

**Antibacterial Evaluation of Ethyl Acetate Extract of some Actinomycetes Isolated from Thai Terrestrial Environment**

**การประเมินฤทธิ์ต้านแบคทีเรียจากสารสกัดหยาบเอทิลอะซิเตทของแอกติโนมัยซีทที่แยกจากสิ่งแวดล้อมทางบกของไทย**

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**ABSTRACT**

Selected 17 actinomycetes isolates were obtained from Thai terrestrial environment and characterized taxonomically based on colony characteristics using isolation agar. Preliminary screening of antibacterial activities of culture broth of 17 isolated actinomycetes strains were examined towards standard Gram-positive and Gram-negative bacterial using agar-well diffusion method. The results showed that culture broth of 7 isolates inhibited growth of Gram-positive bacteria, but not Gram-negative bacteria. Crude metabolites of 7 actinomycetes were extracted from their culture broth by liquid-liquid extraction using ethyl acetate as solvent. The antibacterial activity of ethyl acetate extracts were evaluated against Gram-positive bacteria using disc diffusion method. Three ethyl acetate extracts obtained from AT1L-8, PN51B-4-1 and PN51B-9-6 exhibited antibacterial activity against *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 6538 and ATCC 25923) and methicillin-resistance *Staphylococcus aureus* (DMST 20654). Based on 16S rRNA gene sequence, strain AT1L-8 was categorized as genus *Streptomyces*, whereas strains PN51B-4-1 and PN51B-9-6 were closely related to genus *Actinomadura*.

**บทคัดย่อ**

แอกติโนมัยซีท 17 ไอโซเลทที่แยกจากสิ่งแวดล้อมทางบกของไทยได้ถูกคัดเลือกเพื่อนำมาศึกษาอนุกรมวิธานเบื้องต้นของเชื้อโดยการตรวจสอบลักษณะทางสัณฐานวิทยาบนอาหารแยกเชื้อ การคัดเลือกฤทธิ์ต้านแบคทีเรียเบื้องต้นของอาหารเหลวจากการเลี้ยงเชื้อแอกติโนมัยซีททั้ง 17 ไอโซเลททำโดยการทดสอบกับเชื้อแบคทีเรียแกรมบวกและแกรมลบสายพันธุ์มาตรฐาน โดยวิธีเอกาเวลดิฟฟิซัน ผลการทดลองพบว่าอาหารเหลวจากการเลี้ยงเชื้อจากแอกติโนมัยซีท 7 ไอโซเลทสามารถยับยั้งการเจริญเติบโตของแบคทีเรียแกรมบวก แต่ไม่สามารถยับยั้งการเจริญเติบโตของแบคทีเรียแกรมลบ เมแทบอไลต์หยาบของแอกติโนมัยซีททั้ง 7 ไอโซเลทจากอาหารเหลวจากการเลี้ยงเชื้อได้ถูกสกัดด้วยวิธีการสกัดของเหลวด้วยของเหลวโดยตัวทำละลายเอทิลอะซิเตท ฤทธิ์ต้านแบคทีเรียของสารสกัดหยาบเอทิลอะซิเตทจะถูกนำมาประเมินฤทธิ์ต้านเชื้อแบคทีเรียแกรมบวกโดยวิธีดิสดิฟฟิซัน สารสกัดหยาบจากเอทิลอะซิเตท 3 ไอโซเลท ได้แก่ AT1L-8 PN51B-4-1 และ PN51B-9-6 แสดงฤทธิ์ยับยั้ง *Bacillus subtilis* (ATCC 6633) *Staphylococcus aureus* (ATCC 6538 และ ATCC 25923) และ methicillin-resistance *Staphylococcus aureus* (DMST 20654) จากการวิเคราะห์ลำดับนิวคลีโอไทด์ในช่วงยีนสิบหก เอส อาร์ อาร์เอ็นเอ พบว่าสายพันธุ์ AT1L-8 เป็นเชื้อในสกุลสเตรปโตมัยเซส ส่วนสายพันธุ์ PN51B-4-1 และ PN51B-9-6 น่าจะเป็นเชื้อในสกุลแอกติโนมัยซีท

**Key Words:** Actinomycetes, Antibacterial activity, Ethyl acetate extract

**คำสำคัญ:** แอกติโนมัยซีท ฤทธิ์ต้านแบคทีเรีย สารสกัดเอทิลอะซิเตท

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## Introduction

Bacterial infection is still a serious problem in hospital and community because some bacteria can develop themselves to be antibiotic-resistant strains. Therefore, a new antibacterial drug is urgently needed. Many researchers aim to discover new antibacterial agents from various sources. Actinomycetes are one of the most attractive sources of new bioactive metabolites, especially novel and useful antibiotics (Lazzarini et al., 2001). The actinomycetes have ability to produce a wide variety of secondary metabolites such as enzymes (Berdy, 2005) immunomodulators anticancer and antimicrobial agents (Berdy, 2005; Mann, 2001; Blunt et al., 2006; Bibb, 2005). Actinomycetes are one of the most important bacteria in the terrestrial soil (Berdy, 2005; Takahashi, Omura, 2003). Actinomycetes are a group of Gram-positive bacteria with high G+C ratio, frequently filamentous form and sporulation (Chavan et al., 2013). The actinomycetes, especially *Streptomyces* is a main genus that produces a broad-spectrum of potential antibacterial substances such as streptomycin, neomycin, gentamycin (Hotam et al., 2013; Lam, 2006). The other genera, like *Amycolatopsis*, *Actinomadura* and *Micromonospora* have also been reported to produce a wide range spectrum of antibacterial metabolites (Lam, 2006; Deepika, Kannabiran, 2009)

## Objective of the study

The aim of this study was to evaluate antibacterial activity of crude extract from Thai terrestrial environment. The antibacterial activity of culture broth and ethyl acetate extract were examined against standard strains of Gram-positive and Gram-negative bacteria. The genus of actinomycetes strains which exhibited potential antibacterial activity were

identified using morphological and 16s rRNA gene sequence analysis.

## Methodology

### Isolation of actinomycetes

The 17 actinomycete strains were selected from the collection of actinomycetes isolated from various sources of Thai terrestrial environment, belonging to the Actinobacterial Research Unit of Dr. Chitti Thawai.

### Preparation of standard bacteria for antibacterial assay

The standard strains of bacteria in this study were *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* (ATCC 6538 and ATCC 25923), methicillin-resistance *Staphylococcus aureus* MRSA (DMST 20654), *Escherichia coli* (ATCC 8739 and ATCC 25922), *Klebsiella pneumonia* ATCC 700603, *Pseudomonas aeruginosa* (ATCC 9027 and ATCC 27853) and *Salmonella* Typhimurium (ATCC 13311 and ATCC 14028). All of these bacteria were cultured using Tryptic Soy Broth (TSB) with shaking 150 rpm, at 37°C for 18 hours.

### Morphological characterization

Morphological characteristics of selected actinomycetes were investigated by observation of colony color growing on yeast extract-malt extract agar (ISP2) agar incubation at 30°C for 14 days. Aerial mycelium color and spore morphology were observed under high power light microscope by growing on soil extract agar after incubation at 30°C for 21 days (Shirling, Gottlieb, 1996).

### Cultivation process of isolated actinomycetes

Seed inoculum prepared by transferring the single colony of the 17 isolated actinomycetes into

100 ml of ISP2 broth. The flask was placed in incubator shaker at 200 rpm at 30° C for 14 days. In cultivation process, the isolated actinomycetes from seed inoculum was inoculated in 1L Eelenmeyer flask that contain 500 ml of ISP2 broth with shaking condition at 200 rpm at 30° C for 14 days. After the incubation, the culture broth was centrifuged for separate intact cells from culture broth. The culture broth was used for preliminary screening of antibacterial activity by agar well diffusion method. The culture broth was collected to perform the extraction of secondary metabolite (Hana et al., 2013).

#### **Antimicrobial activity screening of culture broth by agar well diffusion method**

Preliminary screening of antibacterial activity of culture broth was determined by using agar well diffusion method according to CLSI (Clinical and Laboratory Standards Institute, 2012) guideline. The reference bacterial strains were freshly prepared and adjusted to approximately  $10^8$  CFU/ml. Then the bacterial suspension was swabbed on surface of Mueller Hinton Agar (MHA). A hole of 7 mm in diameter was punched with a sterile cork-borer aseptically. Each well were filled with the volume 75  $\mu$ l of culture broth and incubated at 37°C for 24 hours. For all standard bacterial strains, 75  $\mu$ l of 5  $\mu$ g/ml of ciprofloxacin was used as a positive control (Solanki, Nagori, 2012), except for *Staphylococcus aureus* MRSA, 30  $\mu$ g/ml of ciprofloxacin was used (Geetha, Anitha, 2013). Tryptic Soy Broth (TSB) was used as a negative control. After incubation, the diameter zone of inhibition was measured in millimeters (mm.) and the strains of actinomycetes which have potential antibacterial activity were selected (Hana et al., 2013).

#### **Extraction of secondary metabolite**

After cultivation process, cultured medium were filtered by What-man filter paper no.1 to separate intact cell from the culture broth. Then, supernatants were extracted with ethyl acetate using the ratio of supernatant and ethyl acetate 1:1 (v/v). The organic layer was collected and evaporated to concentrate by rotary vacuum evaporator, yielding ethyl acetate crude extracts (Vimal et al., 2009).

#### **Antimicrobial activity evaluation of ethyl acetate extract by disc diffusion method**

The antibacterial susceptibility of ethyl acetate actinomycetes extracts was assayed by using agar disc diffusion method as described by Kirby-Bauer with modification according to CLSI (Clinical and Laboratory Standards Institute, 2012) guideline. The referenced bacterial strains were freshly prepared and adjusted to approximately  $10^8$  CFU/ml. Then the bacterial suspension was swabbed on surface of Mueller Hinton Agar (MHA). The ethyl acetate actinomycetes extracts was dissolved in dimethyl sulphoxide (DMSO), to produce an extract solution at a concentration of 5 mg/ml. Sterile filter paper discs of 6 mm. were impregnated with 20  $\mu$ l of extract solution which was equal to a final concentration of extract at 100  $\mu$ g/disc and allowed to air-dry. For all standard bacterial strains, 20  $\mu$ g/disc of ciprofloxacin was used as a positive control, except for *Staphylococcus aureus* MRSA, 30  $\mu$ g/disc of vancomycin was used as positive control according to CLSI (Clinical and Laboratory Standards Institute, 2007) was used as a positive control while pure DMSO was used as a negative control. The discs that impregnated with ethyl acetate actinomycetes extracts solution were put on the surface swabbed MHA plate and incubated at 37 °C for 24 hours. Antibacterial

activity evaluated by measuring the diameter of inhibition zone (mm) on the surface of plates. The zone diameter of standard antibiotic was used to compare and interpret the susceptibility of isolated actinomycetes extracts (Kumar, Venkata, 2012).

#### **Identification and phylogenetic analysis of isolated actinomycetes**

DNA of the isolated actinomycetes was isolated from single colony directly in ISP2 agar by using Colony PCR method. DNA of the isolated actinomycetes was identified by using 16S rRNA gene sequencing. PCR reagents were performed in Thermal Cycler (Biorad). PCR products were analyzed by agarose gel electrophoresis and visualization under LED transilluminator. The PCR products were purified by PCR purification kit. Purified DNA was submitted with 518F and 800R primer for sequence analysis. 16S rRNA gene sequences were aligned with Extaxon BLAST database. Phylogenetic tree was constructed by the Neighbor-joining (Saito, Nei, 1987) phylogenetic trees in MEGA program version 6 (Kumar et al., 2001). The values of branches of phylogenetic tree were determined using bootstrap (Felsenstein, 1985) analysis base on 1000 resamplings. The method was performed according to previous protocol described by Anansiriwattana et al., 2006.

### **Results**

#### **Preliminary screening for antibacterial activity**

Among the 17 isolates from Thai terrestrial environmental, only culture broth of 7 isolates showed potential antibacterial activity against only Gram-positive bacteria (see Table 1). Culture broth of isolates ATIL-8, PN51B-4-1, PN51B-9-6 and P51R-

8-1 had potential antibacterial activity against *B. subtilis* (ATCC 6633) and *S. aureus* (ATCC 6538 and ATCC 25923) with the diameter of inhibition zone ranging from 10 to 18 mm. The isolate PN47BB-4-1 and PN47BB-4-2 showed antibacterial activity against *S. aureus*, whereas P31SR-4-12 had antibacterial activity against *B. subtilis*. Only culture broth of ATIL-8 exhibited the effective anti-MRSA activity. Thus 7 isolates were selected for further studies.

#### **Morphological characterization**

The morphological characterization of 7 isolates from Thai terrestrial environment was studied by growing on ISP2 and Soil Agar Extract. The aerial mycelium color and spore morphology were shown in Table 2.

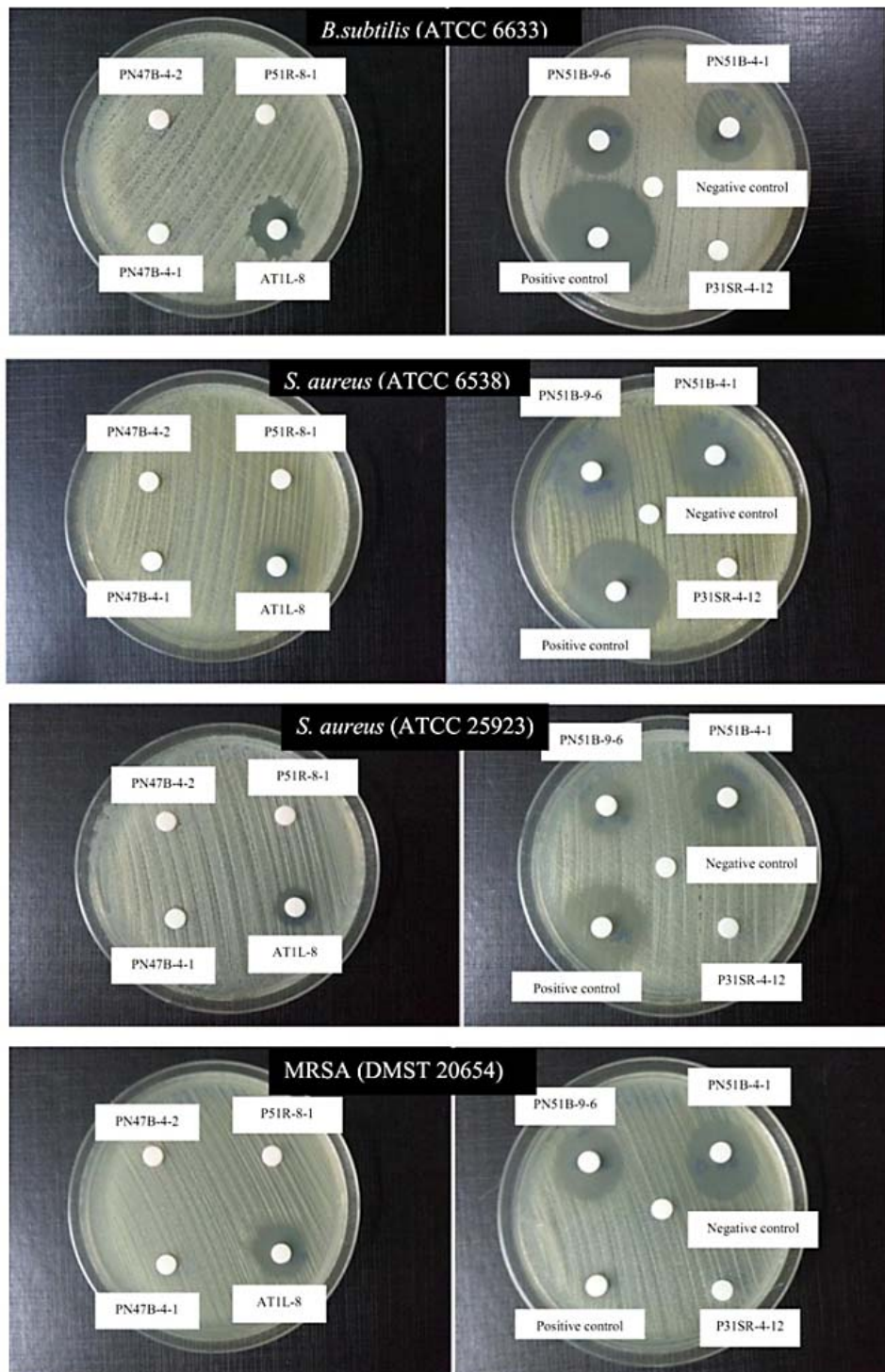
#### **Evaluation of antibacterial activity of ethyl acetate extracts of selected actinomycetes by disc diffusion method**

The ethyl acetate extracts of 7 actinomycetes were tested for antibacterial activity using agar disc diffusion method. The results showed that 3 ethyl acetate extracts obtained from ATIL-8, PN51B-4-1 and PN51B-9-6 had potential antibacterial activities against *B. subtilis* (ATCC 6633), *S. aureus* (ATCC 6538 and ATCC 25923) and MRSA (DMST 20654) (see Figure 1 and Table 3).

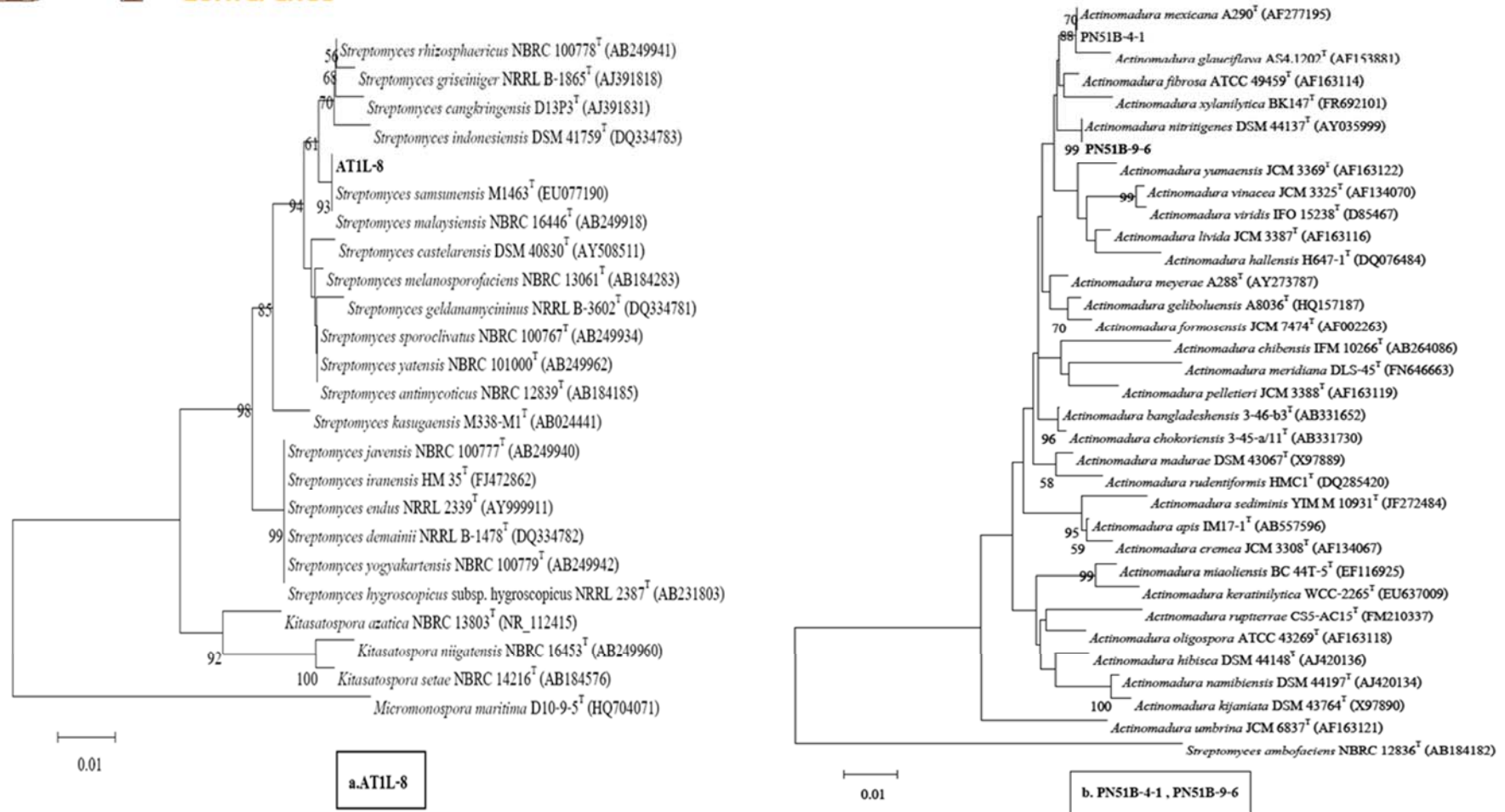
#### **Phylogenetic studies and genus identification**

The 16s rRNA sequence of 3 actinomycetes isolates ATIL-8, PN51B-4-1 and PN51B-9-6 which had potential antibacterial activity were determined using Extaxon BLAST database. The branching pattern was generated by neighbor-joining tree analysis of 16s rRNA gene sequences. The numbers on the branches indicated the percentage bootstrap values of 1,000 replicates only values >50% were indicated. Bar, 0.01 substitutions per nucleotide position. The results were shown in Figure 2a-b.





**Figure 1** Antibacterial activity of 7 ethyl acetate extracts obtained from actinomycetes isolates ATIL-8, PN47B-4-1, PN47B-4-2, PN51B-4-1, PN51B-9-6, P51R-8-1, and P31SR-4-12 using disc diffusion method



**Figure 2** Neighbor-joining phylogenetic tree based on partial 16S rRNA gene sequence of Actinomycetes strains ATIL-8, PN51B-4-1 and PN51B-9-6. Number on the connecting branches represent bootstrap values of 1,000 replications; only bootstrap values greater than 50% are shown.

**Table 1** Antibacterial activity of culture broth obtained from 7 actinomycetes isolates using agar well diffusion method.

Tested Strain	Diameter of clear zone (mm.)							
	ATIL-8	PN47B-4-1	PN47B-4-2	PN51B-4-1	PN51B-9-6	P31SR-4-12	P51R-8-1	Ciprofloxacin*
<i>B. subtilis</i> (ATCC 6633)	18	0	0	17	12	9	12	24
<i>S. aureus</i> (ATCC 6538)	16	9	0	11	10	0	15	20
<i>S. aureus</i> (ATCC 25923)	17	9	10	10	10	0	14	17
MRSA (DMST 20654)	14	0	0	0	0	0	0	22

\* 5 µg/ml of ciprofloxacin was used as a positive control for *B. subtilis* and *S. aureus*, whereas 30 µg/ml of ciprofloxacin was used as a positive control for *S.aureus* MRSA.

**Table 2** Morphological characteristics of 7 isolated actinomycetes from Thai terrestrial environment

Culture	Aerial mycelium	Reverse color	Spore morphology
ATIL-8	White	Light Grayish Yellowish Brown	Simple (S)
PN47B-4-1	Brilliant Orange Yellow	Light Yellow	Verticillate (BIV-S)
PN47B-4-2	Strong Purple Pink	Strong Yellowish Pink	Simple (RA)
PN51B-4-1	White	Yellowish White	Simple (MV-S)
PN51B-9-6	White	Pale Orange Yellow	Verticillate (MV)
P31SR-4-12	Light Bluish Gray	Deep Orange	Verticillate (MV-S)
P51R-8-1	White	Strong Red	Verticillate (MV-S)

(BIV-S): Biverticillus, (MV): Monoverticillus, (MV-S): Monoverticillus-spira, (R): Rectus, (RA): Retinaculum-Apertum, (S): Spira

**Table 3** Antibacterial activity of ethyl acetate extracts (100 µg/disc) obtained from 3 actinomycetes isolates using disc diffusion method (diameter of clear zone in mm)

Tested Strain	Diameter of clear zone (mm.)				
	ATIL-8	PN51B-4-1	PN51B-9-6	Ciprofloxacin* (20µg/disc)	Vancomycin** (30 µg/disc)
<i>B. subtilis</i> (ATCC 6633)	18	23.5	17.5	33	-
<i>S. aureus</i> (ATCC 6538)	10.5	16.5	12.5	24.5	-
<i>S. aureus</i> (ATCC 25923)	6	16	17	22	-
MRSA (DMST 20654)	14.5	18	17	-	15

\*20 µg/ml of ciprofloxacin was used as a positive control for *B. subtilis* and *S. aureus*.

\*\*30 µg/ml of vancomycin was used as a positive control for *S. aureus* MRSA.

### Discussion and Conclusions

In the present study, selected 17 isolates obtained from Thai terrestrial environmental were used for screening of antibacterial activity. Out of 17 isolates, culture broth of 7 isolates (AT1L-8, PN47B-4-1, PN47B-4-2, PN51B-4-1, PN51B-9-6, P31SR-4-12 and P51R-8-1) was found to show antibacterial activity against Gram-positive bacteria. Among those isolates, AT1L-8 exhibited moderate activity against *B. subtilis* and *S. aureus* in comparison with ciprofloxacin. Remarkably, AT1L-8 showed antibacterial capacity (14 mm of clear zone diameter) for methicillin-resistance *Staphylococcus aureus* (MRSA). All seven isolates were selected for further studies. The morphology of each isolates was characterized by growing on ISP2 and Soil Agar Extract. These strains were Gram-positive, filamentous with long spore chain except PN47B-4-2 produced single spore.

Out of seven ethyl acetate extracts of actinomycetes isolates, three isolates ATIL-8, PN51B-4-1 and PN51B-9-6 had antibacterial

activities against Gram-positive bacteria. The active ethyl acetate extract from PN51B-4-1 and PN51B-9-6 showed zone of inhibition  $\geq 15$  mm. The maximum zone of inhibition of 23.5 mm against *B. subtilis* was found for PN51B-4-1. Ethyl acetate extracts of all isolates showed activity against MRSA (14.5-18 mm) at 100 µg/disc concentration. However, none of ethyl acetate extracts showed activity against all tested Gram-negative bacteria.

Notably, the culture broth of PN51B-4-1 and PN51B-9-6 MRSA had not antibacterial activities against MRSA in a screening protocol. This might be due to a low concentration of active substances existed in crude culture broth of PN51B-4-1 and PN51B-9-6 MRSA. For AT1L-8, both crude culture broth and ethyl acetate extract produced similar antibacterial activity against MRSA. This might be due to some active substances had been lost or could not be extracted by ethyl acetate. However, it should be kept in mind that agar well diffusion and disc diffusion methods are different in basis of assay system. Thus, the antibacterial activity of culture broth by agar well antibacterial property must diffusion and ethyl acetate extracts by disc diffusion methods could not be compared directly.



The three active isolates ATIL-8, PN51B-4-1 and PN51B-9-6 was identified as *Actinobacterium* using 16S rRNA studies. The strain ATIL-8 was most closely associated with *Streptomyces samsunensis* M1463<sup>T</sup> and *Streptomyces malaysiensis* NBRC 16446<sup>T</sup> in the neighbor-joining analysis by a high bootstrap value and shared the highest 16S rRNA gene sequence similarity percentage of 100. Based on this information and analysis, ATIL-8 is categorized in genus *Streptomyces*. Strain PN51B-4-1 was most closely associated with *Actinomadura mexicana* A290<sup>T</sup> in the neighbor-joining analysis by a high bootstrap value and shared 16S rRNA gene sequence similarity percentage of 99.69 with *Actinomadura mexicana* A290<sup>T</sup>. For the strain PN51B-9-6 was closely related to *Actinomadura nitritigenes* DSM 44137<sup>T</sup> similarity percentage of 99.85. Based on this information and analysis, PN51B-4-1 and PN51B-9-6 are categorized in genus *Actinomadura*. The results of the present work indicated that among the potential 7 isolated actinomycetes, ethyl acetate extract of ATIL-8, PN51B-4-1 and PN51B-9-6 possessed effective antibacterial property against tested Gram positive bacteria, especially MRSA. The further studies will be performed on these three strains, to find out the mass production of bioactive secondary metabolites using medium and day optimization. The other solvents such as hexane, dichloromethane, chloroform, acetone, and butanol will be used for further extractions. Finally, the structure of bioactive secondary metabolites will be clarified using purification and structure elucidation.

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