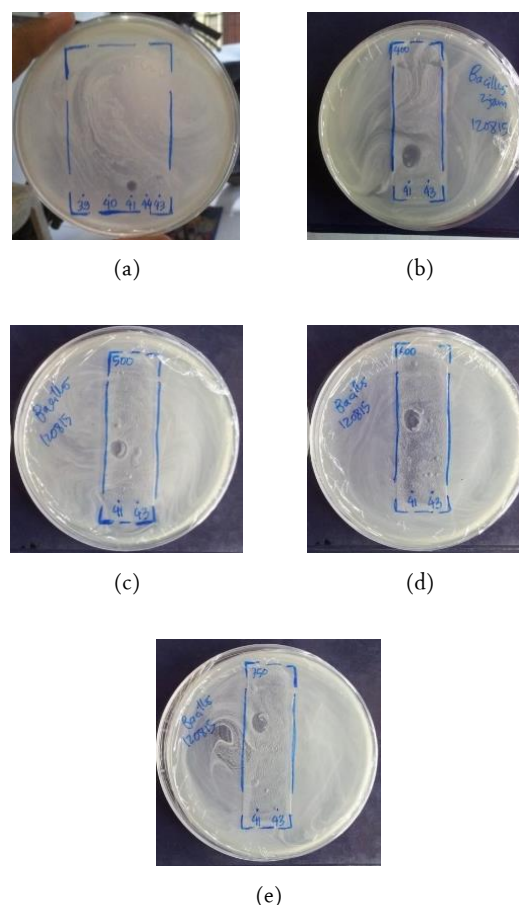


Figure 3. Bioautography visualisation of antibacterial bioactive compounds from endophytic actinomycetes with chloroform ( $\mu\text{L}$ ): methanol ( $\mu\text{L}$ ) = 2000 : 250 (a); 2000 : 400 (b); 2000 : 500 (c); 2000 : 600 (d); 2000 : 750 (e)

tected 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 endo-phytic 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.

## ACKNOWLEDGMENT

This research was supported by Research Grant of Hiber DIKTI 2016 and PPT DIKTI 2017 awarded to RHP number 0581/K3/KM/2016 and number 28/E/KPT/2017 respectively. We would like to thank to LPPM Staff of Universitas Indraprasta PGRI and Chemistry Laboratory at Research Center for Chemistry, Indonesian Institute of Sciences (LIPI), PUSPIPTEK, Serpong for valuable technical assistance.

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