Research Article

Antimicrobial Activities against Pathogenic Bacteria of Marine Actinobacteria Isolated from Mangrove Sediments at Klong Khon Mangrove Forest, Thailand

Jetsda Tongtoht[1], Jintanart Wongchawalit[1] and Ratchanee Mingma[1*]

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ABSTRACT

The mangrove ecosystem is a complex structure that harbors a wide range of microbial communities, including marine actinobacteria that have significant potential for producing bioactive compounds with various biological functions. The investigation of mangrove-associated actinobacteria for antibacterial compounds reveals them as promising candidates for new antibacterial medications and presents opportunities for other biotechnological applications. The objective of this study was to examine the antibacterial properties of culturable marine actinobacteria from mangrove sediments of Klong Khon Mangrove Forest, Thailand. Fifteen actinobacteria were recovered and evaluated the antimicrobial activity using perpendicular streak method. Eight isolates showed the ability to inhibit the growth of Gram-positive bacteria *Bacillus cereus* TISTR 687 and *Staphylococcus aureus* ATCC 27853, with the inhibition zone ranging from 13.5±1.50 to 45.0±2.65 mm. Isolates KK20-01, KK20-27 and KK20-31 had the highest antimicrobial activity against pathogenic Gram-positive bacteria *B. cereus* TISTR 687 and *S. aureus* ATCC 27853, and were chosen to assess the antibacterial efficacy of the cell-free supernatant using the agar well diffusion method. All three isolates exhibited the capacity to suppress the growth of *B. cereus* TISTR 687, and were identified using 16S rRNA gene sequence analysis, which were found to be most closely related to *Streptomyces olivaceus* NRRL B-3009^T (99.93% similarity), *Streptomyces daghestanicus* NRRL B-5418^T (100% similarity) and *Streptomyces parvulus* NBRC13193^T (99.42% similarity), respectively. Therefore, the sediments in the Klong Khon Mangrove Forest provided a rich source of streptomycetes exhibiting antibacterial properties. This demonstrates a great opportunity to carry out further investigation, considering the potential of marine actinobacteria to produce unique biologically active compounds.

Keywords: Actinobacteria, Antimicrobial activity, *Streptomyces*, Mangrove sediment

[1] Department of Science and Bioinnovation, Faculty of Liberal Arts and Science, Kasetsart University Kamphaeng Saen campus, Nakhon Pathom 73140, Thailand

[*] Corresponding author, email: faasrnm@ku.ac.th

Introduction

Actinobacteria, also known as actinomycetes, are gram-positive bacteria that can form branching filamentous structures called mycelium. These structures resemble the mycelium of fungi but are smaller in size. Although actinobacteria are bacteria, they differ from other Gram-positive bacteria as their DNA contains more than 55 percent guanine and cytosine bases [1]. Actinobacteria are incredibly versatile and can be found in various environments, including soil, water sources like freshwater and marine environments, as well as in symbiotic relationships with plants and animals [2]. Actinobacteria are important microorganisms that synthesize a diverse range of beneficial enzymes and secondary metabolites, including immunomodulators, anticancer chemicals, and antibiotics [3]. Approximately 40% of the 33,500 bioactive compounds discovered in bacteria were produced by actinobacteria, with over 10,400 originating from the actinobacteria genus *Streptomyces* [4]. These chemicals have attracted attention due to potential applications in medicine, agriculture, and industry [5].

Mangrove forest ecosystems encompass various coastal habitats such as marshes, estuaries, shoals, canals, lakes, swamps, and muddy areas. Especially in areas that are located in the tropics and subtropics [6]. Mangrove forests are complex ecosystems that are influenced by high salinity, tidal inundation, and organic matter input. These factors significantly impact the capacity of marine microorganisms, including actinobacteria, to evolve and adapt [7]. These unique environmental conditions of mangrove ecosystems also promote the development of marine actinobacteria with unique metabolic capacities that are not commonly found in terrestrial organisms [8]. Several drugs produced from marine actinobacteria, such as salinosporamide A, actinomycin D, marinomycin, vancomycin, mitomycin C and arenimycin, have demonstrated potential in preclinical studies [9]. These compounds show promise for combating bacterial infections and may contribute to the development of new antibiotics. However, the emergence of drug resistance poses a significant challenge to the effectiveness of antibiotics derived from actinobacterial sources. Therefore, investigating the antimicrobial activities of marine actinobacteria from mangrove sediments is a promising research direction that can potentially yield valuable insights and new antimicrobial compounds. It is noteworthy that more than 50% of bioactive chemicals produced by marine actinobacteria have been isolated from marine sediment and sand, followed by marine flora and fauna-associated actinobacteria [10]. Actinobacteria isolated from mangrove sediments in the General Prem Tinsulanonda Historial Park, Songkhla province showed potential antibacterial activity [11]. In China, actinobacteria isolated from mangrove soil samples collected from Futian and Maoweihai, showed antibacterial activities against “ESKAPE” bacteria [12]. In addition, *Streptomyces iconiensis* OUCMDZ-5511 having antimicrobial activity were isolated from mangrove sediment of Thai mangrove forests [13]. The objective of this study was to investigate the antibacterial activities of culturable marine actinobacteria found in the Klong Khon Mangrove Forest, a relatively understudied mangrove forest in Thailand. The exploration of mangrove-associated actinobacteria for antibacterial compounds not only contributes to our understanding of microbial diversity but also holds great potential for addressing the urgent global challenge of antibiotic resistance.

Materials and Methods

Sample collection

Mangrove sediments were collected in November 2020 from tropical mangrove forest at Mangrove Forest Conservation Center (MFCC), Klong Khon sub-district, Muang district, Samut Songkhram province, Thailand (13.331367, 99.969844). Located along the Gulf of Thailand, this mangrove forest is vital to the restoration and conservation of mangrove ecosystems in the region. The sample collection area, which is situated one kilometer from the MFCC (Figure 1), can only be reached by sailing along Klong Khon canal using a long-tail boat. Sediment samples were obtained from a single location at a depth of 10–15 cm during low tide, which would be in contact with the water during the next high tide. The samples were sealed in clean plastic bags and transported to the laboratory for actinobacteria isolation.

Figure 1 Map showing the sampling locations (yellow star) at Mangrove Forest Conservation Center (red star) at Klong Khon Mangrove Forest, Samut Songkhram, Thailand.

Isolation of actinobacteria

Soil samples (10 g) were suspended in 90 mL of sterile 0.85% (w/v) NaCl. Then, serial 10-fold dilutions were made and aliquots (0.1 mL) of each dilution was spread on starch casein (SC) agar [14] containing 1.5% (w/v) NaCl and supplemented with antibiotics ketoconazole (100 μg/mL) and nalidixic acid (25 μg/mL) to inhibit fungal and bacterial growth, respectively. The plates were incubated at 28°C for 14 days. The appearance and growth of the actinobacterial colony were observed every day. Individual colonies were picked up and sub-cultured on International *Streptomyces* Project Medium No. 2 (ISP-2) [15]. The pure colonies were assigned the KK20 code, which denotes the sampling site as Klong Khon Mangrove Forest, along with the year of sample acquisition. The spores and mycelium suspension were stored at a temperature of -20°C in a solution containing 20% (w/v) glycerol.

Cultural characterization

The morphological characteristics of the selected actinobacteria were characterized on ISP-2 agar following incubation at a temperature of 28 °C for 7 days. The color of substrate and aerial mycelium, and soluble pigment production were determined.

Primary screening for antimicrobial activities

Primary screening for the evaluating the antimicrobial activity of actinobacteria was performed by perpendicular streak method [16] against two strains of Gram-positive bacteria (*Bacillus cereus* TISTR 687 and *Staphylococcus aureus* ATCC 27853) and two strains of Gram-negative bacteria (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa*). *P. aeruginosa* was obtained from Microbiology Laboratory, Division of Microbiology, Faculty of Liberal Arts and Science, Kasetsart University, Kamphaeng Saen campus. Each isolate of actinobacteria was streaked in a linear pattern, about 2 cm away from the edge of the ISP-2 agar plate. The plates were incubated at 28°C for 7 days. Bacterial strains that were used for testing were cultured overnight in nutrient broth (NB) at a temperature of 37°C. The cells density was adjusted to 10^8 cells/mL using a spectrophotometer, with an optical density (OD_{600}) of 0.125. Subsequently, the cells were inoculated by a single streak at a 90° angle to the streak of the actinobacterial line. The plates were incubated at 37°C for 24–48 h. The antagonistic activity was determined as a distance of inhibition between the original streaks and the test organism's growth. All the experiments were performed in triplicate and the average values were considered for analysis.

Secondary screening of biological activities

The actinobacteria exhibiting the greatest inhibitory activity against pathogenic bacteria in the primary screening were chosen. A loop full of each selected isolates was inoculated to 250 mL Erlenmeyer flasks containing 50 mL of SC broth, shaking at 130 rpm. The culture medium was collected on the 7^{th} and 14^{th} days of incubation. After centrifugation at 10,000 rpm for 10 min, the supernatant was filtered through sterile filter membrane with pore size of 0.45 μm and collected in sterile centrifuge tube. The effectiveness of actinobacteria cell free supernatant to inhibit pathogenic bacteria was investigated using the agar well diffusion method. The cell suspension of the tested pathogenic bacteria was adjusted to 10^8 cell/mL as mentioned earlier and spread on the surface of Mueller-Hinton agar (Himedia, India) medium with sterile cotton swabs. Subsequently, sterile cork borers with a diameter of 0.6 cm were used to make wells and then filled with 70 μL of supernatant obtained from actinobacterial culture. The positive and negative controls were streptomycin (10 mg/mL) and SC broth, respectively. The plates were incubated at 37°C for 24 h and the inhibition zone diameter was measured in millimeters. All the experiments were performed in triplicate and the average values were considered for analysis.

Taxonomic characterization of actinobacteria

The isolates of actinobacteria that demonstrate the ability to inhibit pathogenic bacteria in secondary screening were selected to identified through 16S rRNA gene sequencing. The isolates were cultured in glucose yeast extract broth (1% glucose, 1% yeast extract) and incubated at 28°C for 5 days. Genomic DNA was extracted using the method described by Také et al. [17]. The 16S rRNA gene was amplified by PCR using primers 1F (5□-TCACGGAGAGTTTGATCCTG-3□) and 1530R (5□-AAGGAGATCCAGCCGCA-3□) [18]. PCR protocol was started with a pre-denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 1 min followed by final-extension at 72°C for 5 min. The PCR product was purified using FavorPrep™ GEL/PCR Purification Kit (Farvogen, Taiwan), according to the manufacturer's instructions. The 16S rRNA was analysis by a commercial sequencing company at Bionics (Seoul, Korea) using universal primers 27F and 1492R. The percentage of DNA similarity was determined using the EzbioCloud database (http://www.ezbiocloud.net) [19]. Phylogenetic tree was conducted according to Neighbor-Joining method [20] using MEGA 11.0 program [21]. Bootstrap analysis was performed with 1000 re-samplings. Sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/index.html) under the accession numbers [GenBank: OQ852728 - OQ852730].

Statistical Analysis

The experiments were carried out in triplicates and the data was expressed as mean value ± standard deviation. The means of all the parameters were examined for significance by software R version 4.4.0. Differences were considered significant at a probability level of $P<0.05$.

Results and Discussion

Isolation of actinobacteria

Actinobacteria was isolated by using SC agar as a selective medium. This medium is suitable for isolation of actinobacteria especially the hyphae forming [14, 15]. The characteristics of actinobacterial colony on SC agar plate were different from those of typical bacteria, such as the colony appearance which is dry and appears to be chalk or powdery. The surface colony of actinobacteria might be tough, rough, leathery, wrinkled or a short velvet-like thread that may be seen with the naked eye. In this study, total of actinobacteria fifteen isolates were obtained. The diversity was relatively low due to the use of freshly sediment without airdrying. Air-drying soil samples prior to the isolation of actinobacteria is essential to reduce the number of fast-growing bacteria in the soil, as actinobacteria have a slow growth rate. However, the number of obtained actinobacteria were consistent with the reports from other researchers. For example, Gong *et al*. [22] had isolated actinobacteria from soil of nine sampling sites at Maowei Sea Mangrove Reserve, China, and the lowest and highest number of obtained actinobacteria were three and sixteen isolates, respectively. Similarly, Rozirwan et al. [23] isolated total of 10 actinobacterial isolates from mangrove sediment in Tanjung Api-Api, South Sumatra,

Indonesia. However, number of isolated actinobacteria from this study were less than many other studies [24-26].

Primary screening of antimicrobial activities

The antimicrobial activity showed that among the 15 actinobacteria isolated, 8 (53.3%) isolates exhibited antibacterial activity against at least one of the tested microorganisms. As shown in Table 1, all active isolates were against *B. cereus* TISTR 687 and six isolates were against *S. aureus* ATCC 27853. None of the actinobacterial isolate exhibited the growth of the Gram- negative bacteria, *P. aeruginosa* and *E. coli* ATCC 25922. A similar study conducted by Abidin *et al*., [26] found that 28 isolates (35.9%) of the total active isolates from Pahang mangrove forest demonstrated antagonistic activity against *B. subtilis* and *S. aureus* (Gram-positive bacteria), but not Gram-negative bacteria. The results of the study were consistent with previous studies that show the antimicrobial activity of actinobacteria were more against Gram-positive bacteria than Gram-negative bacteria [27-30]. Gram-negative bacteria typically possess greater resistance to other antimicrobial compounds than Gram-positive bacteria due to the distinct composition of their cell walls. Both Gram-positive and Gram-negative bacteria possess cell wall peptidoglycans, but the layer of peptidoglycan in Gram-positive bacteria are many times thicker than those of Gram-negative bacteria. Furthermore, the cell walls of Gram-negative bacteria also contain lipopolysaccharide to protect the bacteria from antibiotics [31]. However, the antibacterial activity of compounds, especially those derived from natural sources, is indeed influenced by several factors, including the method of testing, the type of bacteria, and the level of resistance of the tested microbes. Isolate KK20-01 exhibited the greatest inhibition (45.0±2.65 mm) against *B. cereus* TISTR 687, followed by isolate KK20-31 (29.5±3.12 mm) while isolate KK20-27 displayed highest inhibition (28.0±1.32 mm) against *S. aureus* ATCC 27853, followed by KK20-01 (23.0±2.00) (Figure 2). Hence, these three isolates are suitable candidate for investigation of the effectiveness of cell-free supernatant against bacterial pathogens in the next step.

Previously research have reported the actinobacteria isolated from mangrove sediments that possess antimicrobial activity. Sangkanu *et al*. found that among 118 actinobacterial isolates, 15 and 6 isolates exhibited significant activity against *S. aureus* (with inhibition zones ranging from 15.4 to 36.5 mm) and *E. coli* (16.4 - 23.6 mm), respectively. None of the isolates could inhibit *P. aeruginosa* [11]. The actinobacteria isolate M20, which was isolated from rhizosphere of *Avicennia marina* in mangrove forest in India, showed broad spectrum antibacterial activity using cross streak method against *P. aeruginosa* (32 mm), *B. subtilis* (32 mm), *S. aureus* (30 mm), *E. coli* (22 mm) and *P. fluorescens* (12 mm) [32]. Abidin et al. studied the antagonistic activity of actinobacteria from mangrove forest of Pahang, Malaysia. Among 140 isolates, 78 isolates exhibited antibacterial and antifungal activity. Isolate K7-11 displayed strong inhibition (27.0 ± 0.0 mm) against *E. coli* and the largest zone of inhibition against *B. subtilis*, *S. aureus*, *Serratia marcescens* and *Candida albicans* (>30 mm) [26]. It was revealed that mangrove sediments are a promising source of actinobacteria that produce bioactive compounds against pathogenic bacteria.

Table 1 Antagonistic potential of active actinobacteria isolates against pathogenic bacteria.

Isolate	Inhibition zone (mm)			
	***Bacillus cereus* TISTR 687**	***Staphylococcus aureus* ATCC 27853**	***Pseudomonas aeruginosa***	***Escherichia coli* ATCC 25922**
KK20-01	45.0 ± 2.65[a]	23.0 ± 2.00[b]	-	-
KK20-06	18.0 ± 1.73[d]	20.0 ± 0.50[d]	-	-
KK20-07	25.5 ± 6.14[bc]	-	-	-
KK20-14	23.0 ± 1.00[c]	-	-	-
KK20-20	25.5 ± 1.80[bc]	15.0 ± 1.00[c]	-	-
KK20-25	27.0 ± 1.00[bc]	14.5 ± 1.32[d]	-	-
KK20-27	26.0 ± 1.73[bc]	28.0 ± 1.32[a]	-	-
KK20-31	29.5 ± 3.12[b]	13.5 ± 1.50[d]	-	-

This table demonstrates average ± SD (n=3) and different letters in the same column represent significant differences among treatments (Duncan's test, $p < 0.05$). (-) = does not inhibit test bacteria.

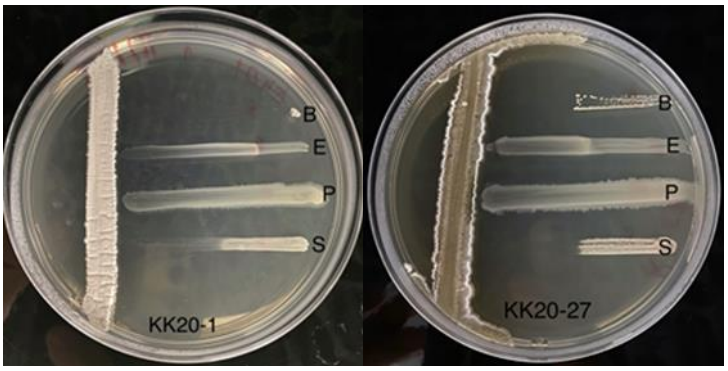

Figure 2 Primary screening using perpendicular streak method for antimicrobial activities of actinobacteria isolates KK20-01 (left) and KK20-27 (right).
Note: B, *B. cereus* TISTR 687; E, *E. coli* ATCC 25922; P, *P. aeruginosa*; S, *S. aureus* ATCC 27853

Secondary screening of biological activities

The efficacy of culture filtrate supernatant from isolates KK20-01, KK20-27, and KK20-31 in inhibiting pathogenic bacteria was assessed after incubation for 7 and 14 days in SC broth. The finding indicated that cell free supernatant of all isolates had inhibition activity against *B. cereus* TISTR 687 but did not demonstrate inhibition against *S. aureus* ATCC 27853, *P. aeruginosa* and *E. coli* ATCC 25922. The results suggested that *B. cereus* TISTR 687 were more susceptibility to bioactive compound produced by selected actinobacteria.

After 7 days of incubation, the cell-free supernatant of isolate KK20-01 showed antibacterial activity against *B. cereus* TISTR 687 with an inhibition zone of 22.5 ± 0.87 mm. On the 14th day,

the inhibition zone increased to 24.5 ± 1.80 mm (Table 2). Furthermore, the cell-free supernatant of two other isolates, KK20-27 and KK20-31, did not exhibit any inhibitory effect on the 7th day. However, an inhibition zone with a diameter of 15 mm was detected on the 14th day against *B. cereus* TISTR 687. The antibacterial activity of the culture filtrate was enhanced in proportion to the duration of incubation. This suggests that increase of incubation time led to increase of inhibition zone. Normally, the production of secondary metabolite of actinobacteria were occurred during in the stationary phase of growth [33]. According to AL-Fassi *et al*. [34], the formation of biomass was increased at the first 4 to 7 days of incubation, whereas the maximal production of antibiotic was recorded in the stationary phase of growth. Actinobacterial species exhibit variability in their generation times and growth curves. For example, *Streptomyces* sp. SUK 48 growing in nutrient broth (pH 7.0, shaking at 160 rpm, 28°C) reached the stationary phase and revealed the antimicrobial activity on day 14 (4.56 h/generation) whereas *Streptomyces* sp. SUK 12 grew faster and reached the stationary phase on day 4 [35]. Growth curves of actinobacterial species can vary depending on factors like nutrient requirements, metabolic pathways and regulatory mechanisms.

The active isolates from the primary screening exhibited different activity when subjected to secondary screening, some active isolates did not show the activity in the secondary screening. According to Bushell et al. [36], during secondary metabolite screening, actinobacterial isolates that exhibit antibiotic action on agar but not in liquid culture are frequently observed. This could be due to differences in medium composition, inoculum size, and incubation time in primary and secondary screening. It is also possible that the active chemicals secreted by actinobacteria become inactive in the culture broth [37].

Table 2 Secondary screening of biological activities of supernatant collected 7th and 14th days of incubation using agar well diffusion method.

Isolates	Inhibition zone diameter (mm)							
	B. cereus		*S. aureus*		*P. aeruginosa*		*E. coli*	
	7	14	7	14	7	14	7	14
KK20-01	22.5 ± 0.87	24.5 ± 1.80[a]	-	-	-	-	-	-
KK20-27	-	15.0 ± 2.00[b]	-	-	-	-	-	-
KK20-31	-	15.0 ± 2.64[b]	-	-	-	-	-	-

This table demonstrates average ± SD (n=3) and different letters in the same column represent significant differences among treatments (Duncan's test, $p < 0.05$). (-) = does not inhibit test bacteria.

Identification of the active actinobacteria

The colony morphology of the active isolates KK20-01, KK20-27, and KK20-31 were investigated after 7 days of cultivation on ISP-2 medium at 28°C. All isolates had gray of mature spore mass. Isolates KK20-01 and KK20-27 exhibited brown substrate mycelia, while isolate KK20-31

displayed brownish yellow substrate mycelia. No diffusible pigment was found. The spore chain was straight to flexuous (Figure 3).

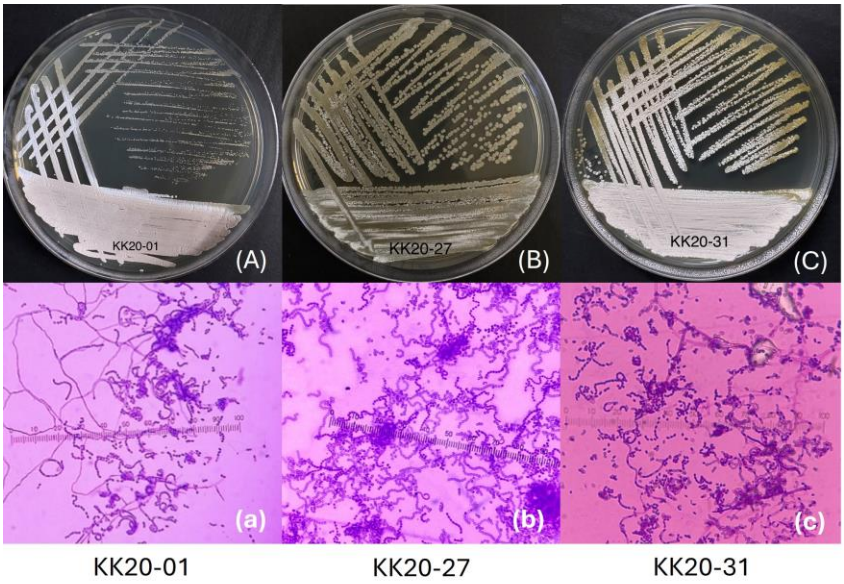

Figure 3 The morphological colony (A-C) and spore chain morphology under a 1000X magnification light microscope (a-c) of marine actinobacteria after 7 days on ISP-2 medium.

Based on the analysis of 16S rRNA sequencing, it was found that isolates KK20-01, KK20-27 and KK20-31 belonged to the genus *Streptomyces* with the similarity percentage higher than 99% (Table 3). The phylogenetic tree of 16S rRNA gene sequences constructed from the Neighbor-Joining (NJ) method also supported the relationships between the isolates with *Streptomyces* sp. (Figure 4). Isolates KK20-01, KK20-27 and KK20-31 showed the highest similarity to *Streptomyces olivaceus* NRRL B-3009^{T} (99.93%), *Streptomyces daghestanicus* NRRL B-5418^{T} (100%) and *Streptomyces parvulus* NBRC 13193^{T} (99.42%), respectively. *Streptomyces* are well known as broad spectrum antibacterial producers. Previous investigations have indicated that these *Streptomyces* strains are capable of producing various bioactive compounds and antibiotics. For example, dixiamycins produced by *Streptomyces olivaceus* OUCLQ19-3 and actinomycin D produced by *S. parvulus* Av-R5 showed antibacterial activity against multi-drug-resistant (MDR) strains [38, 39]. Cyclopentene, an antimicrobial compound produced by *Streptomyces olivaceus* LEP7 inhibited wound inhabiting microbial pathogens *E. coli*, *P. aeroginosa*, *S. aureus*, *Klebsiella* sp., *Acinetobacter* sp., and *Candida* sp. [40]. Moreover, *Streptomyces* was the predominant actinobacteria discovered in mangrove sediments. Sangkanu et al. isolated a total of actinobacteria 118 isolates from mangrove sediments in the South of Thailand, out of which 71 (60.2%) were identified as *Streptomyces.* Out of these, three *Streptomyces* strains showed a broad spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria [11]. Gong et al. also reported that *Streptomyces* was the most abundant genus of isolated actinobacteria from Maowei Sea Mangrove Reserve, China. Three strains, including one of *Streptomyces griseorubens* and two of *Streptomyces parvus*, shown significant efficacy against drug-resistant clinical strains [41]. From

the results of this work, it is shown that actinobacteria from mangrove sediments in Thailand is another promising bioresource of bioactive compounds. This demonstrates a great opportunity to carry out further investigation in this fields, considering the potential of marine actinobacteria to produce unique anti-infective substances for application in biotechnology.

Table 3 Identification results of 16S rRNA genes analysis of isolates KK20-01, KK20-27 and KK20-31.

Isolates	The most closely related strain	Similarity (%)	No. nucleotides difference/ Total no. nucleotides comparison
KK20-01	*Streptomyces olivaceus* NRRL B-3009^{T} (JOFH01000101)	99.93	1/1401
KK20-27	*Streptomyces daghestanicus* NRRL B-5418^{T} (DQ442497)	100.00	0/1380
KK20-31	*Streptomyces parvulus* NBRC 13193^{T} (AB184326)	99.42	8/1378

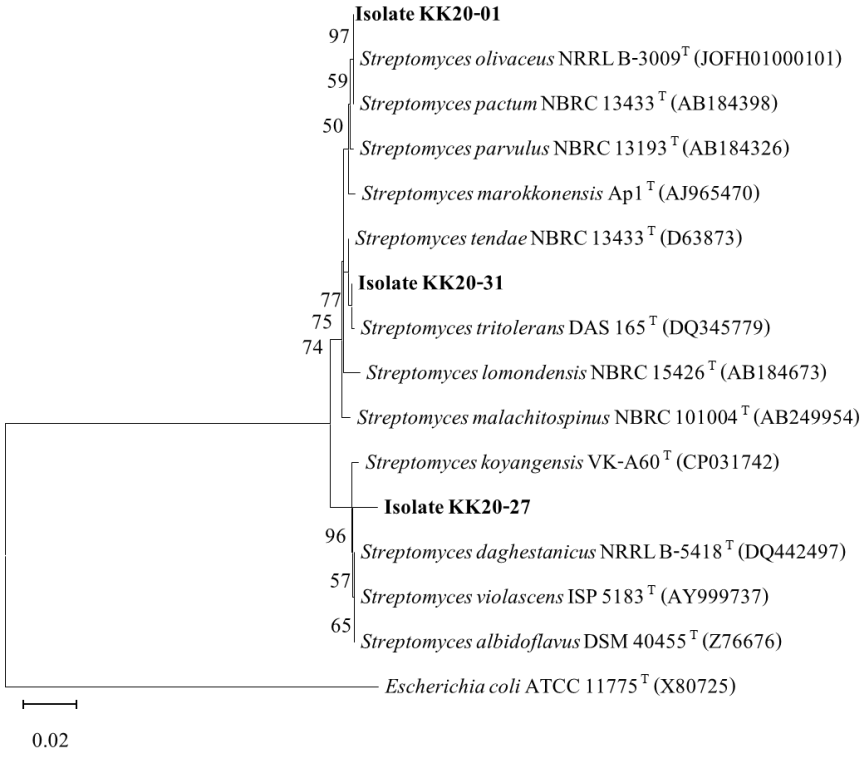

Figure 4 Phylogenetic tree constructed using the neighbors-joining method, showing the phylogenetic position of the actinobacteria strains among related species.

Conclusions

Mangrove sediment harbor fifteen actinobacteria were recovered from mangrove sediments collected from Klong Khon Mangrove Forst, Samut Songkhram province, Thailand, and subjected to primary screening for antimicrobial activity. Eight and six isolates showed antibacterial activity against *B. cereus* TISTR 687 and *S. aureus* ATCC 27853, respectively, while they showed no activity against *P. aeruginosa* and *E. coli* ATCC 25922. Isolates KK20-01, KK20-27 and KK20-31 showed good antibacterial activity, and were further tested in a well-diffusion assay. The cell-free supernatants of all three actinobacterial isolates exhibited inhibitory activities against *B. cereus* TISTR 687. Based on 16S rRNA analysis, isolates KK20-01, KK20-27 and KK20-31 belonged to the genus *Streptomyces*. The present study demonstrated that Klong Khon mangrove forest is a valuable source of actinobacteria with promising antibacterial activity. Further research is required to optimize the culture conditions and extraction the bioactive compounds in order to enhance the yield of bioactive substances, which have potential uses in medicine, agriculture, and biotechnology.

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