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Evaluation of Antibacterial and Antibiofilm Effects from Soil *Streptomyces* spp. against Multidrug-Resistant Bacteria

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ABSTRACT

The global increase in multidrug-resistant (MDR) bacterial infection has rapidly gained concern globally. This study aimed to investigate antibacterial and antibiofilm potential of 25 soil actinomycete strains against MDR strains including *Escherichia coli* strain M4, *Pseudomonas aeruginosa* strain M19, *Klebsiella pneumoniae* strain M19, *Bacillus subtilis* strain M18, and *Methicillin Resistant Staphylococcus aureus* (MRSA). In this study, three actinomycete isolates encoded APM-7, APM-11, and APM-21 showed a strong and broad antibacterial spectrum. The minimum inhibitory concentration (MIC) of extracts derived from these isolates was ranged from 78 µg/ml to 10,000 µg/ml. In addition, The extracts also displayed significant biofilm inhibition values ranging from 6.06 to 72.4%. Based on the results, APM-21 extract had the best antibacterial and antibiofilm activities with the strongest values against MRSA. According to the nucleotide sequencing of the 16S rRNA gene, APM-7, APM-11, and APM-21 strains possessed similar identities with *Streptomyces cyaneus, Streptomyces coerulescens*, and *Streptomyces panayensis*, respectively. Based on Liquid Chromatography Tandem-Mass Spectrometry (LC-MS/MS) analysis, two antibacterial compounds, namely rancimanycin III, and enteromycin were detected in all those three extracts. Interestingly, APM-21 extract also contained two prominent antibacterial substances including paramagnetoquinone C, and caerulomycin I, suggesting their contribution to the most potential activities. Moreover, new insights were provided into a promising candidate for use in an active compound combating strategy to control MDR bacterial strain infection.

1. Introduction

Infection caused by multidrug resistance (MDR) bacteria considerably has a major impact on human well-being and is expected to increase global mortality and world economic turmoil. Among the diverse modulators of MDR strains, the consumption of weak medicines, low-quality of antibiotics, poor infection control, and overuse of drugs-related antibiotics are contributing to the emergence of MDR strains (Manesh and Varghese 2021). Therefore, a comprehensive and multidisciplinary effort is needed to control the resistance by describing the role of antibiotics, the mechanism of action, and the origin of microorganisms (Knight *et al.* 2021). Some previous reports showed that numerous MDR strains were reported in Indonesian hospitals. Virawan *et al.* (2020) stated that *Klebsiella pneumoniae* MDR strains were

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isolated from patients at Soeradji Tirtonegoro General Hospital, Central Java. Additionally, *Escherichia coli* and *K. pneumoniae* strains isolated from patients at Dr. Zainoel Abidin hospital, Nanggroe Aceh Darussalam, Indonesia, were also resistant to beta-lactam antibiotics which results in failure of infection treatment (Hayati *et al.* 2019). In this context, new alternative antibiotic therapy is needed to overcome these resistance cases.

Biofilms, defined as communities of microorganisms incorporated in a surface-adhered organic polymeric matrix, are important for bacterial virulence factors. More than 99% of all microorganisms reside in various ecosystems of the formation modulating stronger adverse environment compared to planktonic bacteria forms (Abebe 2020). Biofilms are widely found in food processing, industry, water distribution, and medical sectors (Reuben *et al.* 2019). After the formation, the bacteria are protected from immune defence, and chemicals such as antibiotics, detergents, and disinfectants. In severe conditions related to the inappropriate use of antibiotics, a strong adaptation of bacteria and the selection of MDR strains are promoted to form biofilm formation (Olsen 2015). Elimination of biofilms requires degradation of the structure or the discovery of antibiotics disrupting the matrix. In this context, investigations are needed on new antibacterials, and antibiofilm compounds that are safe and effective for replacement or rotation of the antibiotics used.

Actinomycetes are a group of filamentous Gram-positive bacteria known to be capable of producing various great antimicrobial compounds, such as streptomycin, actinomycin, macrolides, acetyl-griseoviridine, and desulphurized griseoviridine (Donald *et al.* 2022). These bacteria are found in various sources such as the aquatic environment, plants, animals, and soil. The most abundant source is reported to be derived from soil. Each gram of soil contains about 18×10^3 up to 2.9×10^6 CFU/g dry soil (Ghorbani-Nasrabadi *et al.* 2013). The isolation from a unique environment is expected to obtain potential actinomycetes, especially as a producer of antibacterial compounds. The soil on Muna Island, Southeast Sulawesi, Indonesia, is dry and calcareous, and has not been polluted by chemical pollutants, so that it can be used as a potential source of actinomycetes (Hindersah and Rahmi 2020). However, research on the diversity and bioprospection of soil actinomycetes from this area have not been widely studied. The present research aimed to investigate the antibacterial and antibiofilm effects of soil *Streptomyces* spp. strains which isolated from Muna island, Indonesia against clinical MDR bacterial strain. There was no report regarding the antibiofilm activity against MDR clinical strains of soil *Streptomyces* spp. from Indonesia. Therefore, this study belonged to the first report concerning to these interesting topics.

2. Materials and Methods

2.1. Isolation of Soil Actinomycetes

Soil samples were collected from Muna Island Southeast, Sulawesi, Indonesia [Coordinate: latitude 4°54'48.8"S, longitude 122°39'56.7"E] at a depth of 5-15 cm and were placed in sterile plastic bags. The sample were kept in an icebox, and then aseptically transferred to the Laboratory of Microbiology, IPB University, Indonesia to isolate targeted actinomycetes. As for the isolation technique, the serial dilution method was used for the isolation of soil actinomycetes following the method as described by Singh *et al.* (2016). A total of 1 gram soil samples were suspended in 9 ml of sterile 0.85% NaCl followed by vigorous vortex. Subsequently, 0.1 ml was diluted from 10^{-4} to 10^{-6} and was spread on the Humic Vitamin Agar (HVA) plated medium containing 100 µg/ml nystatin. Plates were incubated at 28-29°C for 7 days before being observed. Single distinct actinomycete colonies were selected, purified, and maintained by subculturing in International Streptomyces Project 2 (ISP-2) medium (composition: 4 g/L yeast extract, 10 g/L malt extract, 4 g/L glucose, and 20 g/L agar), and was preserved at 4°C.

2.2. Screening for Antibacterial Activity by Double Layer Method

Each isolate was re-cultured on ISP-2 agar medium, and incubated for 4 days at 28-30°C and a total of 5 Multi Drugs Resistant (MDR) strains were used for targeted bacterial which were isolated from clinical patients in Dr. Kariyadi Hospital, Semarang, Central Java. The bacterial target belongs to the Gram-negative group including *Escherichia coli* strain M4, *Klebsiella pneumoniae* strain M19, and *Pseudomonas aeruginosa* strain M19. As for Gram positive bacterial targets namely *Bacillus subtilis* strain M18, and methicillin-resistant *Staphylococcus aureus* (MRSA). The bacterial targets were cultured in Nutrient Broth (NB) medium with shaking at 120 rpm, 37°C for 24 h. The test was designed by pouring Nutrient Agar (NA) medium into a petri dish. After the media had

solidified, a semi-solid NA medium was poured over the solid medium. This semi-solid NA medium had previously been inoculated with each bacterial target (OD_{600} of 0.6) for 1% (v/v). After the semi-solid layer had solidified, the soil actinomycetes isolate culture (after 4 days incubation) was taken using a round glass rod (6 mm in diameter) and placed upside down on the media. The plates were incubated at 37°C for 24 h and the presence of antibacterial activity was indicated by the formation of an inhibition zone around actinomycetes colonies.

2.3. Fermentation and Extraction of Active Metabolites

The selected actinomycetes isolates were cultured in ISP-2 liquid medium and incubated for 10 days at 28-30 °C with a shaking at 120 rpm. Subsequently, the culture of the isolate was mixed with ethyl acetate solvent in a 1:1 (v/v) ratio and vigorously shaken at 150 rpm for 2 h. The solvent layer was evaporated using a rotary evaporator at 45°C and the crude extract obtained was stored at 4°C prior to being used (Prastya *et al.* 2019).

2.4. Disc Diffusion Assay

In this study, the actinomycetes extract concentration was used for testing at 20 mg/ml. Each bacterial target was incubated for 24 h on NB media at 120 rpm, 37°C (OD_{600} of 0.6) were inoculated 1.5% (v/v) into Mueller Hinton Agar (MHA) media, and allowed to solidify. An amount of 20 µL of actinomycetes extract was dropped on a sterile disc (6 mm in diameter) and placed on the MHA plate surfaces. The petri dish was further incubated at 37°C for 24 h and the inhibition zone around the disc was observed and its diameter was calculated. In addition. 99% dimethyl sulfoxide (DMSO) and tetracycline (300 µg/ml) were applied as negative and positive controls, respectively (Priyanto *et al.* 2022).

2.5. MIC and MBC determination

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) was carried out using the micro-broth dilution test method on a 96-well microplate (CLSI 2020). The row test on well A was filled with test sample, DMSO (negative control), and tetracycline (positive control) in Mueller-Hinton Broth (MHB) medium with a total volume of 200 µL. Meanwhile, B-H well was filled with 100 µL of MHB medium and the content in well A were then diluted twice starting from well A-H by transferring 100 µL aliquots from well A to well B and so on. 100 µL aliquots from the H well were discarded to obtain equal volume. Subsequently, a total of 100 µL of the targeted bacterial suspension (0.5 McFarland or equivalent to 10^8 CFU/ml) in 0.85% NaCl solution (w/v) was inoculated into each well to obtain a final volume of 200 µL. The microplate was shaken at 150 rpm at 37°C for 24 h, while a clear well at the lowest sample concentration showed the MIC value. MBC value was obtained by dipping 100 µL of culture from well (the culture was taken from the MIC well to well containing the highest extract concentration). The suspension was spread onto the MHA plate. MBC is defined as the concentration of the extract, that can kill all targeted bacteria, characterized by the absence of growth of bacterial colonies in MHA plate.

2.6. Antibiofilm Assessment by Crystal Violet

Antibiofilm activity was carried out using a crystal violet assay (Priyanto *et al.* 2023). Individual wells of sterile microtiter 96 well plates were filled with 100 µL of Brain Heart Infusion (BHI) medium and inoculated with 100 µL of the extract at concentrations of ¼ × MIC, ½ × MIC, 1 × MIC, and 2 × MIC followed serial dilution until well H. Subsequently, each well was inoculated with bacterial target at Mc Farland standard 0.5 bacterial and incubated at 37°C for 2 h under shaking at 120 rpm. Meanwhile, the medium was removed, and washed with 0.85% NaCl. Each well was then flooded with 200 µL of 0.1% crystal violet staining followed by incubation for 30 min at 37°C. Absorbance showing biofilm formation was calculated using ELISA Thermo Scientific Varioskan Flash (ThermoFischer) Elisa reader at a wavelength of 595 nm. In addition, in the well from the biofilm test of the most potential treatment which had been staining crystal violet, we also observed the biofilm matrix pattern using a light microscope (LM). After adding crystal violet, the treatment results were placed on a coverslip, and observed on LM (INV100-FL, BEL Engineering, Italy) by 100 × magnification.

2.7. Eradication of Cells biofilm

The eradication of cell biofilm was conducted using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Biofilm formation of each bacterial target was produced in microtiter 96 well plates before being treated with the sample. In this study, several concentrations of ¼ × MIC, ½ × MIC, 1 × MIC, and 2 × MIC were used. Generally, 100 µL of bacterial suspension in 0.85% NaCl (0.5 McFarland)

was applied to microtiter 96 well plates and added with 100 µL of BHI medium. This was followed by incubation for 5 days at 37°C with shaking at 120 rpm and the medium was periodically changed every 24 h with 200 µL of BHI liquid medium (enriched with 0.25% glucose). After biofilm was produced, the sample in each concentration was added to the plate and incubated again for 24 h under the same conditions. The medium was eliminated and added with 10 µL of 5 mg/ml MTT solution (Roche) followed by incubation at 37°C for 3 h. An amount of 200 µL of 99% DMSO was applied to dissolve the insoluble formazan crystals and absorbance was determined using an ELISA reader at 595 nm (Priyanto *et al.* 2023).

2.8. Scanning Electron Microscope (SEM)

Antibiofilm potential of the most active targeted bacterial (MRSA) was visualized under SEM. The biofilms were developed using all procedures and treatments as described above. Subsequently, the biofilm matrix was harvested, then dropped on the surface of the Silicon Wafer-single polished (Sigma) and incubated for 18 h. This SEM specimen was further coated with sputter-gold particles (Hitachi®, Tokyo, Japan), and observed with a SEM- JEOL JSM-IT200 (JEOL, South Korea) at a working distance of 5µm, and 10 kV of accelerating voltage.

2.9. Molecular Identification of Potential Actinomycetes

The selected actinomycetes isolates were identified based on the 16S rRNA gene sequence. The actinomycetes genomic DNA extraction procedure was performed using the PrestoTM Bacterial gDNA Mini Kit (Geneaid, Taiwan) according to the manufacturer's protocol instructions. A set of primers: 1387R (5'-GGG CGG WGT GTA CAA GGC-3'), and 63F (5'-CAG GCC TAA CAC ATG CAA GTC-3') developed by Marchesi *et al.* (1998) was used to amplify 16S rRNA gene. The 50 µL PCR reaction was prepared by mixing 5 µL of the forward primer (10 µM), 5 µL of the reverse primer (10 µM), 25 µL of GoTaq Green® Master Mix 2X (Promega), 2 µL of DNA template (concentration of 50-100 ng/µL), and 13 µL of nuclease-free water. PCR cycles were carried out for 35 cycles, with PCR conditions of pre-denaturation 94°C for 5 min, denaturation at 94°C for 30 s, annealing at 55°C for 45 s, elongation at 72°C for 90 s, and post-PCR condition at 72°C for 10 min (Priyanto *et al.* 2023). Meanwhile, the results were visualized by electrophoresis with an amplicon target of around 1,300 bp. Amplicons were then sequenced and analyzed using the BLAST nucleotide program (BLAST-N) at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic tree was constructed using MEGA (Molecular Evolutionary Genetic Analysis) software version 11.0 with the neighbor-joining tree method and a bootstrap value of 1,000x. The 16S rRNA sequences of the selected isolates were deposited into NCBI genbank database.

2.10. Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

The selected actinomycetes extracts were identified, and the dominant compounds were analyzed the using Xevo G2- XS QTof (Quadrupole Time-of-Flight) mass spectrometry instrument (Waters, USA) with an electron spray ionization (ESI). The sample separation was carried out using stepwise gradients from 95% A and 5% B to 5% A and 95% B over 16 minutes (A contained 0.1% formic acid + distilled water; B contained acetonitrile + 0.1% formic acid). These chromatographic separation conditions were performed using an LC system in the form of an Ultra Performance Liquid Chromatography (UPLC)/QTof MS analytical system (Waters). The MS conditions included column temperature 40°C, cone voltage 30 V, mass range: 100–1,200 Da, capillary 2kV, source temperature 120°C, cone gas flow 50 L/h, desolvation temperature 500°C, desolvation gas flow 1,000 L/h, and collision energy (ramp: 10–40 eV). Mass spectrometry of each peak was identified using the type of electrospray ionization (ES) Xevo G2-S QTof (Waters) with Quadrupole Time-of-Flight mass spectrometry in positive ion mode. The identified mass and fragment ions were analyzed using the UNIFI software library integrated into the instrument and the natural products atlas website services (https://www.npatlas.org/).

2.11. Statistical Analysis

The data obtained was analyzed in statistical analyses from three experiments and the results were reflected as the mean ± standard deviation. Statistical significance was investigated by one-way analysis or variance (ANOVA) followed by multiple Duncan tests. In this context, the values were considered statistically different when $p<0.05$.

3. Results

3.1. Antibacterial Activity of Actinomycetes

During the isolation process, 25 actinomycete isolates were obtained. Actinomycete colonies showing distinct morphological characteristics were selected and maintained for further antibacterial screening (Figure 1). Out of the 25 total isolates, 21 isolates (84%) showed moderate to strong antibacterial activity against Gram-negative, and Gram-positive MDR bacterial strains (Table 1). Based on the results, three isolates, namely APM-7, APM-11, and APM-21, showed the best antibacterial activity with the widest inhibition zone derived from colonies using the dual culture method (Figure 2). Therefore, these three isolates were selected for the extraction of their active compounds.

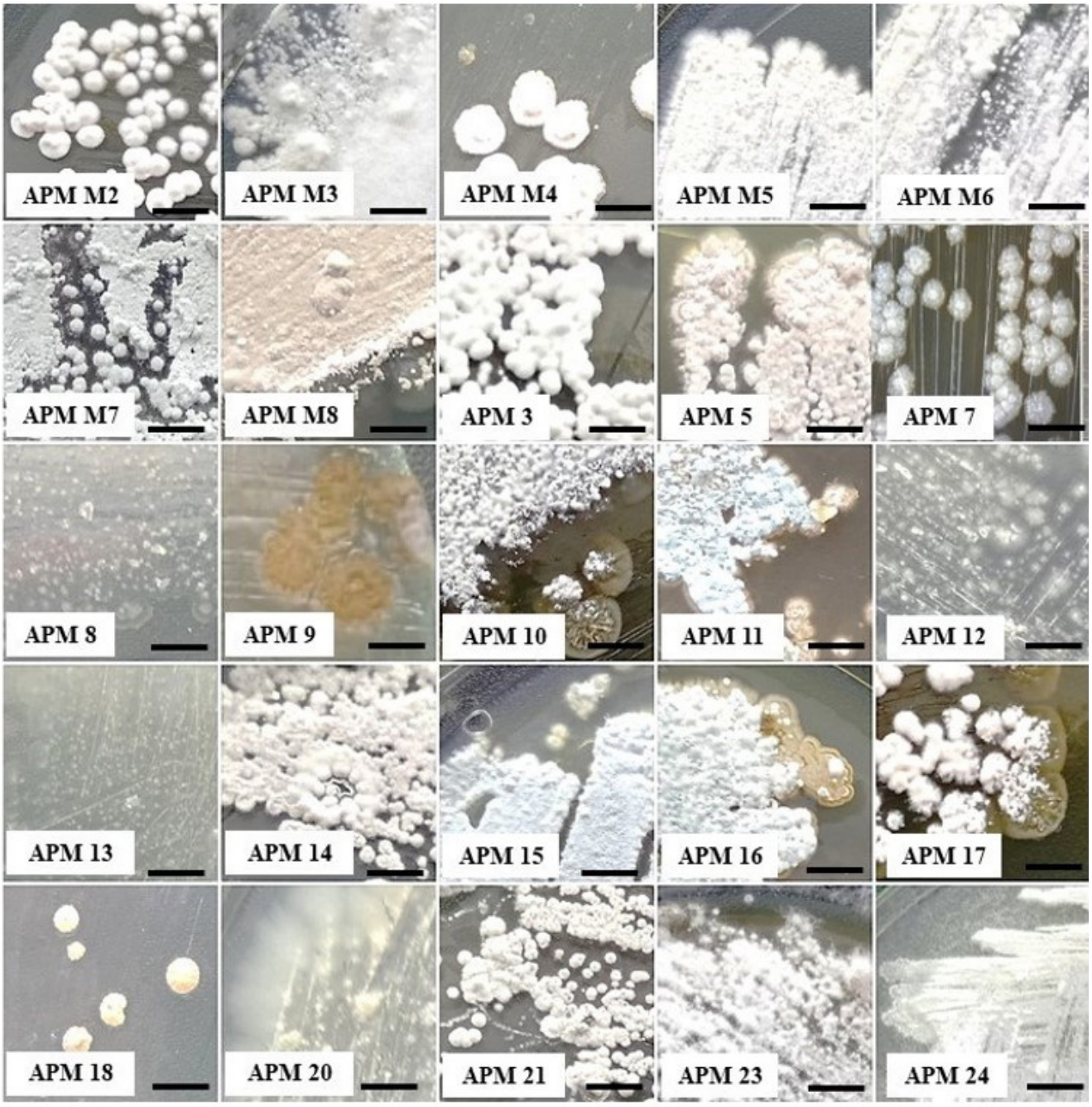

Figure 1. Colony morphology of 25 soil actinomycete strains. Each isolate was grown on ISP-2 medium and incubated for 7 days at 28-29°C prior to being observed. Bars represent 6 mm

Table 1. Antibacterial activity of 25 soil actinomycetes strains against multidrug-resistant clinical isolates

Actinomycetes isolate	Inhibition zone (mm)				
	E. coli strain M4	*P. aeruginosa* strain M19	*K. penumoniae* strain M19	*B. subtilis* strain M18	Methicillin-resistant *S. aureus*
APM 3	-	-	+	-	-
APM 5	+	+	+	-	++
APM 7	++	++	+	++	++
APM 8	-	-	+	-	-
APM 9	+	+	+	+	++
APM 10	-	-	+	-	-
APM 11	++	++	+	++	+++
APM 12	-	-	+	-	-
APM 13	-	-	+	-	-
APM 14	-	-	-	-	-
APM 15	++	++	+	++	+
APM 16	++	++	+	++	++
APM 17	-	-	+	-	-
APM 18	++	++	-	++	++
APM 20	-	-	-	-	-
APM 21	++	++	++	++	++
APM 23	+	+	+	++	+
APM 24	-	-	-	-	-
APM M2	-	-	+	-	-
APM M3	-	-	+	-	-
APM M4	++	++	++	-	-
APM M5	-	-	++	-	-
APM M6	-	-	++	-	-
APM M7	-	-	-	-	-
APM M8	-	+	++	+	++

(-) no activity; (+++) highly active (inhibition zone:>13 mm); (++) moderately active (inhibition zone: 8–13 mm); (+) less active (inhibition zone: ≤8 mm)

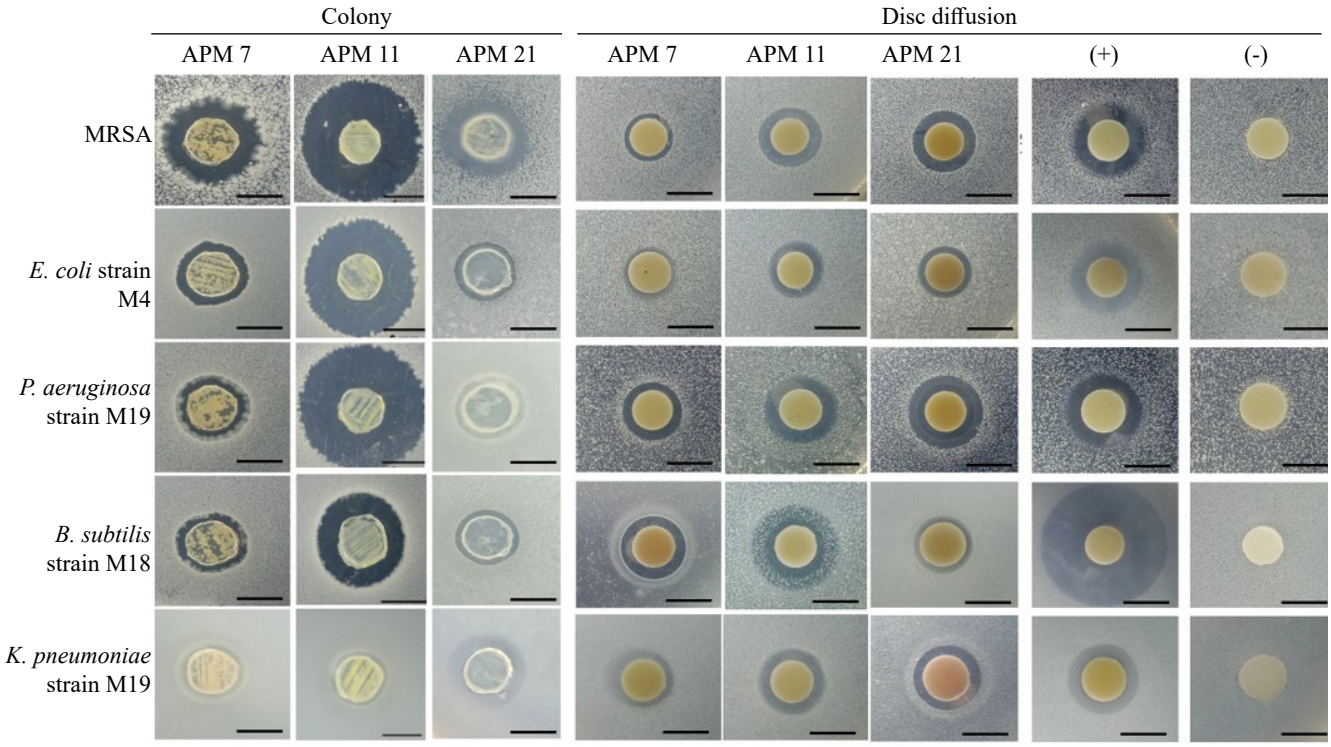

Figure 2. Antibacterial activity of selected actinomycetes strain and its crude extract against multidrug-resistant clinical isolates. Tetracycline (200 µg/ml) and DMSO (1% v/v) were used for positive and negative controls, respectively. Bars represent 6 mm

Results from the extraction process showed that APM-7 isolate produced the highest yield of the crude extract at 0.096%. Based on disc diffusion antibacterial assay, the 3 selected extracts showed different values in inhibiting the growth of five bacterial targets ranging from 7.5±0.5 to 15.0±2.1 mm (Table 2). Among the three extracts tested, APM-7 extract exhibited the best activity against *E. coli* strain M4 and *K. pneumoniae* strain M19. Meanwhile, APM-11 extract showed the highest inhibition zone value against *B. subtilis* strain M18, and MRSA. *P. aeruginosa* strain M19 was inhibited at the best value by the APM-21 extract. These 3 active extracts were subjected to antibacterial assessment to determine the MIC, and MBC values.

3.2. MIC and MBC of Actinomycetes Extracts

The MIC of the three selected extracts was determined for the standard antibacterial study using a broth microdilution assay. The values varied between 78 µg/ml and 10,000 µg/ml, with the lowest MIC value of 78 µg/ml recorded from APM-21 extract against MRSA. The highest MIC (10,000 µg/ml) was shown by APM-7 extract on *K. pneumoniae* strain M19. Meanwhile, APM-11 extract exhibited the lowest (MIC and MBC) values against four bacterial targets including *E. coli* strain M4 (125 µg/ml and 250 µg/ml), *P. aeruginosa* strain M19 (250 µg/ml and 500 µg/ml), *K. pneumoniae* strain M19 (125 µg/ml and 250 µg/ml), and *B. subtilis* strain M18 (250 µg/ml and 500 µg/ml) (Table 3). The lowest MIC and MBC values reflect the most active compound acting as antibacterial agents.

3.3. Antibiofilm Ability

The results of the antibiofilm ability of three selected extracts against all MDR bacterial strains showed that all extracts significantly ($p<0.05$) reduced biofilm formation in a concentration-dependent manner. The highest inhibition value was recorded for APM-21 extract at a concentration of 2×MIC against MRSA with an inhibition value of 72.4%, as shown in Figure 3A-F. The inhibition of pre-established biofilm after APM-21 extract treatment was confirmed using LM and SEM, as shown in Figure 4A and B. The three potential extracts can inhibit biofilm formation with varying effectiveness against the other four tested bacteria, including *E. coli* strain M4, *P. aeruginosa* strain M19, *K. pneumoniae* strain M19, and *B. subtilis* strain M18, respectively (Figure 3A-C). In contrast, the lowest reduction in biofilm was recorded for APM-7 extract at a concentration of ¼ × MIC with inhibition values of 6.02% and 6.20% against *K. pneumoniae* strain M19, and *B. subtilis* strain M18 biofilms, respectively (Figure 3A).

Table 2. Antibacterial activity of selected soil actinomycetes crude extracts against multidrug-resistant clinical isolates by disc diffusion assay

Extracts code	Inhibition zone (mm); (average ± standard deviation)				
	E. coli strain M4	*P. aeruginosa* strain M19	*K. penumoniae* strain M19	*B. subtilis* strain M18	Methicillin-resistant *S. aureus*
APM 7	9.3±0.4^{c}	9.3±0.4^{b}	9.3±0.4^{b}	8.0±0.8^{b}	8.8±0.6^{b}
APM 11	8.8±0.2^{c}	9.2±0.8^{b}	8.5±0.4^{b}	15.0±2.1^{c}	9.5±0.4^{b}
APM 21	7.3±0.4^{b}	9.5±1.7^{b}	7.7±0.8^{b}	7.5±0.5^{b}	8.5±1.5^{b}
Tetracycline	10.0±0.3^{d}	9.7±2.6^{b}	8.7±3.2^{b}	16.7±0.4^{c}	9.8±1.2^{b}
DMSO	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}

Extract used at 20 mg/ml, and tetracycline along with DMSO are applied at 300 µg/ml, and 1% (v/v), respectively; Different superscript letters in the same column showed significantly different

Table 3. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of selected soil actinomycetes crude extracts

Extracts code	MIC and MBC values (µg/ml)									
	E. coli strain M4		*P. aeruginosa* strain M19		*K. penumoniae* strain M19		*B. subtilis* strain M18		*S. aureus* strain M18	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
APM 7	625^{c}	1250^{c}	625^{c}	1250^{c}	10000^{c}	>10000^{d}	2500^{d}	5000^{d}	156^{c}	312^{c}
APM 11	125^{b}	250^{b}	250^{b}	250^{b}	125^{b}	250^{b}	250^{b}	500^{b}	250^{d}	500^{d}
APM 21	625^{c}	1250^{c}	625^{c}	1250^{c}	2500^{c}	5000^{c}	625^{c}	1250^{c}	78^{b}	156^{b}
Tetracycline	8^{a}	16^{a}	8^{a}	16^{a}	31^{a}	62^{a}	4^{a}	8^{a}	4^{a}	8^{a}

Different superscript letters in the same column showed significantly different

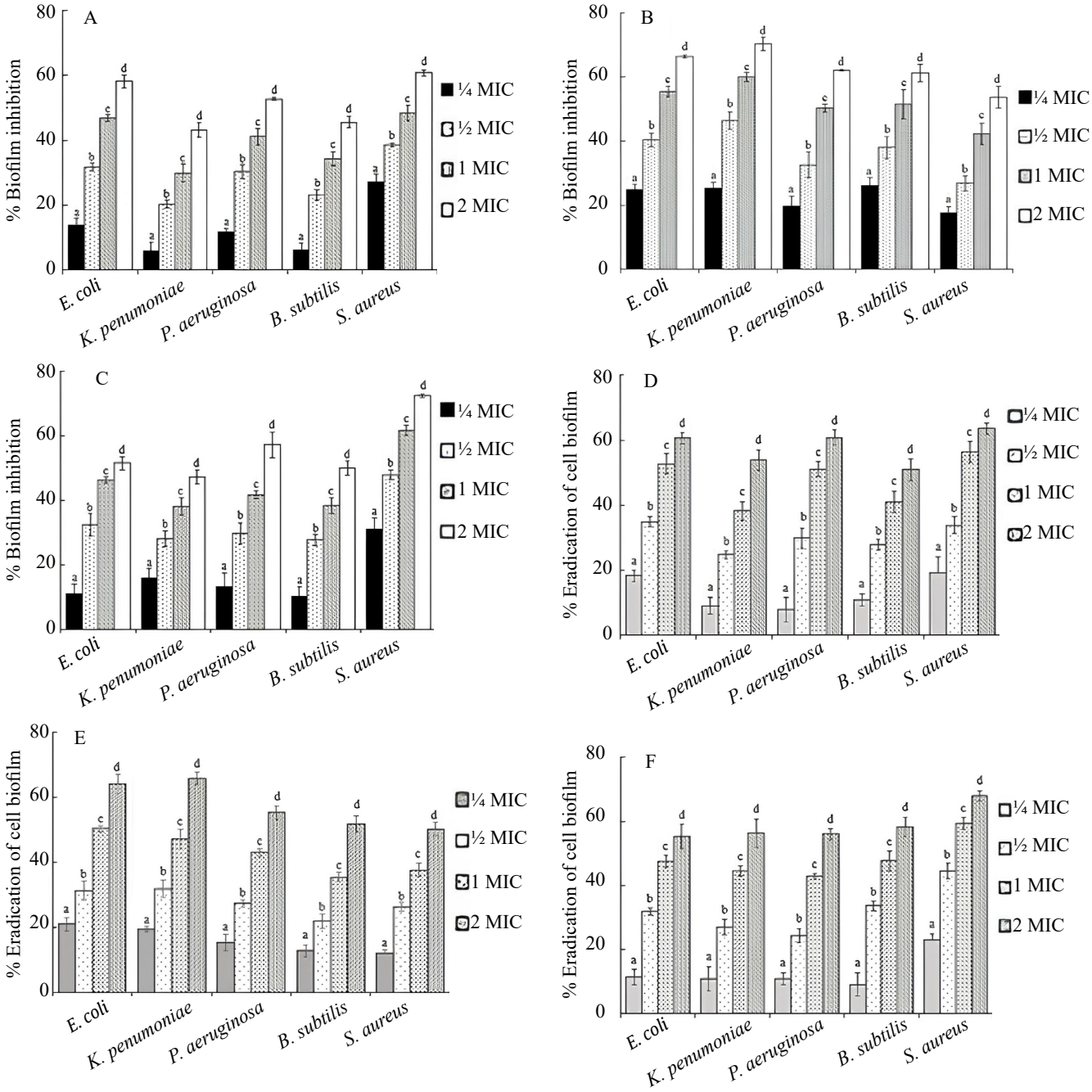

Figure 3. Effect of selected actinomycetes crude extract on the biofilm inhibiton (A-C) and eradications cells biofilm (D-F) from multidrug-resistant clinical isolates cells biofilm. Inhibition activity of crude extract from APM-7 (A), APM-11 (B), and APM-21 (C). Eradication of cells biofilm capacity of crude extract from APM-7 (D), APM-11 (E), and APM-21 (F). Different superscript letters in the same bacterial tested value showed significantly different

3.4. Eradication of Cells Biofilm

The percentage of eradicated cells in biofilm in the presence of the three selected extracts is presented in Figure 3D-F. There was a significant difference in the cell's biofilm exposed to all three test samples at a concentration of 2×MIC compared to ¼ × MIC treatment. Additionally, APM-21 at a concentration of 2×MIC reduced cell biofilm with the highest value of 68% against MRSA. This corresponding extract also recorded the best activity in reducing *B. subtilis* strain M18 biofilm by 58.31%. For APM-11 extract at a concentration of 2×MIC, biofilm of *E. coli* strain M4, and *K. pneumoniae* strain M19 was eradicated with values of 64.21%, and 65.92%, respectively. At the

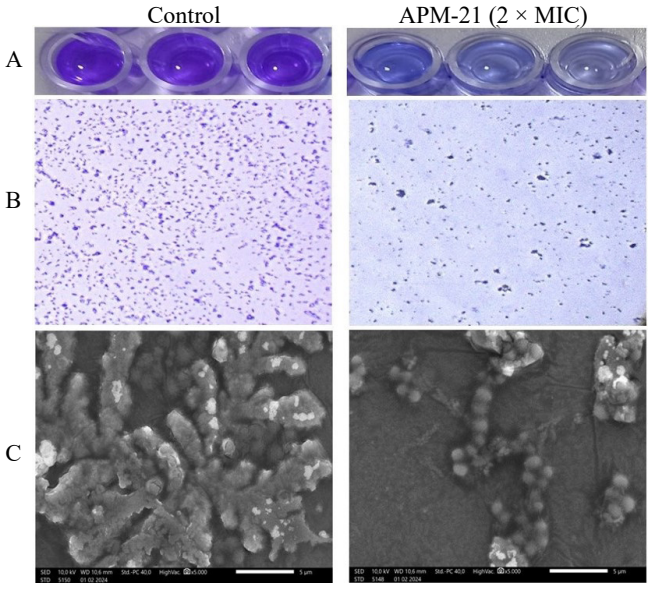

Figure 4. Antibiofilm activity after APM-21 treatment using crystal violet assay. (A) The crystal violet stained biofilm of MRSA after APM-21 treatment in a microtiter plate. (B) Micrographs of the disrupted biofilm of MRSA after APM-21 treatment on the glass surface observed by LM. (C) SEM observations of the disrupted matured biofilms of MRSA by APM-21 extract

lowest concentration of ¼ × MIC, APM-7 extract had poor eradication of cell's biofilm at 9.00% and 7.82% against *K. pneumoniae* strain M19, and *P. aeruginosa* strain M19, respectively.

3.5. Molecular Identity of Selected Isolates

Based on 16S rRNA gene analysis, three potential isolates were identified as *Streptomyces* spp. with a high similarity value (>98%), as shown in Table 4. Further analysis reported that APM-7, APM-11, and APM-21 isolates had high similarity with *Streptomyces cyaneus* strain NBRC 13346, *Streptomyces coerulescens* strain AS 4.1597, and *Streptomyces panayensis* strain 21, respectively. The phylogenetic tree constructed showed that the three potential isolates and their homologous species belong to the same clade of *Streptomyces* spp. (Figure 5). The 16S rRNA sequences of the selected isolates were accessible in the NCBI Genbank database under accession numbers: OR066163.1, OR066164.1, and OR066165.1.

3.6. LC-MS/MS Analysis of 3 Potential Actinomycetes Extracts

The LC-MS/MS analysis of three selected extracts showed seven recognized putative compounds dominant in each extract (Figure 6A-C). These compounds were identified through analysis using the Unify software integrated with the instruments having a comparison of MS fragmentation and library of the Npatlas website. Additionally, the mass spectra of the peaks in the Unify and Npatlas database were compared by considering retention time, molecular formula, and molecular mass (Table 5). Meanwhile, four compounds were identified in APM-7 and APM-21 extracts namely rancinamycin III, enteromycin, bromoisorumbrin, and maremycin. APM-21 extract also contained paramagnetoquinone C, and Caerulomycin I. On the other hand, APM-11 extract, in addition to rancinamycin III, and enteromycin, also contained ashimide B and thiazohalostatin.

4. Discussion

Cureently, novel, active, and broad-spectrum antibiotics are obtained from diverse microbial sources including actinomycetes acting as prominent producers of active metabolites in soil habitats. Isolation of novel strains from extreme and unexplored areas is an important strategy to uncover the potential of new active metabolites (Singh *et al.* 2014). In this context, Muna Island is a region with natural views of karst hills, dry soil and calcareous rock (Purwaningsih *et al.* 2022). This harsh environment allows microbial colonization with the ability to synthesize unique antibacterial compounds. With the development of MDR bacteria and the ongoing effort to combat infectious diseases, there is a highly increasing demand for novel and effective antibiotics from various sources including actinomycetes (Chevrette *et al.* 2019). Therefore, this research was conducted to search for active metabolites against multidrug-resistant bacteria produced by soil actinomycetes.

The initial screening for antibacterial activity showed that the 21 isolates had varying antibacterial activity, as shown in Figure 2. The presence of antibacterial activity was shown by the formation of an inhibition zone around the actinomycetes colonies where bacterial cells are targeted and killed by active compounds produced by actinomycetes. A total of 3 isolates encoding APM-7, APM-11, and APM-21 were identified to inhibit the growth of all five bacterial targets as indicated by the largest inhibition zone. The differences in the antibacterial activity can be influenced by factors such as compound structure, the concentration of active compounds, and the resistance of each targeted bacterial strain. Three potential actinomycete isolates with the capacity to

Table 4. The identity of three selected isolates according to 16S rRNA sequence

Isolate code (accession number)	Closest relative species (accession number)	Query cover (%)	E-value	Similarity (%)
APM 7 (OR066163.1)	*Streptomyces cyaneus* strain NBRC 13346 (NR112525.1)	100	0.00	98.85
APM 11 (OR066164.1)	*Streptomyces coerulescens* strain AS 4.1597 (NR116637.1)	100	0.00	99.59
APM 21 (OR066165.1)	*Streptomyces panayensis* strain 21 (MF462923.1)	100	0.00	99.25

Figure 5. Genetic relationship between our three selected actinomycetes isolates and other related actinomycetes strains based on 16S rRNA gene sequence

inhibit all bacterial targets can produce broad-spectrum antibacterial compounds (Charousová *et al.* 2017). Therefore, the isolates were selected for intensive analysis to extract the active compounds following antibacterial and antibiofilm assessment.

Antibacterial activity from three selected actinomycetes extracts showed that the inhibition zone diameters ranged from 7.3±0.4 mm to 9.5±1.7 mm and from 7.5±0.5 mm to 15.0±2.1 mm against MDR Gram-negative and Gram-positive bacterial strains, respectively (Table 2). Compared with previous investigations, actinomycetes isolated from soils exhibited remarkable antibacterial activity against both Gram-negative and Gram-positive bacteria (Al-Anshari *et al.* 2019). Compared with previous studies on Indonesian actinomycetes strains, our results showed stronger antibacterial activity based on the disc diffusion method. An extract from *Streptomyces* sp. strain H11 isolated from mangrove soil had inhibition zones of 5.8±1.4 and 4.96 mm against *E. coli* strain M4 and *S. aureus*, respectively (Rozirwan *et al.* 2020). However, another extract from actinomycetes isolated from soil rhizosphere showed stronger activity with inhibition zones of 9.23 mm and 8.9 mm against *E. coli* and *S. aureus,* respectively (Rante *et al.* 2020). It is important to note that some previous research was tested against antibiotic-sensitive strains. Additionally, the chemical and physical characteristics of the soil,

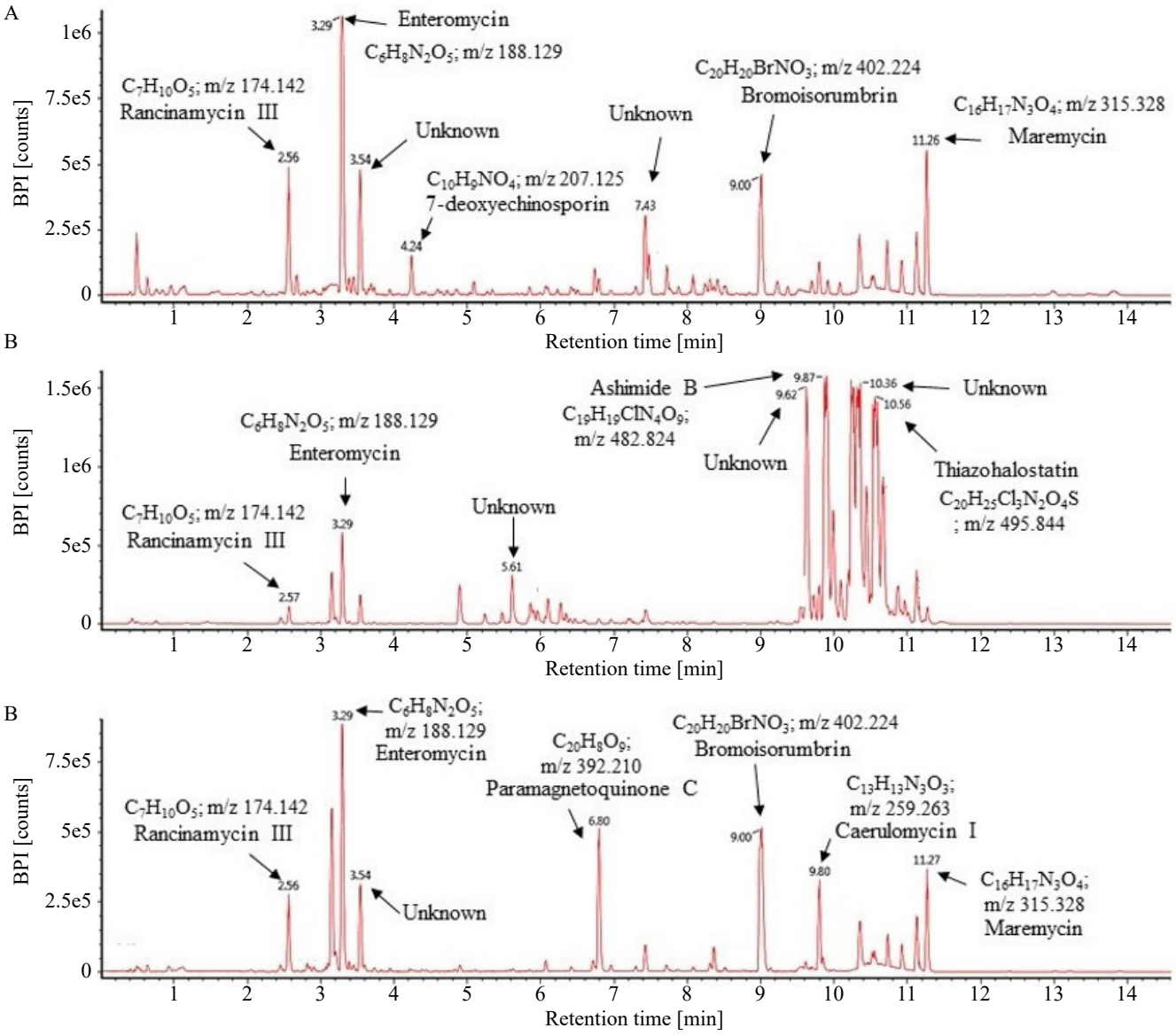

Figure 6. LC-MS/MS chromatogram profile of selected actinomycetes crude extract. (A) APM-7, (B) APM-11, and (C) APM-21

as well as the microbial taxa, varied depending on the environmental condition. Microbes must adapt and develop to withstand different stress conditions and possess the potential to produce novel active compounds with specific bioactivities (Abdel-Haliem *et al.* 2013). Therefore, the three potential actinomycetes from Muna Island soil represent an unexplored source to discover potential active compounds. A few reports demonstrated antibacterial activity from actinomycetes active compounds against MDR bacterial strains (El-Sayed *et al.* 2023), but they have not been previously reported derived from local Indonesian actinomycetes strains.

Antibacterial capacity of natural products could be classified based on the range of MIC values. According to Saraiva *et al.* (2011), an extract is classified as very active, active, moderate, and less active with MIC values of lower than 100 µg/ml, 100-500 µg/ml, 500-1,000 µg/ml, and greater than 1,000 µg/ml, respectively. APM-7 extract was categorized as active against MRSA and showed moderate activity against *E. coli* strain M4 and *P. aeruginosa* strain M19. In addition, APM-11 extract was categorized as active against all bacterial targets. APM-21 extract was highly active against MRSA and showed moderate activity against *E. coli* strain M4, *P. aeruginosa* strain M19, and *B.*

Table 5. LC-MS/MS profile from dominant compounds of selected actinomycetes strains crude extracts

Retention time (minutes)	Compounds	Other sources	Bioactivity	References
		APM-7 extract		
2.56	Rancinamycin III	*S. lincolnensis*	Antibacterial	Argoudelis *et al.* 1976
3.29	Enteromycin	*Micromonospora* sp.	Antibacterial	Igarashi *et al.* 2021
3.54	Unknown	-	-	-
4.24	7-deoxyechinosporin	*S. pseudogriseolus;*	Antibacterial,	Fatima *et al.* 2019; Xu
7.43	Unknown	*Amycolatopsis speibonae*	Antifungal	*et al.* 2019
9.00	Bromoisorumbrin	-	-	-
11.26	Maremycin	*Auxarthron umbrinum*	Cytotoxicity	Clark *et al.* 2011
		Streptomyces sp.	Antifungal	Liu *et al.* 2019
		APM-11 extract		
2.56	Rancinamycin III	*S. lincolnensis*	Antibacterial	Argoudelis *et al.* 1976
3.29	Enteromycin	*Micromonospora* sp.	Antibacterial	Igarashi *et al.* 2021
5.61	Unknown	-	-	-
9.62	Unknown	-	-	-
9.87	Ashimide B	*Streptomyces* sp.	Cytotoxicity	Shi *et al.* 2019
10.36	Unknown	-	-	-
10.56	Thiazohalostatin	*Actinomadura* sp.	Cytoprotective	Yamagishi *et al.* 1993
		APM-21 extract		
2.56	Rancinamycin III	*S. lincolnensis*	Antibacterial	Argoudelis *et al.* 1976
3.29	Enteromycin	*Micromonospora* sp.	Antibacterial	Igarashi *et al.* 2021
3.54	Unknown	-	-	-
6.80	Paramagnetoquinone C	*Actinoallomurus* sp.	Antibacterial	Iorio *et al.* 2017
9.00	Bromoisorumbrin	*Auxarthron umbrinum*	Cytotoxicity	Clark *et al.* 2011
9.80	Caerulomycin I	*Actinoalloteichus*	Antibacterial	Ambavane *et al.* 2014
11.27	Maremycin	*cyanogriseus*	Antifungal	Liu *et al.* 2019
		Streptomyces sp.		

subtilis strain M18. The lowest antibacterial MIC value categorized as very active was observed in the APM-21 extract against MRSA (78 µg/ml) (Table 3). This result was lower than previous study using the *S. lienomycini* BOGE18 extract with MIC value of 250 µg/ml against MDR *S. aureus* strain WS12 (El-Sayed *et al.* 2023). Based on this report, the potential isolate APM-21 exhibited stronger antibacterial activity as showed by the lower MIC value. Compared to *S. koyangensis* strain SHP 9-3 at 7.81 µg/ml), the MIC value against *S. aureus* is slightly larger showing weaker activity (Pahira *et al.* 2023). However, previous study used non-MDR clinical isolates of *S. aureus* strain. Based on antibacterial assessments, the isolate coded as APM-21 was very active against MRSA. This was attributed to the morphological differences with the presence of lipopolysaccharide in Gram-negative bacteria as the hydrophobic layer that results impermeability to lipophilic compounds. On the other hand, Gram-positive bacteria have only an outer peptidoglycan layer which is not effective as a permeability barrier, making them more susceptible to antimicrobial substances (Singh *et al.* 2016).

Antibiofilm activity of three potential actinomycete extracts were further reported. Biofilm is considered the dominant microbial lifestyle and is present in various biotic and abiotic suface. The structure plays a crucial role in antibiotic resistance mechanisms to protects microbial cells from host immunity and prevent the penetration of antibiotics (Patel 2005). In this study, antibiofilm activity of the extract was investigated from actinomycetes strains isolated from a region in Indonesia on MDR bacterial strains. The results showed that APM-11 extract at the concentration of 2× MIC exhibited the best antibiofilm ability against biofilms formed by *E. coli* strain M4, *P. aeruginosa* strain M19, *K. pneumoniae* strain M19, and *B. subtilis* strain M18 with inhibition values ranging from 61.18 to 72.27% (Figure 3B). APM-21 extract at the same concentration had the highest inhibition value of 72.4% against MRSA biofilm formation (Figure 3C). This study also investigated the eradication of biofilm cells. Meanwhile, APM-11 and APM-21 showed the highest percentage of eradication cells biofilm (Figure 3D-F). Previous studies have reported antibiofilm activity from various actinomycetes strains, such as *Streptomyces*

sp., *Streptomyces* sp. SBT343, and *S. lienomycini* BOGE18 against *S. aureus* (MRSA) (Pusparajah *et al.* 2021), *S. epidermidis* (Balasubramanian *et al.* 2017), *S. aureus* WS12 MDR strain (El-Sayed *et al.* 2023) with different antibiofilm capacities. However, the extracts showed a lower inhibition antibiofilm value than *S. lienomycini* BOGE18 extract against *S. aureus* MDR strain, possibly due to differences in the sources of actinomycetes leading to variation of active compounds and bioactivities.

The selected isolates were identified using nucleotide sequencing as *S. cyaneus* (APM-7), *S. coerulescens* (APM-11), and *S. panayensis* (APM-21). Previous research reported that the three identified *Streptomyces* spp. showed antibacterial activity against both Gram-positive and Gram-negative bacteria. Several compounds that may be responsible for the antibacterial activity of the three *Streptomyces* spp. isolates have been identified, such as cirramycin-B, di-(2-ethylhexyl) phthalate and cephamycin (Fukase *et al.* 1976; Driche *et al.* 2015; Atta *et al.* 2011). Antibacterial substances have specific mechanisms in inhibiting bacterial cell growth, including inhibition of cell wall synthesis, alterations to plasma membrane integrity, inhibition of essential metabolite synthesis, damage to nucleic acids, disruption of protein synthesis, and disruption of cellular energy production (Eshboev *et al.* 2024).

In an ongoing investigation of the potency of three actinomycete isolates, further analysis was carried out to identify the chemical constituents present in the three actinomycete extracts through LC-MS/MS. The results showed that three extracts contained two putative compounds, namely rancinamycin III and enteromycin (Figure 6A-C). These compounds have been identified in several actinomycetes strains with potent antibacterial activity (Table 5) (Argoudelis *et al.* 1976; Igarashi *et al.* 2021). Therefore, the strong antibacterial activity of the three extracts is likely attributed to the presence of these compounds. APM-7 extract also contained other putative compounds, namely 7-deoxyechinosporin, bromoisorumbrin, and maremycin, reported to possess antibacterial, cytotoxic, and antifungal activities, respectively (Clark *et al.* 2011; Fatima *et al.* 2019; Xu *et al.* 2019; Liu *et al.* 2019). In addition, APM-11 extract contained other putative compounds, namely ashimide and thiazohalostatin, known for cytotoxic and cytoprotective activities, respectively (Yamagishi *et al.* 1993; Shi *et al.* 2019). APM-21 extract, which exhibited the strongest antibacterial activity against MRSA, also contained two other putative compounds, namely paramagnetoquinone C and caerulomycin I, known antibiotics derived from actinomycetes with potent antibacterial activities (Ambavane *et al.* 2014; Iorio *et al.* 2017). The results suggested that the antibacterial and antibiofilm properties of the selected extracts might be promoted by the presence of active compounds.

In conclusion, soil *Streptomyces* spp. encoding APM-7, APM-11, and APM-21 were reported to show potential antibacterial and antibiofilm activities against MDR clinical isolates. The MIC of antibacterial compounds against the test pathogens showed potent activity as an antibacterial agent against MDR strains, specifically MRSA. Meanwhile, naturally occurring *Streptomyces* spp. had the potential to produce active constituents against target bacteria and disrupt their biofilm structures enabling the discovery of alternative antibiotics. Further characterization and structure elucidation of the active compounds must be carried out to provide deep insight into the development of antibiotic candidates derived from Indonesian *Streptomyces* spp. isolates.

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