BIODIVERSITAS
Volume 25, Number 5, May 2024
Pages: 2221-2229

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d250540

Antibiotic production by an endophytic *Streptomyces* isolated from the medicinal plant *Poikilospermum suaveolens*

ALIMUDDIN ALI[1,♥], MUHARRAM PASSITTA[2], HERLINA RANTE[3], ELLY WAHYUDIN[3], NANA JUNIARTI NATSIR DJIDE[3], ROLAND JANSEN POLITAN[4], ELYZA AIMAN NUR[4], SATORU SHIGENO[4,5], SATOSHI OHTE[4,5], KEISUKE KOBAYASHI[4,5], KANJI HOSODA[6], HIROSHI TOMODA[6], TAICHI OHSHIRO[4,5]

[1]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Makassar. Jl. Daeng Tata, Makassar 90222, South Sulawesi, Indonesia. Tel.: +62-411-864936, Fax.: +62-411-880568, ♥email: muddin_69@unm.ac.id
[2]Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Negeri Makassar. Jl. Daeng Tata, Makassar 90222, South Sulawesi, Indonesia
[3]Faculty of Pharmacy, Universitas Hasanuddin. Jl. Perintis Kemerdekaan KM 10, Makassar 90245, South Sulawesi, Indonesia
[4]Department of Microbial Chemistry, Graduate School of Pharmaceutical Sciences, Kitasato University. Tokyo 108-8641, Japan
[5]Medicinal Research Laboratories, Graduate School of Pharmaceutical Sciences Kitasato University. Tokyo 108-8641, Japan
[6]Laboratory of Drug Discovery, Graduate School of Pharmaceutical Sciences, Kitasato University. Tokyo 108-8641, Japan

Manuscript received: 10 January 2024. Revision accepted: 25 May 2024.

Abstract. *Ali A, Passitta M, Rante H, Wahyudin E, Djide NJN, Politan RJ, Nur EA, Shigeno S, Ohte S, Kobayashi K, Hosoda K, Tomoda H, Ohshiro T. 2024. Antibiotic production by an endophytic* Streptomyces *isolated from the medicinal plant* Poikilospermum suaveolens. *Biodiversitas 25: 2221-2229.* This study investigates the antibiotic production potential of endophytic actinomycetes isolated from *Poikilospermum suaveolens* plants. Samples were collected from various parts of the plant in karst ecosystems. Endophytic actinomycetes were cultured and screened for antimicrobial activity using agar diffusion and liquid microdilution methods. One of the 11 isolated strains exhibited significant antibiotic activity against clinically relevant organisms identified as belonging to the *Streptomyces* genus, specifically *Streptomyces mirabilis* strain NRRL ISP-5169. Submerged fermentation of this strain over 9 days showed strong antagonistic effects against Gram-positive bacteria. Spectroscopic analyses confirmed the presence of actinomycin D as the bioactive compound. This study provides on the previously unexplored potential of endophytic actinomycetes from *P. suaveolens* as a promising resource for antibiotic discovery.

Keywords: Actinomycin, antimicrobial, *Poikilospermum suaveolens*, *Streptomyces-endophytic*

INTRODUCTION

Actinomycetes, Gram-positive bacteria with filamentous growth and high G+C DNA content, are found ubiquitously in air, terrestrial, and aquatic habitats (Barka et al. 2015). Particularly, the genus *Streptomyces* is renowned for producing the majority of known microbial antibiotics (de Lima Procópio et al. 2012). This species synthesis of a wide array of biologically active metabolites, including antitumor, immunomodulatory, hypercholesterolemic, antiparasitic, insecticidal, and anti-inflammatory compounds (Al-Ansari et al. 2019; Quinn et al. 2020; Selim et al. 2021). In clinical practice, *Streptomyces* contribute to nearly 75-80% of these compounds (Carvalho et al. 2016; Crnovčić et al. 2013)

Moreover, it was previously reported that exploring actinomycetes produces novel bioactive metabolites for clinical utilization has had restricted success, and repeated isolation of known compounds has become a foremost challenge (Qin et al. 2009). Numerous investigations have focused particularly on endophytic actinomycete within medicinal plants and the biotechnological utilization of these microorganisms (Janardhan et al. 2014). Endophytes are currently relevant in the biotechnology and industrial fields due to their ability to generate secondary metabolites as antimicrobial, biocontrol, immunosuppressant, and antitumor agents (Gouda et al. 2016). Furthermore, they also play a role in natural antioxidants, antibiotics, antidiabetic agents, and insecticidal product development (Yadav et al. 2018). These reports show actinomycetes, including medicinal plant isolates, are a potential source of new antimicrobial metabolites (Passari et al. 2015). According to previous studies, several actinomycetes are able to form intimate relations with plants and consequently colonize the inner tissues. Almost all plants have been discovered to experience infestation from one or more endophytes (Qin et al. 2009; Golinska et al. 2015).

As previously reported, actinomycetes isolated from medicinal plants have been identified as sources of many secondary biologically active metabolites (Gos et al. 2017; Savi et al. 2015); these metabolites are potentially associated with the medicinal activities of the host plants (Saini et al. 2016). However, there have been no reports on endophytic actinomycetes isolated from *Poikilospermum suaveolens*.

In this study, the endophytic actinomycetes isolated from a medicinal plant, *P. suaveolens*, analyzed the profile of metabolites of endophytic actinomycetes and purified a bioactive compound against clinical pathogens.

MATERIALS AND METHODS

Sample collection and Isolation of endophytic Actinomycetes

This study used 5-10 cm long plant tissue (root, leaf, and stem bark) obtained from limestone soil ecosystem in Maros District (South Sulawesi, Indonesia) and used as source material for endophytic Actinomycetes isolation. The plant tissues were cut with a cutter blade, placed in clean polyethylene bags, washed with tap water to remove debris and soil and cut into 2-4 cm long pieces. Finally, the plant tissues were cut into small pieces (0.2×2 cm^2). Subsequently, the surface of their plant tissues was sterilized using serial treatment of 70 % EtOH for 10 minutes, treated with 5% sodium hypochlorite for 10 minutes, and washed thrice with sterilized distilled water. After air-drying, their specimens (15 mm) were mashed with a sterile pestle and mortar. This was followed by adding about 1 mL of an aliquot of each plant tissue's suspension onto a starch casein (SC) agar plate (1% soluble starch, 0.2% K_2HPO_4, 0.2% NaCl, 0.2% KNO_3, 0.03% casein, 0.001% $FeSO_4.7H_2O$, 0.005% $MgSO_4.7H_2O$, pH 7±0.1). After sterilization, the media was supplemented with 100 µg/mL nystatin, spread using a hockey-stick glass spreader, and then incubated. The endophytic actinomycetes colonies appearing after an incubation period of 1-3 weeks at 30°C were purified. Colonies displaying characters peculiar to Actinomycetes morphologies were selected, purified, and utilized for further studies. Subsequently, the strain pure cultures were stored at -80°C as 15% (v/v) glycerol stock.

Validation of the surface sterilization protocol

Therefore, to validate the surface sterilization process and prove the strain was indeed obtained from plant specimen internal tissue, about 0.1 mL of the soaked suspension was spread on SC agar plate. Subsequently, the plates were incubated for a week at 30°C and observed for actinomycetes growth. The protocol was considered effective in cases where no colony growth was observed on SC agar after the final incubation time.

Preliminary screening on the isolated actinomycete strains for antibacterial activity

This screening was conducted using an agar diffusion assay. The pure culture strain was streaked on starch nitrate (SN) agar plates and incubated at 30°C for 9 days. The agar block (6 mm diameter and 3 mm thickness), cut out from pure Actinomycete culture plate, was transferred on nutrient agar (NA) plates that had been inoculated with the test microbes *Micrococcus luteus* FDA209P, *Bacillus subtilis* PC1219, *Staphylococcus aureus* KB212, *Pseudomonas aeruginosa* IFO12689, and *Escherichia coli* JM109. Furthermore, the positive control consisted of agar blocks cut from a blank plate containing chloramphenicol (20 mg/mL), whereas the negative control comprised agar blocks cut from a blank plate containing sterilized water. Following incubation of the assay plate for 24 hours at 30°C, the diameter of the clear zone surrounding each agar block was measured. This process was repeated three times, and strains exhibiting the greatest inhibition activity during each repetition were selected for subsequent investigation.

Secondary screening of the isolated actinomycete strains for antibacterial activity

After preliminary screening, the actinomycete strains with the strongest inhibition activity were subjected to further screening for antibacterial-producing ability. For this screening, 25 mL of a selected strain spore suspension (about 10^6-10^7 spores mL^{-1}) was transferred to 75 mL of Starch Nitrate broth and incubated for 9 days at 30°C. This was followed by centrifugation for 15 min at 7,000 rpm, then pellet separation from supernatant. The supernatant was subsequently extracted with ethyl acetate, shaken vigorously for an hour, and then separated with a separation funnel. The organic layer was then concentrated in a rotary evaporator, and the dried crude extract's antibacterial activity against test microbes was evaluated using a paper disc diffusion assay, as de Zoysa et al. (2019) described.

In this screening process, the bacterial cultures under test (at a concentration of 10^7-10^8 colony-forming units per milliliter) were evenly spread onto the surface of solidified nutrient agar media and subsequently swabbed using a sterile cotton swab. Simultaneously, sterile paper discs with a diameter of 6 mm were impregnated with 25 µL of crude extract, prepared as a 1 mg/mL solution in sterile 10% dimethyl sulfoxide (DMSO), and positioned on the nutrient agar plates. Tetracycline (30 µg/disc) was the positive control, while a 10% DMSO solution was used as the negative control. Following a 24-hour incubation period at 30°C, the inhibition zone surrounding each paper disc was observed and quantified. This protocol was replicated three times, and measurements were taken after each experiment iteration.

Metabolite production of the selected actinomycete strains

The selected actinomycete strain was inoculated into four 100 mL Erlenmeyer flasks containing 250 mL of seed starch nitrate-broth medium. The Erlenmeyer flask, inoculated and set in agitation on a rotary shaker at 100 rpm, underwent incubation at 30°C for 5 days to cultivate the seed culture. Subsequently, for the production of active compounds, the culture was inoculated by transferring 400 mL of the seed culture to a fermenter with a capacity of 5 L containing 4,000 mL of production medium. Fermentation was performed on the fermenter at 30°C for 14 days. After the fermentation time was achieved, the supernatant was separated from the biomass using a thin cloth and filtered with filter paper. The supernatant was then extracted using ethyl acetate (1:1 v/v), while the macerate was concentrated with a rotary evaporator. This was followed by collecting the semisolid macerate for active compound purification.

Purification and identification of bioactive metabolite

The active crude extracts were dissolved in ethyl acetate and fractionated using vacuum column chromatography (Merck, silica gel G 60 F254 7730) with hexane, ethyl acetate, and methanol. Meanwhile, the active combined fractions were partially purified using

preparative TLC (Merck, Silica gel GF 60, 0.5 mm) with ethyl acetate and methanol (7:4) as mobile phase.

Various analyses were performed to identify the chemical structure of bioactive compound: Diverse NMR spectra were acquired using the NMR System 400 MHz spectrometer (Agilent Technologies, Santa Clara, CA, USA). Mass spectrometry (MS) analysis utilized the AccuTOF LC-plus JMS-T100LP system (JEOL, Tokyo, Japan). Ultra-Fast Liquid Chromatography (UFLC) analysis (Prominence, Shimadzu, Kyoto, Japan) was performed under the specified conditions: the column used was Shim Pack XR-ODS (Shimadzu), measuring 2.0 x 75 mm; column temperature was maintained at 50°C; the solvent composition underwent a 6-minute linear gradient transition from 5.0% acetonitrile in 0.10% phosphoric acid to 95% acetonitrile in 0.10% phosphoric acid; the flow rate was set at 0.55 ml/min; and detection was performed UV at a wavelength of 210 nm. Finally, based on spectral data, the compound's chemical structure was searched and identified using CAS SciFinder^{n} (American Chemical Society, Columbus, OH, USA).

Antimicrobial activity of bioactive compound by agar diffusion assay

Antimicrobial evaluations against six microorganism species were conducted using the agar diffusion method with paper disks (Uchida et al. 2016). Briefly, the media for the microorganisms were as follows: Nutrient Agar (Sanko Junyaku, Tokyo, Japan) for *Bacillus subtilis* PCI219, *Staphylococcus aureus* FDA209P, *Micrococcus luteus* KB212, *Escherichia coli* JM109, and *Pseudomonas aeruginosa* IFO12689; and a medium comprised of 4.0% glucose, 1.0% peptone, and 1.5% agar for *Saccharomyces cerevisiae* S288c. A paper disk (inner diameter: 6 mm; Toyo Roshi Kaisha, Tokyo, Japan) containing a sample (10 µg) was placed on the agar plate. Bacterial cultures were incubated at 37°C for 24 hours, while *C. albicans* was incubated at 27°C for 48 hours. The antimicrobial activity was presented by measuring the diameter (mm) of the inhibitory zone.

Antimicrobial activity by liquid microdilution assay

Antimicrobial assessments against three *Mycobacterium* species were conducted using liquid microdilution (Hosoda et al. 2019). Briefly, suspensions of *M. avium*, *M. intracellulare*, and *M. abscessus* were adjusted to $4.0x10^{6}$-$1x10^{7}$ cfu/mL in Middlebrook 7H9 broth supplemented with 0.05% Tween 80 and 10% ADC enrichment. Each well of a 96-well microplate (Corning) added a 95 µL aliquot of the suspension, with or without the inclusion of test drugs (5 µL in MeOH or water), and the plates were then incubated at 37°C for 72-120 hours. MTT reagent (5.5 mg/mL MTT, 5 µL) was subsequently introduced to each well, and the cells were incubated for 16 hours. Following cell lysis with a buffer solution (40% N, N-dimethylformamide, Nacalai Tesque, Kyoto, Japan; 20% SDS, Wako Pure Chemical Industries; 2% CH_3COOH, Kanto Chemical, Tokyo, Japan; 95 µL), the absorbance of the lysate was quantified at 570 nm using an absorbance spectrometer.

Selected actinomycetes strain characterization

The selected actinomycetes strain was studied based on colony growth pattern, spore chain ornament, aerial color, and substrate on various *International Streptomyces Project* (ISP) media. Meanwhile, the strain's ability to utilize nitrogen and carbon was evaluated on ISP9 medium (Shirling and Gottlieb 1966). The isolate's growth in several sodium chloride concentrations (2 to 9%), optimal temperatures (4, 25, 37, 40, and 45°C), and pH (5, 6, 7, 8, and 9) were also evaluated.

Phylogenetic tree of selective strain 16S rDNA sequences

The strain's total genomic DNA extraction was conducted using a method described by Green and Sambrook (Green and Sambrook 2014). Furthermore, the 16S rDNA sequence was PCR-amplified using the (16S rRNA): 27f (5'-AGAGTTTGATCCTGGCTCAG-3')/1492r (5'-GGTTACCTTGTTACGACTT-3') primers. The amplification was performed for 30 cycles (97°C for 5 minutes, 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 2 minutes, and final extension at 72°C for 10 minutes). Subsequently, the DNA fragments' amplicon was sequenced using ABI 3100 sequencer model and following the manufacturers' directions (ABI PRISMA 3100 Genetic Analyzer User's Manual). The sequences were aligned with the most closely related strains of the 16S rRNA gene sequence within the Actinomycetes group, retrieved from GenBank using the BLAST program (http://www.ncbi.nlm.nih.gov). Additionally, multiple sequence alignments were performed with Clustal X program (Thompson et al. 1997), while phylogenetic tree calculation was conducted through the Neighbor-joining method (Hong et al. 2020), inferred by using suitable programs of the PHYLIP (phylogeny inference package) version 3.68 (Felsenstein 2008). Meanwhile, individual branches were identified through bootstrap analysis, employing 1000 random samplings. This study deposited the partial 16S rRNA gene nucleotide sequences in the DNA databank (https://www.ncbi.nlm.nih.gov/) under the corresponding accession number OP218017.

RESULTS AND DISCUSSION

Actinomycetes isolation and antibacterial potential screening

A total of 11 actinomycetes isolates were recovered from *P. suaveolens* plant tissues based on the colony color morphological trait (Table 1). Colonies exhibiting similar characteristics were regarded as the same strain to avoid duplication. According to the isolate's morphological characterization, nine *Streptomyces* spp. and two *non-Streptomyces* were recovered from *P. suaveolens* plant.

Table 1. Preliminary and secondary screening of actinomycetes isolated from *P. suaveolens* plant tissue

Stain code	Inhibition zone												Presumptive genus	Plant tissue
	Gram-positive						Gram-negative							
	B.sub		*S.aur*		*M.lut*		*E.col*		*P.aer*		*S.typ*			
	P	S	P	S	P	S	P	S	P	S	P	S		
MRK01NP	-		-		-		-		-		-		*Streptomyces*	Stem bark
MRK01PY	+++	+++	+++	+++	-		-		-		-		*Streptomyces*	Root
MRK05NP	-		-		-		-		-		-		*Streptomyces*	Root
MRK08NP	-		-		-		-		-		-		*Non-Streptomyces*	Stem bark
MRK17NP	+	-	++	-	-	-	-		-		-		*Streptomyces*	Root
MRK18NP	-		-		-		-		-		-		*Streptomyces*	Stem bark
MRK20NP	-		-		-		+	+	++	+	+	+	*Streptomyces*	Leaves
MRK24NP	-		-		-		-		-		-		*Non-Streptomyces*	Root
MRK32NP	++	++	+	+	-	-	-		-		-		*Streptomyces*	Root
MRK34NP	-		-		-		-		-		-		*Streptomyces*	Root
MRK57NR	-		-		-		-		-		-		*Streptomyces*	Root

Note: *Bacillus subtilis*, *Micrococcus luteus*, *S. aureus*, *E. coli*, *P. aeruginosa*, P: preliminary screening, S: Secondary screening. (+++/>20 mm): very strong inhibition; (++/10-20 mm): moderate inhibition; (+/5-10 mm): weak inhibition; (−): no inhibition

Table 2. The selective strain MRK01PY's morphological and physiological traits on different media

Media	Morphological of hyphae		Growth
	Aerial hyphae mass color	Reverse side color (Substrate hyphae)	
ISP2	White	Brown	+++
ISP4	White	Yellow	+
ISP5	Grey	Brown	+
MHA	Grey	Brown	+++
NA	White	Yellowish	+++
SNA	Grey	Yellow	+++
MGYA	Grey	Yellowish	+++

Carbon source utilization

Glucose	Sucrose	Arabinose	Xylose	Fructose
+++	++	-	+	++

Nitrogen source utilization

KNO_3	$(NH_4)_2SO_4$	$NaNO_3$	Urea	DL-amino-n-butyric acid
+++	+++	-	+	++

NaCl tolerance (%)

0	2.5	5	7.5	10
+++	++	+	-	-

pH growth

5	6	7	8	9
-	+	+++	++	-

Temperature

4	25	37	40	45
-	++	+++	++	-

Note: The symbol, + representing the positive reaction/presence of growth, – representing the negative reaction/absence of growth

The active strains exhibited adequate growth in all tested media, producing aerial mycelia with white to whitish-grey color, with ISP2 being the most suitable medium. In addition, diffusible pigments were absent in all tested media except strain MRK01PY. This implies the pigment diffuses into the surrounding medium on any medium used. A subsequent light microscopic analysis showed the strains possessed spiral spore chains (Figure 1).

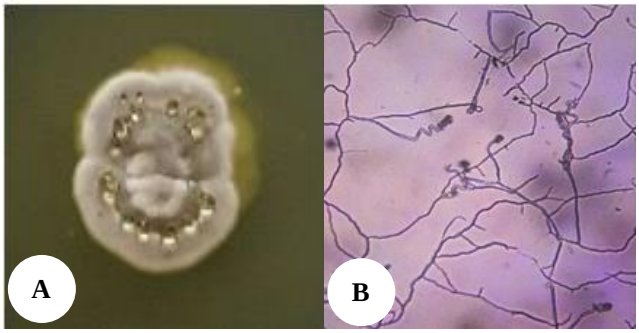

Figure 1. Morphological characteristics (A) and spore chain ornament of active strains MRK01PY (B) producing antibacterial metabolite examined by using a light microscope (400X)

The biochemical and physiological evaluation showed the strain was able to grow within NaCl solution of 0 to 5% concentration. Actinomycetes grouped based on NaCl tolerance as slight (2-3%), moderate (5-20%) or extreme (above 12%) tolerance. In addition, maximum growth occurred at pH 8, while no activity was observed at pH 9. The growth temperature range was determined on ISP2 broth with a temperature gradient incubator (25-40°C). Meanwhile, utilization of the sole carbon and nitrogen sources was analyzed using the medium (ISP9). Subsequently, selective strain growth was observed under several carbon sources, including sucrose, glucose, fructose, and xylose; however, arabinose was not utilized. Also, Urea, DL-amino-n-butyric acid and KNO_3, $(NH_4)_2SO_4$, were the sole nitrogen sources, but $NaNO_3$ is not utilized (Table 2).

Active strain phylogenetic tree

Concerning molecular characterization, the active strains' 16S rRNA gene nucleotide sequences were determined from the strands of the 16S rRNA gene sequences and assigned to GenBank. Subsequently, the sequences obtained were subjected to similarity searches to determine the strain's phylogenetic relationships by aligned tests using the Neighbor-joining method (Figure 2).

Isolation of bioactive compounds from crude extract

The crude extract (1.79 g) was applied onto a silica gel vacuum column chromatography (silica gel G 60, 40 g, 7.0×35 cm) pre-eluted with hexane to isolate the bioactive compound. The separation was performed through gradient elution using 50 mL of ethyl acetate-methanol solutions (ranging from 100% ethyl acetate to 100% methanol). Subsequently, the elution fractions were collected in small test tubes. Eleven fractions (F1F11) were obtained and dried under vacuum. Among these, five fractions (F7-F11) were eluted with ethyl acetate-methanol solvent systems at ratios of 40-60, 30-70 v/v, 20-80 v/v, 10-90 v/v, and 0-100 v/v, respectively, exhibited significant antimicrobial activities and were combined as active materials (570 mg). These materials were then diluted in methanol and subjected to ultra-fast liquid chromatography (UFLC) to yield a single pure active peak.

Structure elucidation of the compound and antibacterial activity

Moreover, the actinomycete strain MRK01PY produced bioactive compounds in the above TLC analyses and bioassays. Therefore, we investigated the secondary metabolites of the actinomycete strain MRK01PY with UFLC analysis. NMR and LCMS analyses were performed to identify the typical peak's chemical structure. Based on spectral data, including ^{1}H NMR and ^{13}C NMR (Figure. 3), LCMS analysis (Figure 4), and the results obtained by ACS SciFinder^{n}, the chemical structure of the typical peak was decided as actinomycin D.

Antimicrobial activity of the crude extract of MRK01PY strain

During screening actinomycetes strains for their antimicrobial activity, it was observed that compounds derived from the MRK01PY strain exhibited highly antagonistic effects against Gram-positive bacteria, specifically *B. subtilis* and *S. aureus*, except for *M. luteus*; meanwhile, it had no inhibitory effect on the tested Gram-negative bacteria and fungi (Table 3). However, it is important to highlight that certain Gram-positive microbes, *Mycobacterium* spp., are sensitive to antimicrobial agents.

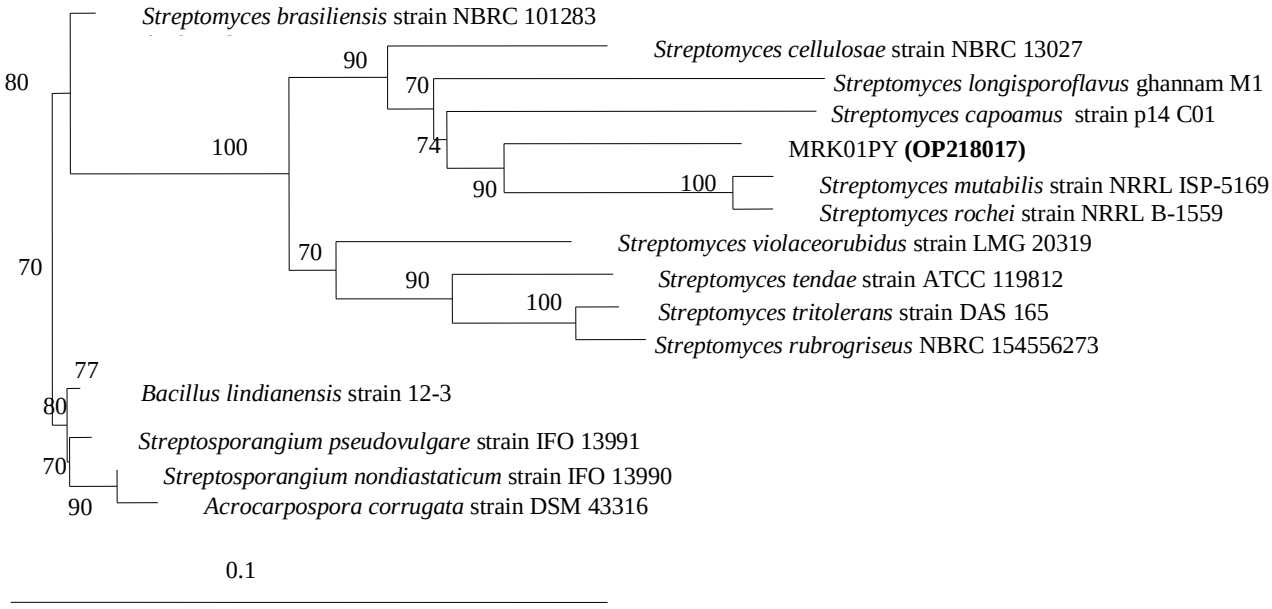

Figure 2. Neighbor-joining phylogenetic tree, inferred from 16S rRNA gene sequences. A phylogenetic tree shows the endophytic actinomycetes strains' phylogenetic relationship with related genera. Meanwhile, bootstrap values are expressed as percentages of 1000 replications, and values ≥50% are shown at branch points. The score bar represents 1 nucleotide substitution per 100 nucleotides

Table 3. Antimicrobial activity of strain MRK01PY's pure compound

Microorganisms	**Antimicrobial activity of extract of MRK01PY**
	Inhibition zone (mm) by agar diffusion assay (10 µg/6 mm thin disk)
Bacillus subtilis PCI219	25.35
Staphylococcus aureus KB212	27.60
Micrococcus luteus FDA209P	-
Escherichia coli JM109	-
Pseudomonas aeruginosa IFO12689	-
Saccharomyces cerevisiae S288c	-
	Growth inhibition by liquid microdilution assay (5 µg/sample)
Mycobacterium abscessus ATCC19977	+
Mycobacterium avium JCM15430	+
Mycobacterium intracellulare JCM6384	+

Note: -: no activity was detected, +: activity was detected

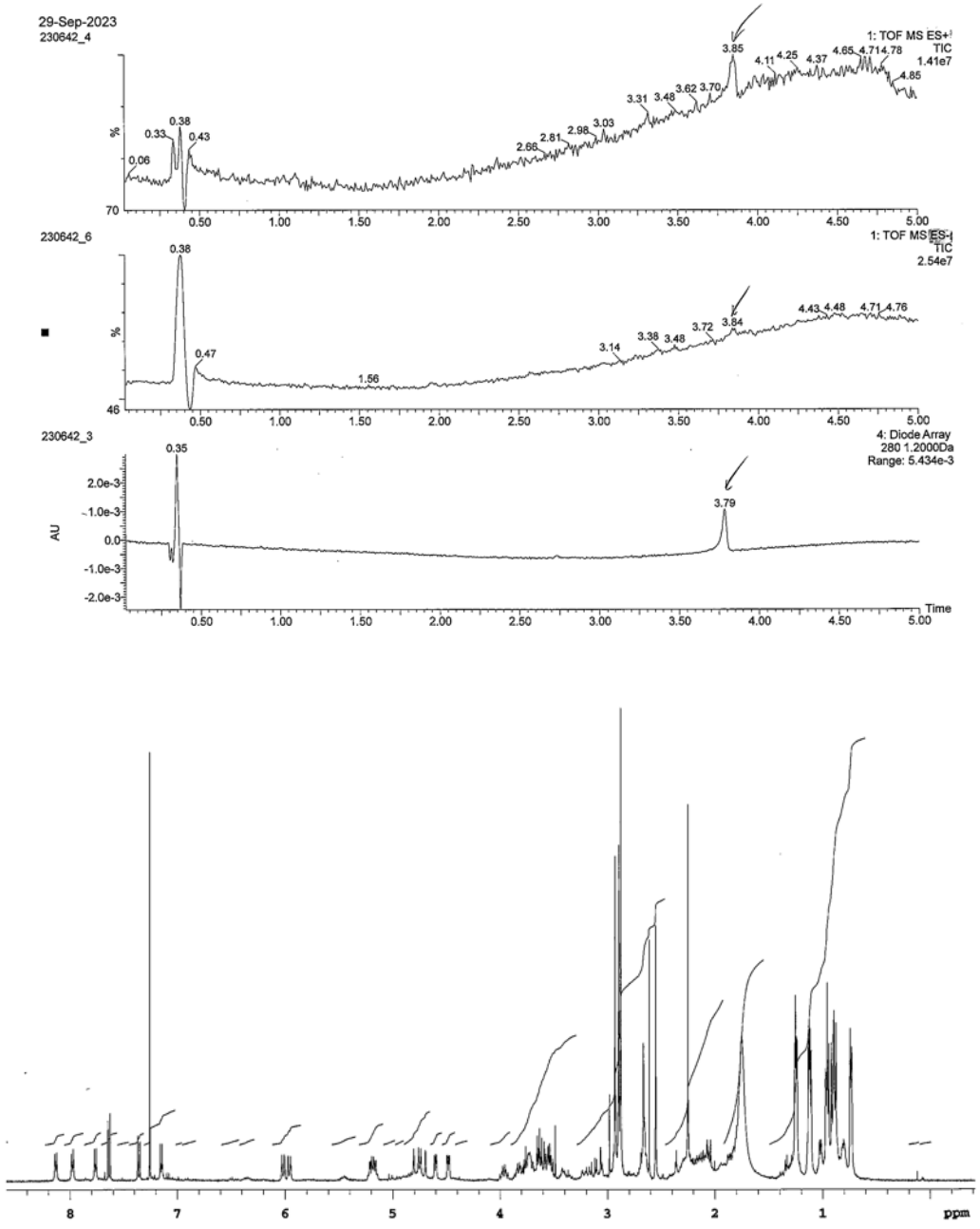

Figure 3. ^{1}H NMR spectra of actinomycin D (400 mHz, in $CDCl_3$-*d*)

Discussion

Moreover, actinomycetes play a crucial role in producing diverse metabolites for medicinal applications, ongoing research continually explores new microbial sources and metabolite types, particularly on medicinal plants. This study investigates actinomycetes found in plants located in limestone soil areas. Through initial screening, eleven strains were isolated from various plant organs, predominantly from roots, followed by stem bark, with one strain originating from leaves. Morphological examination revealed that all strains exhibited a spiral-shaped dominance in their spore chains, a characteristic typically associated with the genus *Streptomyces*. *Streptomyces* is the predominant genus found in various host plants (Shan et al. 2018). The antagonistic analysis identified four strains capable of inhibiting bacterial growth. Among these, three strains (MRK01PY, MRK17NP, and MRK32NP) exhibited inhibition against Gram-positive bacteria, varying degrees of inhibition from weak to very strong. Conversely, one strain (MRK20NP) specifically demonstrated inhibition against Gram-negative bacteria. Actinomycetes derived from plants have been intensively studied due to their potential as sources of various biologically active secondary metabolites, with studies documenting their association with the plant host (Behie et al. 2017; Trujillo et al. 2015).

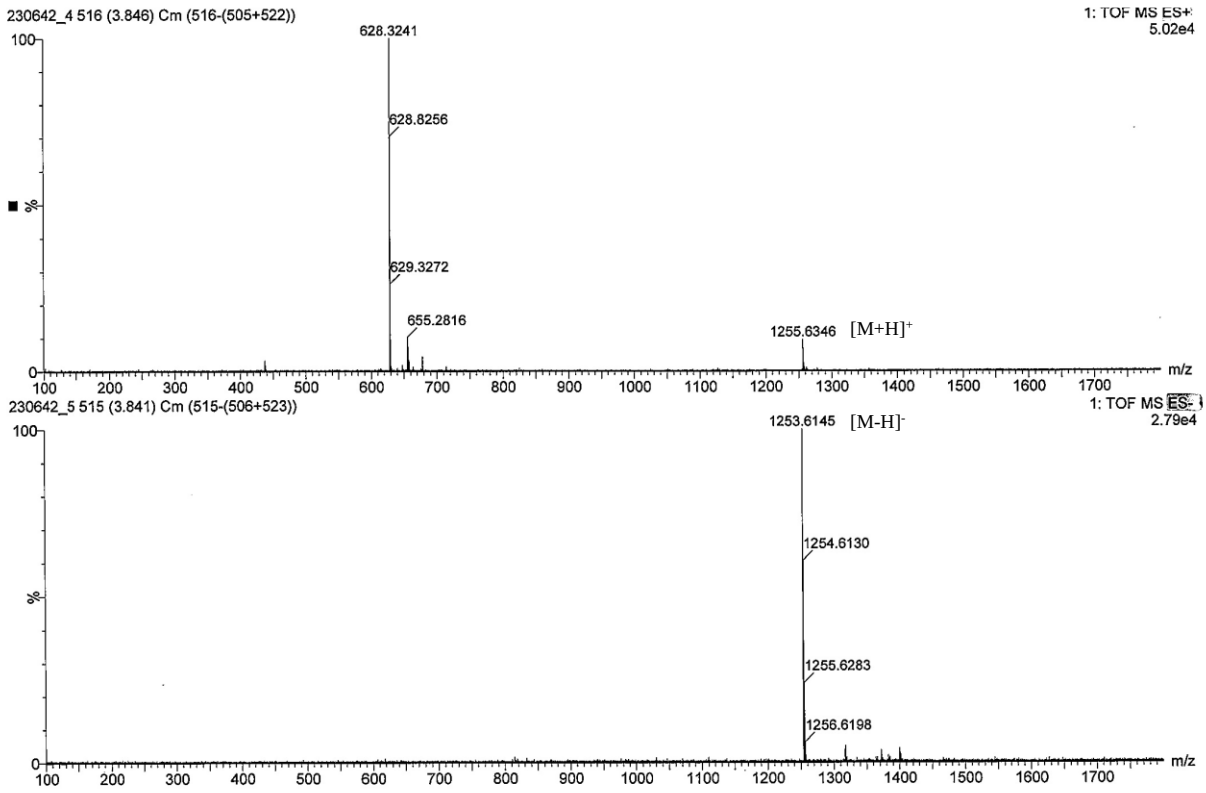

Figure 4. LCMS of actinomycin D. Method: cortecs column (2.1× 50 mm), 40%-100% CH_3CN/H_2O gradient for 5 minutes, flow 0.3 mL/min

Plants possess diverse anatomical and chemical defense mechanisms, the effectiveness of which depends on environmental and biotic factors. Notably, there is substantial evidence indicating the colonization of plant roots and rhizospheres by several actinomycetes species. Furthermore, it has been proposed that antibiotic production by actinomycetes may confer protection to host plants against phytopathogens (Tarkka et al. 2008). This observation highlights the diverse antimicrobial capabilities of the strains isolated from their host plant.

Therefore, this recent study investigated endophytic actinomycetes isolated from plants growing in limestone-rich environments to assess their antimicrobial properties. The findings revealed that the antimicrobial activities of these isolates were comparatively lower than those of their non-antimicrobial counterparts. Additionally, no isolates with broad-spectrum antimicrobial activity were identified. However, one isolate was noted for producing a yellow soluble pigment with significant antibacterial activity. The data indicated that secondary metabolites produced by these actinomycete isolates primarily inhibited the growth of Gram-positive bacteria while having low to no effect on Gram-negative bacteria. This suggests that the secondary metabolites possess specific targets that influence organismal growth. These results confirm previous studies which have suggested that the majority of *Streptomyces* spp. strains isolated from plants exhibit the highest inhibitory activity against Gram-positive bacteria (Baskaran et al. 2011). Furthermore, another study revealed the antimicrobial activity of actinomycetes against *Escherichia coli*, *Staphylococcus aureus, Pseudomonas vulgaris,* and *Staphylococcus epidermidis* while showing no activity against *Candida albicans* and *Trichophyton rubrum* (Ganesan et al. 2017). These findings contribute to our understanding of the diverse antimicrobial capabilities of actinomycetes in their host plant.

During the primary screening, variations in the inhibition activity of the active strain were observed compared to the secondary screening, particularly evident in strain MRK17NP. Additionally, it was noted that the strains exhibited a decrease in inhibition activity following the secondary screening for antimicrobial potential. This loss of inhibition activity in strain metabolites may be attributed to the production of different active secondary metabolites during cultivation on liquid and solid media (Charousová et al. 2015). Furthermore, some metabolites may be lost during the extraction process with organic solvents, which could result in the inactivation of its active components.

Based on morphological observations of the strain's 21-day-old culture grown on ISP-2 medium, it was observed that the sporophores exhibited monopodial branching and produced oval-shaped spores arranged in an open spiral pattern. Additionally, the aerial mycelium occasionally displayed a discernible yellow soluble pigment, with an orange hue that tended towards brown. These morphological characteristics led to the classification of the endophytic actinomycete MRK01PY within the

Streptomyces genus. The phylogenetic analysis of the strain and representative and closely related strains revealed that all strains belong to the *Streptomyces* genus.

Comparative analysis of the 16S rRNA gene sequences identified the selective *Streptomyces* sp. strain MRK01PY as closely related to *Streptomyces mirabilis* strain NRRL ISP-5169. Interestingly, strain NRRL ISP-5169 shares similarities with *Streptomyces* sp. IA1, isolated from Saharan soil and known to produce actinomycin. Moreover, strain IA1 also produces a yellow pigment (Toumatia et al. 2015). Both desert soils and limestone environments share extreme environmental conditions. Studies indicate consistent biogeographical patterns, where certain groups of actinomycetes are found in both area substrates, suggesting potential dispersal mechanisms or shared ecological niches (Selim et al. 2021).

The selected strain was subjected to the extract antimicrobes assay against bacteria and fungi. The crude extract from the *Streptomyces* sp. MRK01PY isolate's ethyl acetate extraction displayed the highest inhibitory activity against Gram-positive bacteria, producing yellow diffusible pigment. Consequently, this isolate was identified as a potent strain and selected for further investigation in active metabolite production. It is well-established that secondary metabolites and antibiotics from actinomycetes are naturally extracellular. The antimicrobial activity of an organism is generally influenced by its habitat's nature and substrate composition variations. Moreover, differences in test organisms and strains often lead to variations in antimicrobial activity (Kumar et al. 2014).

Furthermore, the *Streptomyces* sp. MRK01PY isolate exhibited heightened antibacterial activity against both Gram-positive and Gram-negative pathogenic bacteria compared to other antagonistic isolates. Similar variations in antimicrobial activity have been reported previously (Tomaseto et al. 2020). In the present study, strain MRK01PY produced extracellular secondary metabolites that demonstrated significant antimicrobial activity against various bacterial pathogens when extracted using ethyl acetate. Several previous studies have also identified ethyl acetate as an effective solvent for extracting antimicrobial compounds from actinomycetes, particularly within the *Streptomyces* genus (Makky et al. 2021; Saadouli et al. 2020).

Actinomycin D constitutes a significant component derived from the yellow soluble pigment synthesized by the selected strain, MRK01PY. This compound is important due to its potential biomedical applications and biological activities. Actinomycin D is a well-known compound renowned for its diverse biological activities. For instance, actinomycin D isolated from strain T33 demonstrated potent antifungal activity against various pathogens, including Magnaporthe grisea, *Fusarium oxysporum f. sp. cucumerinum, Rhizoctonia solani, Valsa mali* (with an IC50 value of 1.7 µg/mL), and *Dothiorella gregaria* (Yin et al. 2019). Actinomycin D is recognized as a potent bioactive compound with therapeutic potential for treating various human ailments, including bacterial infections and cancer (Liu et al. 2016). Although numerous *Streptomyces* species produce actinomycin (Amin et al. 2021; Keller et al. 2010), this study marks the first report of an actinomycin-producing *Streptomyces* sp. isolated from an endophyte associated with the *P. suaveolens* plant.

According to available databases, actinomycins are chromopeptide lactones obtained from various *Streptomyces* strains (Praveen et al. 2008), characterized by a phenoxazinone chromophore (actinocin) linked by amide bonds to two pentapeptide lactone rings (Sakiyama et al. 2014). Since its discovery, actinomycin D has emerged as a significant molecular and cell biology compound, exhibiting potential cytotoxicity against numerous cancer cell lines (Sharma et al. 2019). Its distinct structure and biological characteristics allow it to intercalate into duplex DNA, inhibiting DNA-dependent RNA polymerase activity and subsequently impeding protein synthesis (Mukhtar et al. 2012).

Therefore, the findings of this study underscore the significance of plant-limestone soil-associated actinomycetes as an important source of antibiotic substances, highlighting their potential as valuable producers of secondary metabolites. Furthermore, a metabolite was isolated from the fermented broth and identified as actinomycin. This study represents the inaugural report of actinomycin production by endophytic *Streptomyces* isolated from the *P. suaveolens* plant, thus contributing novel insights into the biosynthetic capabilities of these microbial communities.

ACKNOWLEDGEMENTS

We thank Ms. Noriko Sato and Dr. Kenichiro Nagai (Graduate School of Pharmaceutical Sciences, Kitasato University) for the NMR spectra and MS data measurements. This work was financially supported by The Indonesian Ministry of Research, Technology, and Higher Education under grant PNBP-PGB 2019 UNM No: 4713/UN36/KP and JSPS KAKENHI Grant numbers 18KK0219 (Fund for the Promotion of Joint International Research (Fostering Joint International Research (B)) (HT) and MEXT Scholarship (EAA).

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