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Antimicrobial compounds from intracellular and extracellular secondary metabolites of Actinobacteria InaCC A759

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ABSTRACT The World Health Organization (WHO) has determined a list of pathogens that require the development of new antimicrobials due to resistance problems; these include *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus*. In addition, *Mycobacterium smegmatis* has been used for antimycobacterial discovery to address the increasing burden of tuberculosis. In this study, optimization of antimicrobial activity, secondary metabolite profiling, and strain identification was conducted on Actinobacteria InaCC A759. Intracellular and extracellular extracts of Actinobacteria InaCC A759 were found to have different antimicrobial activities. The minimum inhibitory concentration (MIC) values of the extract to inhibit the growth of *M. smegmatis*, *E. coli*, and *P. aeruginosa* were 50, 25, and 100 µg/mL (intracellular), and 25, 25, and 100 µg/mL (extracellular), respectively. However, neither extract was able to inhibit the growth of *S. aureus*. Metabolite profiling using High resolution-mass spectrometry (HR-MS) resulted in differences in the major compound between the two extracts of Actinobacteria InaCC A759, namely n-acetyltyramine ($C_{10}H_{13}NO_2$/179.0945) (24.24%) (intracellular) and palmitic acid ($C_{16}H_{32}O_2$/273.27034) (86.92%) (extracellular). Based on molecular analysis of the 16S rRNA gene, Actinobacteria InaCC A759 is identical to the *Streptomyces olivaceus* strain FoRh46. The antimicrobial activity and secondary metabolites profiles of *Streptomyces olivaceus* InaCC A759 have not been previously reported.

KEYWORDS Actinobacteria; Antimicrobial agents; Metabolite profiling; N-acetyltyramine; Palmitic acid

1. Introduction

Antimicrobial resistance (AMR) is still become global problem, causing 700,000 deaths globally per year. Several approaches can be taken to address AMR, including reduce the overuse and misuse of antimicrobials (IACG 2019; Serwecińska 2020). Antimicrobials discovery and development is also necessary to overcome AMR problems. Since 2017, new antimicrobials have been developed, with a total of 252 currently in the pre-clinical development phase and 8 drugs that have entered the clinical phase (World Health Organization 2019a,b).

In 2017, World Health Organization (WHO) has determined a list of pathogens that require the development of new antimicrobial. The main priority is the development of tuberculosis drugs. However, several pathogens are in the order of critical and high priority, such as *Pseudomonas aeruginosa*, Enterobacteriacae, and *Staphylococcus aureus* (World Health Organization 2017). *Escherichia coli* as an Enterobacteriacae is still a significant cause of death due to infection (MacKinnon et al. 2020).

For antimycobacterial activity, *Mycobacterium smegmatis* can replace *Mycobacterium tuberculosis* as test bacteria (Arthur et al. 2019). In addition, *M. smegmatis* is also can be used to understand the cellular processes of pathogenic mycobacteria, the physiological conditions, and stress adaptation factors of *M. tuberculosis* (He and De Buck 2010; T et al. 2020).

Actinobacteria has been known as one of the antimi-

crobials producers, mainly from the genus *Streptomyces*. Various antimicrobials have been produced from *Streptomyces* sp., such as streptomycin, clindamycin, lincomycin, ivermectin, nystatin, and tetracycline (Mast and Stegmann 2019; Quinn et al. 2020). However, many factors can affect the production of antimicrobials from Actinobacteria, including the medium used for production or type of producing species. Antimicrobials are secondary metabolites produced by Actinobacteria, and can be found either intracellular or extracellular. Secondary metabolites that are intracellular or extracellular can have different strengths of biological activity (Retnowati et al. 2018; Damayanti et al. 2021). Extracellular metabolites are easier to obtain than intracellular metabolites as they do not require cell rupture to isolate it (Pinu and Villas-Boas 2017).

Based on initial screening of the antimicrobial activity of extracellular extracts from sixteen strains of Actinobacteria collection from the Indonesian Culture Collection (InaCC), Actinobacteria InaCC A759 had the potential to inhibit the growth of *M. smegmatis*. Actinobacteria InaCC A759 was explored further to see its potential as an antimicrobial agent against *M. smegmatis*, *P. aeruginosa*, *E. coli*, and *S. aureus*. Secondary metabolites of Actinobacteria InaCC A759 were extracted from intracellular and extracellular. The activities of the two extracts were compared, and then secondary metabolite profiling was performed using non targeted High Resolution-Mass Spectrometry (HR-MS). Metabolite profiling using HR-MS has been widely reported to screen the presence of novel compounds from secondary metabolites of microorganisms (Kim et al. 2016; Kibret et al. 2018). HR-MS is an important tool in secondary metabolite profiling due to its good accuracy in distinguishing isobaric/isomeric compounds (Watson 2013). In this research, metabolite profiling was carried out to determine the secondary metabolite compounds contained in the intracellular and extracellular extracts, and its novelty.

This study also identified the species of Actinobacteria InaCC A759 using 16S rRNA gene sequencing analysis. As is well known, the differences in species and the locations founds of Actinobacteria can affect the diversity of its secondary metabolites (Selim et al. 2021). Differences in activated biosynthetic gene clusters, particularly Non Ribosomal Peptide Synthetase (NRPS) and Polyketide Synthase (PKS) also determine the novelty potential of compounds produced by Actinobacteria (Wei et al. 2018). Therefore, we also identified the NRPS and PKS genes from Actinobacteria InaCC A759.

2. Materials and Methods

2.1. Actinobacteria and tested bacteria

Actinobacteria InaCC A759 strain is a collection from InaCC, The National Research and Innovation Agency, Cibinong, Indonesia. The antibacterial activity test of the Actinobacteria InaCC A759 extract was carried out on the *P. aeruginosa* (ATCC 27853), *E. coli* (ATCC 25922), *S. aureus* (ATCC 29213), and *M. smegmatis* strain mc2 155 (ATCC 700084). The bacteria used for the test were obtained from the microbiology laboratory of the Medical Faculty, Universitas Muhammadiyah Semarang, Indonesia.

2.2. Production and extraction of intracellular and extracellular secondary metabolites

The culture process and extracellular extraction of secondary metabolites of Actinobacteria were undertaken using the methods that have been used previously (Rakhmawatie et al. 2021). Intracellular extracts prepared from cell biomass of Actinobacteria InaCC A759 extracted by liquid-solid extraction with methanol solvent (Merck, Germany) at a ratio of 1:5 (w/v), and using a slow speed stirrer for 30 min. Separation of methanol extract from the Actinobacteria InaCC A759 cells was attempted by centrifugation at 6,000 rpm, 22 °C for 15 min (Damayanti et al. 2021). The methanol solvent was then extracted using chloroform (Merck, Germany) at ratio of 1:1 (v/v). At last, vacuum rotary evaporator (Buchi, Switzerland) at 40 °C used to dry the crude methanol-chloroform (intracellular) and ethyl acetate (extracellular) extract.

2.3. Antimicrobial susceptibility testing of Actinobacteria InaCC A759 extracts

The media used for the susceptibility testing is Mueller Hinton Broth (MHB) (Clinical and Laboratory Standards Institute (CLSI) 2020). For the susceptibility testing against *M. smegmatis*, the control drugs used were rifampicin and isoniazid with two-fold dilution concentration ranging from 0.625–1.0 µg/mL. For the susceptibility testing against *P. aeruginosa*, *E. coli*, and *S. aureus*, gentamicin was used as the control, with concentration range of 0.625–1.0 µg/mL. The antibacterial activity of Actinobacteria InaCC A759 extract against *M. smegmatis*, *P. aeruginosa*, *E. coli*, and *S. aureus* was performed using the Resazurin Microplate Assay (REMA) method with concentration range of 6.25–100 µg/mL. An extract concentration of <100 ug/mL is considered a highly active concentration for optimizing antimicrobial development (Silva et al. 2013). The incubation time for *M. smegmatis* is 48 h (Rakhmawatie et al. 2019), while for other bacteria is 18 h (van Rensburg et al. 2021), with both performed at the temperature of 37 °C.

2.4. Molecular identification and phylogenetic analysis of 16S rRNA, NRPS, and PKS gene

The DNA extraction of Actinobacteria InaCC A759 and 16S rRNA gene amplification method was analyzed using the method from previous studies (Rakhmawatie et al. 2021). The change in this research is the use of PCR master mix MyTaqTM HS Red Mix (Bioline, UK) for the amplification of 16S rRNA, NRPS, and also PKS.

For the NRPS gene amplification, A3F and A7R primer were used at the concentration of 10 µM, and PKS gene amplification using K1F and M6R primer. Amplification was carried out using previous method

(Rakhmawatie et al. 2021). The results of DNA amplification of the NRPS and PKS genes were then visualized using 1% agarose gel, 1× TAE buffer, 50 volts for 60 min. The NRPS gene band will be detected at a size of about 700–800 bp, while the PKS gene at a size of around 1,400 bp.

Amplification product of 16S rRNA, PKS, and NRPS Actinobacteria InaCC A759 genes was sent for sequencing analysis to Genetika Science, Tangerang, Indonesia. A homology sequence data match was performed using NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and phylogenetic tree was constructed using the Neighbor Joining Method (Kumar et al. 2016).

2.5. Non-targeted HR-MS metabolite profiling

Intracellular and extracellular extracts of Actinobacteria InaCC A759 were dissolved in methanol to a concentration of 4,000 g/mL, then filtered using a 0.22 m nylon filter membrane (Djinni et al. 2014). Chemical profiling of secondary metabolites were performed using non targeted UHPLC HR-MS (Thermo Scientific™ Ultimate™ 3000 RSLCnano) coupled with mass detector (Thermo Scientific™ Q Exactive™ High-Resolution Mass Spectrometer), and using columns and methods according to previous studies (Rakhmawatie et al. 2021). Detection of secondary metabolites from Actinobacteria InaCC A759 extract was carried out using Thermo Scientific® Compound Discoverer Software.

3. Results and Discussion

3.1. Antimicrobial susceptibility test result of Actinobacteria InaCC A759 extracts

Three types of wild type pathogenic bacteria were used in this study, among others Gram-negative bacteria (*P. aeruginosa* and *E. coli*), and Gram-positive bacteria (*S. aureus*). Apart from being able to replace *M. tuberculosis* as an antimycobacterial test bacteria, the use of *M. smegmatis* is also important because an increase in the number of immunodeficiency patients can increase the risk of *M. smegmatis* to cause infection. *Mycobacterium smegmatis* is also difficult to eradicate because of their higher natural resistance to antimicrobial (Brown-Elliott et al. 2012; Van Ingen et al. 2012).

Antimicrobial activity screening of the intracellular and extracellular extracts of Actinobacteria InaCC A759 yielded information that the two types of extracts had no difference in the growth inhibitory of *P. aeruginosa*, *E. coli*, and *S. aureus*. The intracellular and extracellular extracts of Actinobacteria InaCC A759 were unable to inhibit the growth of *S. aureus* at the highest test concentration (100 µg/mL). Meanwhile, both extract of Actinobacteria InaCC A759 was able to inhibit the growth of *E. coli* (MIC of 25 µg/mL) better than its activity to inhibit *P. aeruginosa* (MIC 100 µg/mL). For the test bacteria *M. smegmatis*, the intracellular extract (MIC of 25 µg/mL) produced by Actinobacteria InaCC A759 had higher activity than the extracellular extract (MIC of 50 µg/mL) (Table 1).

3.2. Phylogenetic analysis results of 16S rRNA, NRPS, and PKS gene

Based on the molecular detection of the 16S rRNA gene followed by phylogenetic tree analysis, the Actinobacteria InaCC A759 was identified as *Streptomyces olivaceus* strain FoRh46 with a similarity of 100.00%. The comparison strain used in the phylogenetic analysis was *Streptomyces sp.* antimicrobial producer (Figure 1).

Meanwhile, for the results of the analysis of secondary metabolite-producing genes, Actinobacteria InaCC A759 was detected to have NRPS and PKS genes. Based on the Blastx results, the NRPS of Actinobacteria InaCC A759 showed 99.57% similarity to the amino acid adenylation domain-containing protein of *Streptomyces olivaceus* (Figure 2). For the PKS phylogenetic analysis, Actinobacteria InaCC A759 has 57.65% of PKS similarity to the type I modular polyketide synthase of *Streptomyces malaysiensis* (Figure 3).

Streptomyces olivaceus has been known to produce antimicrobial compounds. For example, the intracellular ethyl acetate extract of *S. olivaceus* LEP7 was reported to inhibit the growth of *S. aureus*, *E. coli*, *P. aeruginosa* when produced using starch casein media (Rajaram et al. 2020). The antimicrobial activities of secondary metabolites of the *S. olivaceus* JB1 was reported against phytopathogen strains including *Bacillus megaterium*, *Bacillus thuringiensis*, *Leclercia adecarboxylata*, *Pseudomonas punonensis*, and *Mucor circinelloides* (Um et al. 2022).

Whole genome sequencing analysis has also been carried out on *S. olivaceus* SCSIO T05 and resulted in the detection of Biosynthetic Gene Clusters (BGCs) for the production of mycemycins, terpenoids xiamycins, PKS rishirilides and PKS lobophorin. Other BGCs from *S. olivaceus*

TABLE 1 The result of susceptibility testing of Actinobacteria InaCC A759 extract against tested bacteria using two-fold microdilution assay.

Tested Bacteria	Control Drug Used	MIC of Control Drug (µg/mL)	MIC of Intracellular Extract (µg/mL)	MIC of Extracellular Extract (µg/mL)
P. aeruginosa ATCC 15422	Gentamicin	0.125	100	100
E. coli ATCC 23848	Gentamicin	0.0625	25	25
S. aureus ATCC 29213	Gentamicin	0.125	>100	>100
M. smegmatis ATCC 700084	Rifampicin	0.0625	25	50
	Isoniazid	>100		

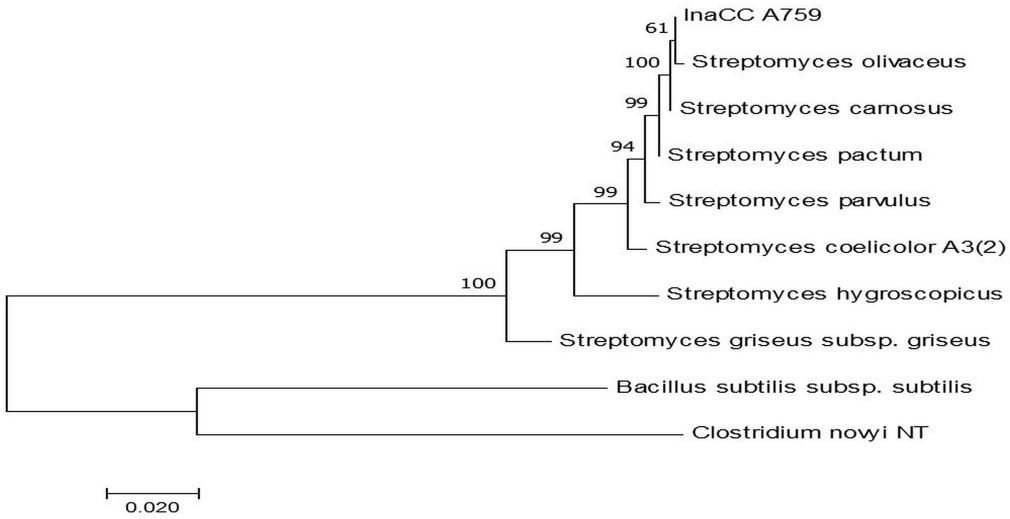

FIGURE 1 The results of the 16S rRNA phylogenetic analysis of the Actinobacteria InaCC A759 using Neighbor-Joining Method MEGA 11.0.

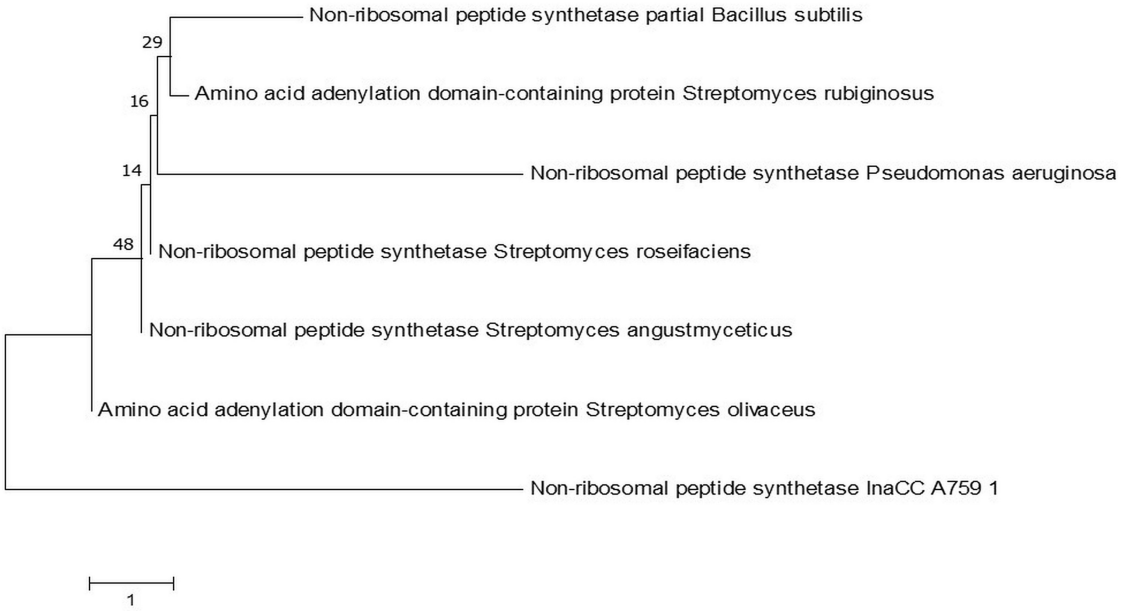

FIGURE 2 The results of the NRPS phylogenetic analysis of the Actinobacteria InaCC A759 using Neighbor-Joining Method MEGA 11.0.

SCSIO T05 including four PKS (Type I, Type II and Type III) and six NRPS. Twenty-one BGCs are also predicted to produce terpenes, bacteriocin, and lanthipeptide (Zhang et al. 2019). For the PKS phylogenetic analysis, Actinobacteria InaCC A759 has 57.65% of PKS similarity to the type I modular polyketide synthase of *Streptomyces malaysiensis*. It seems that the PKS genes of *S. olivaceus* and *S. malaysiensis* have similarities, especially in the production of PKS lobophorin compounds. Lobophorin compounds can be synthesized by *S. olivaceus* JB1 (Um et al. 2022), *S. olivaceus* SCSIO T05 (Sun et al. 2018), and *Streptomyces* sp. B-81 related to *S. malaysiensis* (Clavo et al. 2022).

In this study, lobophorin compounds were not synthesized by Actinobacteria InaCC A759, possibly due to differences in the culture media used. This study used starch, yeast, and peptone nutrients while *S. olivaceus* JB1 used K media (12 g of LB, 12 g of PDB, 1 g of TSB, and 18 g of agar per 1 L of sterilized water) to produce lobophorin (Um et al. 2022). *Streptomyces olivaceus* SCSIO T05 also used a different culture medium to produce lobophorin compounds, namely RA medium (high sucrose as carbon source and potassium nitrate as nitrogen source) (Sun et al. 2018). In addition to lobophorin, *Streptomyces* sp. B-81 related to *S. malaysiensis* can also synthesize PKS divergolides A (3), B (4) and C (naphthoquinone macrolide) compounds (Clavo et al. 2022). However, all these PKS compounds were also not found in the intracellular and extracellular extracts of Actinobacteria InaCC A759.

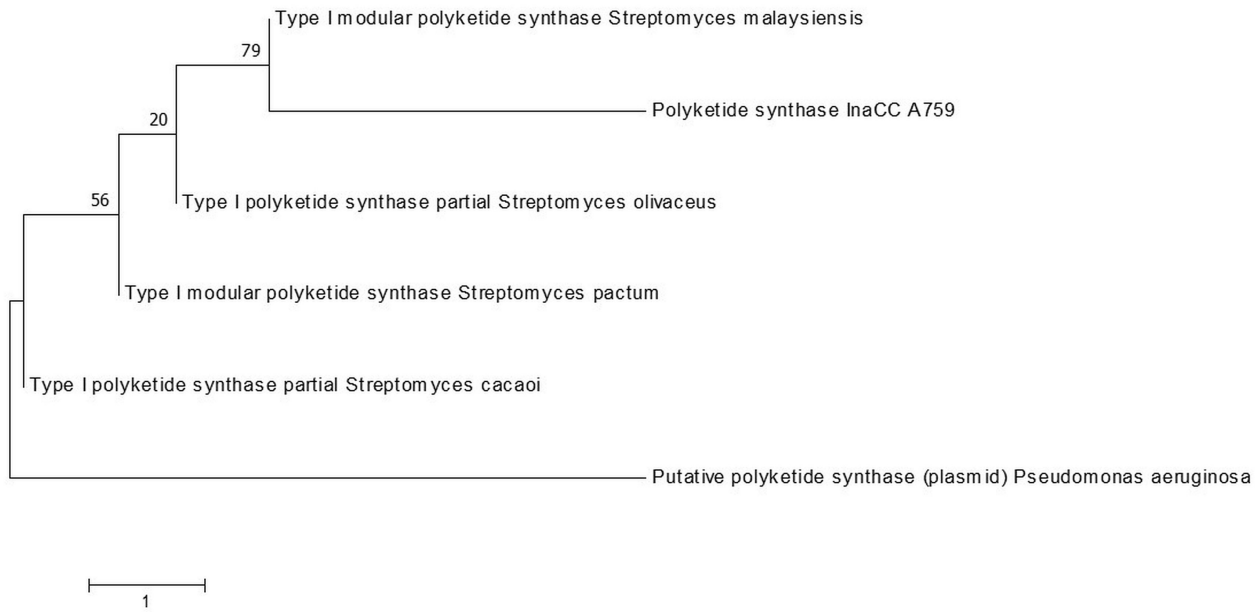

FIGURE 3 The results of the PKS phylogenetic analysis of the Actinobacteria InaCC A759 using Neighbor-Joining Method MEGA 11.0.

3.3. Non-targeted HR-MS metabolite profiling results

The types of compounds that have an area of ≥ 0.5% in extracellular extracts are more varied than the types of compounds in intracellular extracts. In the extracellular extract, n-acetyltyramine ($C_{10}H_{13}NO_2$/179.0945) was the dominant compound with an area of 24.24%. This compound is also present in the intracellular extract with a smaller amount of 0.75% (Table 2). The dominating compound for intracellular extract was palmitic acid ($C_{16}H_{32}O_2$/273.27034) with an area of 86.92% (Table 3). Several other compounds were also present in both types of extracts, including cyclo(phenylalanylprolyl) ($C_{14}H_{16}N_2O_2$/244.1241), cyclo(leucylprolyl) ($C_{11}H_{18}N_2O_2$/210.13718), and (-)-caryophyllene oxide ($C_{15}H_{24}O$/220.18327).

Based on the results of antibacterial activity and metabolite profiling, there are differences in the characteristics of intracellular and extracellular extracts of Actinobacteria InaCC A759. Intracellular extract of Actinobacteria InaCC A759 was obtained from secondary metabolites present in the cells. The extraction process was carried out by shaking the Actinobacteria InaCC A759 cells using methanol as a solvent with the aim of cell leakage and protein denaturation (Mushtaq et al. 2014). Furthermore, chloroform is used for liquid-liquid extraction of methanol extract, so that the polar primary metabolites can be eliminated (Pinu et al. 2017). Primary metabolites are usually metabolites produced for the basic needs of microbes, and are usually polar, such as amino acids and nucleotides (Seyedsayamdost 2019). The extracellular extract of Actinobacteria InaCC A759 was extracted from the supernatant/culture media using ethyl acetate as solvent. Extracellular extracts are considered better to evaluate the changes in bacterial metabolism due to environmental changes. The extraction process of extracellular extract is also considered simpler than intracellular extracts (Pinu and Villas-Boas 2017). Intracellular and extracellular extracts produced by microorganisms can also have differences in terms of compound content. Based on non-targeted HR-MS analysis, there are major differences in the dominant compounds between the two types of extracts.

3.4. Discussion

Based on the results of metabolite profiling, Actinobacteria InaCC A759 in this study was not identified to produce new compounds. Apart from all, Actinobacteria InaCC A759 is still considered potential to produce secondary metabolites that have antimicrobial activity. The dominant compound produced from both the intracellular and extracellular extracts of Actinobacteria InaCC A759 can be used for the development of antimicrobial compounds.

From the dominant compounds present in both types of Actinobacteria InaCC A759 extract, the n-acetyltyramine alkaloid compound is a non-specific compound that can be produced by Actinobacteria. Some species such as *Nocardia nova*, *Nocardiopsis alba*, *Rhodococcus coprophilus*, *Streptomyces albidoflavus*, *Streptomyces flavoviridis*, *Streptomyces griseoflavus*, *Streptomyces hydrogenans*, and *Streptomyces setonii* can produce n-acetyltyramine. This compound has been reported to have antimicrobial activity against *E. coli*, but unable to inhibit the growth of *S. aureus* (dos Santos et al. 2022). The activity of n-acetyltyramine in inhibiting virulence factor activated-quorum sensing of *Chromobacterium violaceum* ATCC 12472 and *P. aeruginosa* was also reported. In that study, n-acetyltyramine was produced by *Vibrio alginolyticus* strain M3-10 (Reina et al.

TABLE 2 Secondary metabolite prediction in intracellular extract of Actinobacteria InaCC A759, based on peak chromatogram profile of non-targeted HR-MS analysis*.

No	Compound Prediction (Chemical Formula/Molecular Weight)	Retention Time (Min)	% Similarity Index	% Peak Area
1	**N-Acetyltyramine ($C_{10}H_{13}NO_2$/179.0945)**	13.06	94.4	24.24
2	[Similar to: Pestalotin; ΔMass: -27.0470 Da] ($C_{13}H_{23}NO_3$/241.1675)	19.38	95.5	13.74
3	**Cyclo(leucylprolyl) ($C_{11}H_{18}N_2O_2$/210.1365)**	14.26	93.1	13.69
4	[Similar to: NPEO; ΔMass: 126.1411 Da] ($C_{10}H_{14}O_3$/182.0940	17.98	91.3	8.37
5	Anthranilamide ($C_7H_8N_2O$/136.0636)	9.85	90.8	5.1
6	[Similar to: 2-(2-Thienyl)-1,4-dihydroquinazolin-4-one; ΔMass: 108.9987 Da] (C_7H_5NO/119.0370)	3.36	98.7	3.87
7	**Cyclo(phenylalanyl-prolyl) ($C_{14}H_{16}N_2O_2$/ 244.1208)**	15.52	95.4	3.45
8	**(Similar to:(-)-Caryophyllene oxide ($C_{15}H_{24}O$/220.1824)**	24.49	93.6	3.15
9	4-Hydroxyephedrine ($C_{10}H_{15}NO_2$/163.0995)	17.02	76	3.07
10	[Similar to: Ritodrine; ΔMass: 94.0420 Da] ($C_{11}H_{15}NO_2$/193.1101)	14.37	96	1.62
11	Acetophenone (C_8H_8O/120.0575)	13.06	84.4	1.26
12	1-(2-Morpholinophenyl)dihydro-1H-pyrrole-2,5-dione ($C_{14}H_{16}N_2O_3$/260.1158)	13.75	70.4	1.18
13	[Similar to: Taurocholic acid; ΔMass: 107.9887 Da] ($C_{24}H_{41}NO_4$/407.3030)	20.31	76.2	1.15
14	6-Methoxyquinoline ($C_{10}H_9NO$/159.0682)	6.45	80.4	1.13
15	Amobarbital ($C_{11}H_{18}N_2O_3$/226.1314)	12.78	54.7	1.01
16	[Similar to: Erucamide; ΔMass: 80.0994 Da] ($C_{15}H_{31}NO_2$/257.2351)	22.43	76.7	0.9
17	Mesalamine ($C_7H_7NO_3$/153.0424)	11.8	69.8	0.82
18	[Similar to: Cyclosporine A; ΔMass: 975.6736 Da] ($C_{12}H_{22}N_2O_2$/226.1678)	12.33	65.4	0.82
19	12-Aminododecanoic acid ($C_{12}H_{25}NO_2$/ 215.1883)	18.58	67.1	0.81
20	[Similar to: Edaravone; ΔMass: -42.0103 Da] ($C_{12}H_{12}N_2O_2$/216.08956)	15.63	69.3	0.7

*Secondary metabolites shown in this table were compounds with high peak area only (> 0.5%), bold print indicates secondary metabolites that are also present in extracellular extracts. This HR-MS analysis was used analytical column C18 1.9 µm; 1×50 mm. The mobile phases were A (0.1% formic acid in H_2O) and B (0.1% formic acid in acetonitrile), and the flow rate was 10 µL/min with a gradient flow of 5–95% B for 22 min.

TABLE 3 Secondary metabolite prediction in extracellular extract of Actinobacteria InaCC A759, based on peak chromatogram profile of non-targeted HR-MS analysis*.

No	Compound Prediction (Chemical Formula/Molecular Weight)	Retention Time (Min)	% Similarity Index	% Peak Area
1	Palmitic Acid ($C_{16}H_{32}O_2$/273.27034)	22.81	84.1	86.92
2	NP-011220 ($C_{11}H_{18}N_2O_2$/210.13717)	11.53	99.5	2.48
3	**Cyclo(phenylalanyl-prolyl) ($C_{14}H_{16}N_2O_2$/ 244.1241)**	13.76	97.9	1.04
4	**Cyclo(leucylprolyl) ($C_{11}H_{18}N_2O_2$/210.13718)**	11.95	99.3	0.83
5	**N-Acetyltyramine ($C_{10}H_{13}NO_2$/179.09428)**	8.26	96.9	0.75
6	**(-)-Caryophyllene oxide ($C_{15}H_{24}O$/220,18327)**	23.37	91.1	0.73
7	N1-[2-oxo-6-(1H-pyrrol-1-yl)-2H-chromen-3-yl]acetamide ($C_{15}H_{12}N_2O_3$/268.08861)	21.92	80.9	0.61
8	6-Hydroxy-1-(hydroxymethyl)-5-{2-[(4-methoxybenzyl)amino]-2-oxoethyl}-1,4a-dimethyldecahydro-2-naphthalenyl propylcarbamate ($C_{27}H_{42}N_2O_6$/490,3109)	0.96	79.1	0.5

*Secondary metabolites shown in this table were compounds with high peak area only (> 0.5%), bold print indicates secondary metabolites that are also present in extracellular extracts. This HR-MS analysis was used analytical column C18 1.9 µm; 1×50 mm. The mobile phases were A (0.1% formic acid in H_2O) and B (0.1% formic acid in acetonitrile), and the flow rate was 10 µL/min with a gradient flow of 5–95% B for 22 min.

2019). However, it seems that not all n-acetyltyramine produced by Actinobacteria has antimicrobial activity because it depends on its concentration. In n-acetyltyramine produced by *Streptomyces* sp. LHW2432, a concentration

of 100 µg/mL was unable to inhibit the growth of *Bacillus mycoides*, methicillin-resistant *S. aureus* (MRSA), *M. smegmatis*, *C. albicans*, and *E. coli* (Liu et al. 2020).

The dominant compound found only in intracellular extracts, namely palmitic acid, is one of the free fatty acids (FFAs) known to have antimicrobial activity. Free fatty acids have broad spectrum activity, including the use for combination/adjuvant antibiotic therapy (Casillas-Vargas et al. 2021). An example is the success of nonanoic and decanoic acid as combination therapy against *M. tuberculosis*, with mechanism enhance the effects of streptomycin, rifampicin, and isoniazid (Chong et al. 2021). As a single compound, palmitic acid from *Psychrolutes marcidusas* is the most active component that can inhibit the growth of *M. tuberculosis* H37Ra and *M. smegmatis* (MIC value of 250–500 µg/mL). The antimicrobial effects is actually odds with current understanding of the fatty acid parameters required for bioactivity in terms of chain length and degree of unsaturation (Dasyam 2014).

Free fatty acids are chains of carbon atoms bonded to hydrogen atoms. The number of carbon atoms varies from 10 to 28. Palmitic acid is saturated FFAs with a total of 16 carbons. The antimicrobial activity of FFAs itself still needs to be studied further. Free fatty acids have a target action on bacterial cell membranes, by blocking cell energy metabolism in the mechanism of electron transport disorders and oxidative phosphorylation. One of research stated that MRSA is reported to have increased susceptibility to FFAs (Song et al. 2020). In contrary, several Gram positive and Gram negative strains can be naturally resistant to FFAs, for example *S. aureus* which uses FFAs to up regulate proteins that strengthen cell walls and reduce their hydrophobicity (Desbois and Smith 2010). Several studies have shown that FFAs can be made in the form of liposomal drug delivery in order to have antibacterial activity. This liposomal form is known to have antibacterial activity against *P. aeruginosa*, *S. aureus*, several drug-resistant strains such as *Staphylococcus epidermidis* and *Enterococcus faecalis* (Casillas-Vargas et al. 2021). Palmitic acid from chloroform intracellular extract has also been reported to be isolated from *Streptomyces* sp. GMR22. In that study, the content of palmitic acid in the intracellular extract reached 42.74% and had antibiofilm activity against *C. albicans* (Nirwati et al. 2022).

In addition to n-acetyltyramine and palmitic acid, several other compounds were also present in both types of extracts. Cyclo(phenylalanyl-prolyl) dan cyclo(leucylprolyl) is a pyrrolosesquiterpenes compound produced by Actinomycetes (Tian et al. 2017). Report of antibacterial activity of pure compound cyclo(phenylalanyl-prolyl) dan cyclo(leucylprolyl) not yet found. However, there is an in silico study that shows the antibacterial activity of cyclo(phenylalanyl-prolyl). In silico, cyclo(phenylalanyl-prolyl) compounds have potential as antibacterial with possible mechanisms of action as membrane integrity antagonists, glycopeptide-like antibiotics, inhibiting membrane permeability, and inhibiting DNA synthesis (Husain and Wardhani 2021). For other sesquiterpene compounds, caryophyllene oxide is not a typical compound of secondary metabolites of microorganisms. Caryophyllene oxide is also a compound commonly found in plant extracts, and may be involved in the antibacterial activity against some microbes. Strong inhibition due to caryophyllene oxide occurred in *Klebsiella pneumoniae*, *E. coli*, and *Bacillus cereus*, but weak inhibition also occurred in *S. aureus* and *P. aeruginosa* (Dahham et al. 2015; Chassagne et al. 2021).

Among the compounds contained in the extracellular extract, other important compounds were also found, such as mesalamine which is an anti-inflammatory agent in the gastrointestinal tract. Mesalamine is known to reduce *P. aeruginosa* biofilm formation and decrease the capacity of these bacteria to remain in inflamed cells (Dahl et al. 2017). However, mesalamine has no activity against *Mycobacterium paratuberculosis* (Sung and Collins 2008).

Secondary metabolite compounds from microorganisms are usually difficult to synthesize chemically, therefore total amount of compounds and the ease for the purification process are important things to ensure the availability of the pure compounds. For example, the purification process of n-acetyltyramine is reported to be quite short and cost effective to be developed as a pharmaceutical product (Heidari and Mohammadipanah 2018). Meanwhile, palmitic acid from secondary metabolites of *Streptomyces* sp. is quite dominant and can be detected between 17.22–62.69% depending on the culture media and extract solvent used (Voytsekhovskaya et al. 2018).

The study's results stated differences in the MIC values of the intracellular and extracellular extracts of Actinobacteria InaCC A759 for the test bacteria. Although the compound with antibacterial activity can be predicted from both types of extracts, the pure compound responsible for the difference in activity needs to be investigated further. Unlike plant extracts which have an antibacterial mechanism of action, especially in disrupting membranes, microbial extracts have a more varied mechanism of action due to the diversity of types of compounds (Álvarez-Martínez et al. 2021). The mechanism action of antibacterial can be studied using traditional methods after purification of the active compound. Currently, new methods such as thermal proteome profiling or affinity chromatography have been developed for easy targeting the mechanism action of mixtures compounds in extracts (Hudson and Lockless 2022), considering that the compound purification process requires more resources. Therefore, further research can be carried out to determine the antibacterial mechanism action of Actinobacteria InaCC A759 extract, thereby optimizing its development as an antibacterial.

4. Conclusions

Intracellular and extracellular extracts of Actinobacteria InaCC A759 showed no different in antimicrobial effects against *P. aeruginosa* and *E. coli*. However, neither both extracts were able to inhibit the growth of *S. aureus* at the highest test concentration of 100 µg/mL. The two ex-

tracts had different antibacterial activity against *M. smegmatis*, with the intracellular extract performed stronger activity than the extracellular extract (25 µg/mL vs. 50 µg/mL). Metabolite profiling had revealed the differences in the dominant compounds between the two extracts. The major compound of the intracellular extract was n-acetyltyramine ($C_{10}H_{13}NO_2$/179.0945) (24.24%), while the major compound from the extracellular extract was palmitic acid ($C_{16}H_{32}O_2$/273.27034) (86.92%). Both two types of Actinobacteria InaCC A759 extracts can be continued for further research in the context of developing antibacterial agents.

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Authors' contributions

MDR, M, TW designed the study. MDR, KR, MMCU, MHA, LC carried out the laboratory work. MDR, M, TW, PL analyzed the data. MDR, KR wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The author declare that they have no competing interest.

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