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Exploring Actinobacteria from Karst Cave on Sumba Island: A New Source of Antimycobacterial Compounds

(Menerokai Aktinobakteria daripada Gua Karst di Pulau Sumba: Sumber Baharu Sebatian Antimikobakteria)

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ABSTRACT

Multidrug-resistant (MDR) mycobacteria are considered a major challenge in tuberculosis treatment, creating an urgent need for novel antimycobacterial drugs. Actinobacteria, known for their ability to produce bioactive compounds, are considered promising sources for new drug discovery. In this study, 87 actinobacteria isolates were successfully obtained from five samples collected in a karst cave on Sumba Island, Indonesia. The isolates were screened for antimycobacterial activity against *Mycobacterium smegmatis* wild-type (WT-M.smeg), rifampicin-resistant (RIF^R-M.smeg), isoniazid-resistant (INH^R-M.smeg), and multidrug-resistant (MDR-M.smeg) strains. Sixteen extracts were found to inhibit WT-M. smeg, with three extracts from isolates KRST 02-20, KRST 03-10, and KRST 05-08 showing potent activity against all resistant strains (≥95% growth inhibition). The extract of isolate KRST 03-10 was observed to exhibit the most significant inhibition, with IC_{50} values of 9.63 µg/mL (WT-*M.smeg*), 29.64 µg/mL (RIF^R-*M.smeg*), 10.89 µg/mL (INH^R-*M.smeg*), and 27.76 µg/mL (MDR-*M.smeg*). Molecular identification showed that this isolate has the highest similarity (98.91%) with *Streptomyces cinereoruber* NBRC 12756. Gas chromatography-mass spectrometry (GC-MS) analysis identified 2,4-di-tert-butylphenol as a compound with potential antituberculosis activity, while liquid chromatography-high-resolution mass spectrometry (LC-HRMS) detected nocardamine, L-α-palmitin, erucamide, and 2-anisic acid, all known for their antimicrobial activity. An unidentified compound, NP-011220 ($C_{11}H_{18}O_2$), was also detected in high relative abundance. Further research is needed to evaluate the activity of the most promising isolate, KRST 03-10, against *Mycobacterium tuberculosis and* to purify its active compounds.

Keywords: Isoniazid-resistant; *Mycobacterium smegmatis*; multidrug-resistant; rifampicin-resistant; tuberculosis

ABSTRAK

Mikobakteria rintang pelbagai ubat (MDR) dianggap sebagai cabaran utama dalam rawatan tuberkulosis, mewujudkan keperluan mendesak untuk ubat antimikobakteria baharu. Aktinobakteria yang dikenali kerana keupayaannya menghasilkan sebatian bioaktif, dianggap sebagai sumber yang berpotensi untuk penemuan ubat baharu. Dalam kajian ini, 87 pencilan aktinobakteria telah berjaya diperoleh daripada lima sampel yang dikumpulkan di gua kars di Pulau Sumba, Indonesia. Pencilan telah disaring untuk aktiviti antimikobakteria terhadap strain *Mycobacterium smegmatis* jenis liar (WT-M. smeg), rintang rifampisin (RIFR-M.smeg), rintang isoniazid (INHR-M.smeg) dan rintang pelbagai ubat (MDR-M.smeg). Enam belas ekstrak didapati menghalang WT-M.smeg dengan tiga ekstrak daripada pencilan KRST 02-20, KRST 03-10 dan KRST 05-08 menunjukkan aktiviti yang kuat terhadap semua strain yang rintang (perencatan pertumbuhan ≥95%). Ekstrak pencilan KRST 03-10 menunjukkan perencatan yang paling ketara dengan nilai IC50 9.63 µg/mL (WT-M.smeg), 29.64 µg/mL (RIFR-M.smeg), 10.89 µg/mL (INHR-M.smeg) dan 27.76 µg/mL (MDR-M.smeg). Pengenalpastian molekul menunjukkan bahawa pencilan ini mempunyai persamaan tertinggi (98.91%) dengan *Streptomyces cinereoruber* NBRC 12756. Analisis kromatografi gas-spektrometri jisim (GC-MS) mengenal pasti 2,4-di-tert-butilfenol sebagai sebatian dengan aktiviti antituberkulosis yang berpotensi, manakala kromatografi cecair-spektrometri jisim resolusi tinggi (LC-HRMS) mengesan nokardina, L-α-palmitin, erukamida dan asid 2-anisik, semuanya dikenali dengan aktiviti antimikrobnya. Sebatian yang tidak dikenali, NP-011220 (C11H18O2) juga dikesan dalam kelimpahan relatif yang tinggi. Kajian lanjut diperlukan untuk menilai aktiviti pencilan yang paling berpotensi, KRST 03-10 terhadap *Mycobacterium tuberculosis* dan untuk menulenkan sebatian aktifnya.

Kata kunci: *Mycobacterium smegmatis*; rintang isoniazid; rintang pelbagai ubat; rintang rifampicin; tuberkulosis

INTRODUCTION

Tuberculosis (TB) is considered a major global health threat, causing an estimated 1.25 million deaths worldwide in 2023 (World Health Organization 2024). Approximately 400,000 people were reported to have developed multidrug-resistant or rifampicin-resistant TB (MDR/RR-TB) (World Health Organization 2023). The increase in MDR/RR-TB cases highlight the critical need for novel antimycobacterial drugs with unique mechanisms of action, particularly to combat resistance to first-line drugs such as isoniazid and rifampicin. The antimycobacterial activity of isoniazid is mediated through its activation by the catalase–peroxidase enzyme encoded by the *katG* gene, which produces a nicotinoyl-NAD complex that inhibits the enoyl–ACP reductase enzyme (*inhA*), thereby disrupting mycolic acid synthesis (Singh et al. 2020). RNA transcription is inhibited by rifampicin through binding to the *β*-subunit of the RNA polymerase enzyme encoded by the *rpoB* gene (Singh et al. 2020). The emergence of *Mycobacterium tuberculosis* strains resistant to first-line therapy is caused by chromosomal gene mutations that alter the genetic structure and reduce drug efficacy (Jagielski et al. 2013; Tseng et al. 2015; Victoria et al. 2021). However, the number of newly approved agents for TB treatment remains low, while resistance to rifampicin, isoniazid, and multidrug-resistant TB (MDR-TB) continues to increase. This situation highlights the urgent need for effective interventions, including the exploration of novel sources for the discovery of potent anti-TB agents. One approach to developing such agents involves the identification of compounds from natural products, particularly secondary metabolites produced by actinobacteria (Selim, Abdelhamid & Mohamed 2021).

Actinobacteria are considered promising candidates for anti-TB drug discovery due to their ability to produce a wide range of bioactive compounds and antibiotics (Selim, Abdelhamid & Mohamed 2021). Members of the genus *Streptomyce*s are known to be producers of bioactive secondary metabolites, including antituberculosis agents such as streptomycin (Sakula 1988), clarithromycin (Zhang et al. 2022), kanamycin (Umezawa et al. 1957), caprazamycin B (Igarashi et al. 2003), and capreomycin (Boeck 1962). To fully exploit the therapeutic potential of actinobacteria, it is important to profile and identify their secondary metabolites accurately. In this study, volatile and non-volatile compounds were identified using gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-high-resolution mass spectrometry (LC-HRMS), respectively, to obtain a comprehensive chemical profile of the isolate.

Actinobacteria, which are responsible for producing over 55% of all known antibiotics, are widely distributed across diverse ecosystems (Ngamcharungchit et al. 2023). However, discovering novel bioactive compounds from soil-derived actinobacteria is challenging due to the high occurrence of similar metabolites. One strategy is to explore unique environments, such as karst caves, which are known to harbor unique microbial communities, including actinobacteria. Karst regions are characterized by unique landscapes and water systems formed through the dissolution of soluble rocks, creating underground water systems and caves. These landscapes are typically composed of limestone, dolomite, gypsum, marble or rock salt (Williams 2008). Caves, in particular, are considered to present extreme environmental conditions, including darkness and limited nutrient availability, making them a suitable habitat for the discovery of novel microbial species (Zhu et al. 2019). Actinobacteria are considered a dominant microbial population in several cave ecosystems, highlighting their potential as a source of novel bioactive compounds (Zhu et al. 2019).

Although karst regions are widespread in Indonesia, research on actinobacterial diversity and its bioactive potential remains limited, particularly in less-explored areas like Sumba Island. Thirty-nine actinobacterial isolates belonging to *Dactylosporangium* and *Micromonospora* were isolated from karst cave soils on Simeulue Island, with several isolates being found to exhibit antibacterial activity (Putri & Sumerta 2020). Additionally, only three actinobacteria isolates were successfully isolated from the plant rhizosphere in the karst ecosystem of Gorontalo (Retnowati et al. 2024), while eight isolates were reported to be collected from the Maros-Pangkep karst area (Rante et al. 2024). However, studies on the diversity of actinobacteria in karst caves on Sumba Island remain scarce. In this study, actinobacteria from karst cave samples on Sumba Island were isolated and cultivated, and their antimycobacterial activity was assessed against rifampicin-resistant, isoniazid-resistant, and multidrug-resistant *M. smegmatis*, and their metabolites were profiled and identified.

MATERIALS AND METHODS

MATERIALS

Samples were collected from Kapungbung Cave, located in Wanggameti Village, Sumba Island (120º16’703” E, 10º03’496” S), at an elevation of 983 m above sea level. Five samples were obtained, consisting of a stalactite fragment (KRST 01), cave soil (KRST 02 and KRST 03), and cave wall materials (KRST 04 and KRST 05). The culture media used for actinobacterial isolation and cultivation included humic-vitamin agar (HVA), Middlebrook 7H9 and 7H10 media (M7H9 and M7H10), Mueller-Hinton broth (MHB), yeast-starch agar (YSA), and yeast-starch broth (YSB).

PREPARATION AND ISOLATION OF ACTINOBACTERIA

The samples collected from the karst cave were placed in sterile plastic bags, transported to the laboratory, air-dried at room temperature for 5-7 days, and then incubated at 60 °C for 20 min. The dried samples were ground using a mortar

and pestle, then sieved. Actinobacteria were isolated using the sodium dodecyl sulfate-yeast extract (SDS-YE) method (Hayakawa & Nonomura 1989). A volume of 100 µL from each dilution was spread onto HVA medium supplemented with 50 µg/mL cycloheximide and 20 µg/mL nalidixic acid (Hayakawa & Nonomura 1987). The grown colonies were picked and further purified on YSA medium. The pure isolates were then preserved in 20% (v/v) glycerol and stored at -80 °C (Putri & Sumerta 2020).

CULTIVATION OF DRUG-SENSITIVE AND MULTIDRUG-RESISTANT *Mycobacteria*

M. smegmatis wild-type (WT-M.smeg), rifampicin-resistant (RIF^R-M.smeg), isoniazid-resistant (INH^R-M.smeg), and multidrug-resistant (MDR-M.smeg) strains were cultivated in M7H9 broth (Sigma-Aldrich, USA) supplemented with 0.5% glycerol and 0.05% Tween 80 (Arthur et al. 2019). Cultures were incubated at 37 °C with shaking at 180 rpm for 24 h.

METABOLITE PRODUCTION AND EXTRACTION

All actinobacterial isolates were cultivated in YSB medium and incubated for 14 days at 30 ºC with shaking at 180 rpm. Extraction was performed by adding ethyl acetate at a 1:1 (v/v) ratio. The organic phase was collected, and the crude extracts were concentrated using a rotary evaporator and stored at -20 ºC for further analysis (Praptiwi et al. 2023).

SCREENING OF ANTIMYCOBACTERIAL ACTIVITY

Antimycobacterial screening was performed using the resazurin reduction microplate assay (REMA). WT-M. smeg was cultured in M7H9 broth at 37 °C with shaking at 180 rpm for 24 h. The cell suspension was diluted 1:1000 to obtain a concentration equivalent to 0.5 McFarland standard. Subsequently, 2 µL of each extract was added to 48 µL of the cell suspension and incubated for 24 h at 37 °C. The positive control contained 2 µL of DMSO and 48 µL of cell suspension, while the negative control consisted of 2 µL of DMSO and 48 µL of sterile M7H9 broth. Resazurin solution was added as a growth indicator (1:1, v/v) to each well. Fluorescence was measured at an excitation of 530 nm and an emission of 590 nm using a Varioskan™ Lux Multimode Microplate Reader (Thermo Fisher Scientific, USA) (Nurkanto et al. 2024). The screening assays were performed in triplicate, and data were analyzed using Microsoft Excel. Extracts showing ≥85% inhibition against WT-M.smeg were selected for further evaluation against RIF^R-M.smeg, INH^R-M.smeg, and MDR-M.smeg strains using the same REMA protocol. The quality of the screening system was assessed by calculating the Z′-factor and signal-to-background ratio (S/B) according to established procedures following previously described method by Zhang, Chung and Oldernburg (1999).

DETERMINATION OF IC_{50}

Crude extracts that inhibited ≥95% of RIF^R-M.smeg, INH^R-M.smeg, and MDR-M.smeg were selected for IC_{50} determination. *M. smegmatis* strains were cultured in M7H9 broth at 37 °C with shaking at 180 rpm for 24 h. The extracts were prepared at an initial concentration of 320 µg/mL and serially diluted twofold until reaching a final concentration of 0.3 µg/mL. Then, 2 µL of each extract was added to 48 µL of the cell suspension and incubated for 24 h at 37 °C. Each assay was performed in triplicate. Cell viability was assessed using resazurin staining, and fluorescence was measured at 530 nm (excitation) and 590 nm (emission) using a Varioskan™ Lux multimode microplate reader (Thermo Fisher Scientific, USA). IC_{50} values were determined using nonlinear regression analysis in GraphPad Prism version 10 (GraphPad Software, USA) (Nurkanto et al. 2024).

MOLECULAR IDENTIFICATION AND PHYLOGENETIC CONSTRUCTION OF POTENTIAL ISOLATES

The actinobacterial isolates showing the highest antimycobacterial activity were identified using a molecular approach by amplifying the 16S rRNA gene with the universal primer pairs 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). Genomic DNA was extracted using a lysis solution following the method of Putri and Sumerta (2020). PCR amplification was carried out using a Thermo Scientific™ Arktik Thermal Cycler (Thermo Fisher Scientific, USA). The 16S rRNA gene fragment was sequenced via Sanger sequencing at First BASE Laboratories, Malaysia. The resulting sequences were assembled and analyzed using ChromasPro software and compared with type strains available in the EzTaxon database (http://eztaxon-e.ezbiocloud.net) (Yoon et al. 2017). DNA sequences of closely related species were aligned, and a phylogenetic tree was constructed using the neighbor-joining method with 1000 bootstrap replicates in MEGA 11 software (Tamura, Stecher & Kumar 2021).

PROFILING OF VOLATILE COMPOUNDS IN ACTINOBACTERIAL EXTRACTS USING GC-MS

Volatile compounds present in the ethyl acetate extract were analyzed using a GC-MS system (QP2010 Ultra, Shimadzu, Japan), equipped with an Rtx-5MS column. The column oven was initially set at 60 °C and held for 5 min, then increased to 300 °C at a rate of 15 °C per min, followed by a final hold of 10 min. The injection temperature was set at 250 °C in splitless mode (1.0 min sampling). The GC was operated under linear velocity control (30.0 kPa, total flow 10.5 mL/min, column flow 0.68 mL/min, linear velocity 30.2 cm/s). The purge flow was 3.0 mL/min, and the split ratio was 10:1, with both high-pressure injection and carrier gas saver functions disabled. The system was

equilibrated for 3.0 min before analysis (Masrukhin et al. 2021). Compound identification was performed by comparing the obtained mass spectra with those in the National Institute of Standards and Technology (NIST, version 2.9) mass spectral library database.

PROFILING OF NON-VOLATILE COMPOUNDS IN ACTINOBACTERIAL EXTRACTS USING LC-HRMS

Non-volatile compound analysis was performed using an untargeted metabolomic approach with a liquid chromatography system (Thermo Scientific, Rockford, IL, USA) (Hermawan et al. 2025). Approximately 5 mg of extract was dissolved in 1 mL of LC–MS grade methanol. The metabolites were separated on a phenyl-hexyl Accucore column (100 mm × 2.1 mm, 2.6 µm particle size) using a gradient elution of mobile phase A (water) and mobile phase B (acetonitrile), each containing 0.1% formic acid. A 5 µL aliquot of each sample was injected at a flow rate of 0.3 mL/min, and the column temperature was maintained at 40 °C. The mass spectrometer was operated with heated electrospray ionization (HESI) at a voltage of 3500 V. Total ion chromatograms (TICs) were exported using Xcalibur software (Thermo Scientific, Rockford, IL, USA) and processed with Compound Discoverer software (Thermo Scientific, Rockford, IL, USA) for metabolite identification. Detected compounds were matched against the MzCloud and ChemSpider databases (Hermawan et al. 2025).

RESULTS AND DISCUSSION

DISTRIBUTION OF ACTINOBACTERIAL ISOLATES FROM KARST CAVE SAMPLES ON SUMBA ISLAND

A total of 87 actinobacterial isolates were obtained from five karst cave samples collected on Sumba Island. The isolates were distributed among different substrates, with 9 isolates being recovered from KRST 01, 26 from KRST 02, 27 from KRST 03, 13 from KRST 04, and 12 from KRST 05. Among these, 63 isolates produced aerial mycelia (Figure 1(A) & 1(B)), whereas 24 isolates produced soluble pigments on YSA medium (Figure 1(B)). The highest number of isolates was recovered from cave soil samples (KRST 02 and KRST 03), while the lowest number was obtained from a stalactite fragment (KRST 01). The predominance of actinobacteria in cave soil suggests that this substrate provides a more favorable environment for their growth.

The type of substrate plays an essential role in determining microbial diversity and composition in cave environments (Zhu et al. 2019). Karst cave soils are typically characterized by a thin soil layer, limited nutrients, minimal water, contains acid-insoluble rock materials, and undergoes a slow soil formation process (Liu et al. 2020). In general, cave soils contain higher levels of nutrients and organic compounds than stalactites and cave walls, which may contribute to a higher abundance of actinobacteria. Actinobacteria represent one of the most abundant microbial groups inhabiting karst cave sediments and rocks (Zhu et al. 2019), with *Streptomyces* being the dominant genus, followed by *Micromonospora* and *Nocardiopsis* (Farda et al. 2022).

The actinobacterial isolates from the karst cave exhibited diverse morphological characteristics, particularly in the production of aerial mycelia and soluble pigments. The presence of aerial mycelia indicates the occurrence of sporulation, a common adaptive strategy that enables actinobacteria to survive in nutrient-limited environments (Beskrovnaya et al. 2021). Meanwhile, the production of soluble pigments suggests the ability to synthesize secondary metabolites, which are often associated with antimicrobial or other bioactive properties (Sarmiento-Tovar et al. 2024).

FIGURE 1. Colony morphology of KRST 05-08 and KRST 02-09 cultured on YSA medium

INHIBITORY POTENTIAL OF ACTINOBACTERIAL EXTRACTS AGAINST DRUG-SENSITIVE AND DRUG-RESISTANT *M. smegmatis*

The resazurin assay of the actinobacterial extracts showed that, out of 87 ethyl acetate extracts, 16 extracts (18.39%) exhibited >40% inhibitory activity against WT-M.smeg. *M. smegmatis* is commonly used as a surrogate organism in tuberculosis research due to its genetic and physiological similarities to *M. tuberculosis*, as well as its non-pathogenic nature and rapid growth rate (Lelovic et al. 2020). Among these, six extracts (KRST 01-03, KRST 02-10, KRST 02-20, KRST 03-10, KRST 04-05, and KRST 05-08) showed strong inhibitory activity (>85%) against WT-M.smeg (Table 1). Notably, isolates KRST 02-20, KRST 03-10, and KRST 05-8 maintained high inhibitory activity (>95%) against drug-resistant strains, including INH^R-M.smeg, RIF^R-M.smeg, and MDR-M.smeg (Table 1).

The distinct environmental conditions of karst cave soils are believed to enhance the production of bioactive compounds, particularly antimycobacterial metabolites from actinobacteria. Previous studies have reported that diverse bioactive metabolites are produced by actinobacteria isolated from karst caves (Rangseekaew & Pathom-Aree 2019). Several of these compounds have been purified, structurally characterized, and shown to exhibit antibacterial and anticancer activities, with *Streptomyces* recognized as the most prolific producer (Rangseekaew & Pathom-Aree 2019). Notable bioactive metabolites derived from cave-associated actinobacteria include xiakemycin A, produced by *Streptomyces* sp. CC8-201 isolated from karst soil in China, which has been shown to inhibit *Staphylococcus aureus* (Jiang et al. 2015). Hypogeamicins A, B, C, and D, produced by *Nonomuraea specus* isolated from the Hardin Cave System in Tennessee, USA, have been reported to exhibit potential anticancer properties (Derewacz et al. 2014). Additionally, huanglongmycin A-C was isolated from *Streptomyces* sp. CB09001 obtained from karst cave soil in Xiangxi, China, has been reported to possess anticancer activity (Jiang et al. 2018).

IC_{50} DETERMINATION OF SELECTED ACTINOBACTERIAL EXTRACTS

The antimycobacterial activities of the three selected isolates (KRST 02-20, KRST 03-10, and KRST 05-8) were evaluated based on their IC_{50} values against WT-M.smeg. The results indicated that only the extracts from isolates KRST 05-8 and KRST 03-10 exhibited IC_{50} values below 20 µg/mL, at 15.77 µg/mL and 9.63 µg/mL, respectively. In contrast, isolate KRST 02-20 showed a higher IC_{50} value of 52 µg/mL, indicating weaker activity compared to KRST 05-8 and KRST 03-10. An IC_{50} value below 20 µg/mL is generally considered promising in preliminary screening for anti-*Mycobacterium* compounds (Dos Santos et al. 2018). Based on these findings, only isolates KRST 03-10 and KRST 05-8 were selected for further evaluation to determine their IC_{50} values against drug-resistant strains, including RIF^R-M.smeg, INH^R-M.smeg, and MDR-M.smeg strains.

Rifampicin and isoniazid were used as standard antibiotics for comparison. Rifampicin showed an IC_{50} value of 0.70 µg/mL against WT-M.smeg, with markedly reduced activity against RIF^R-M.smeg (359.9 µg/mL) and MDR-M.smeg (302 µg/mL). Meanwhile, isoniazid exhibited an IC_{50} value of 14.98 µg/mL against WT-M.smeg, but its activity decreased substantially against INH^R-M.smeg (661.3 µg/mL) and MDR-M.smeg (769.4 µg/mL). Evaluation of the two selected extracts showed that the extract of isolate KRST 03-10 possessed a stronger antimycobacterial potential than that of KRST 05-8. The extract of isolate KRST 03-10 exhibited consistent inhibitory activity against all three resistant strains, with IC_{50} values of 29.64 µg/mL (RIFR-M.smeg), 10.89 µg/mL (INHR-M.smeg), and 27.76 µg/mL (MDR-M.smeg) (Figure 2). In contrast, KRST 05-8 showed a marked reduction in activity against the resistant strains, with IC_{50} values of 150.8 µg/mL (RIF^R-M.smeg), 71.96 µg/mL (INH^R-M.smeg), and 155.5 µg/mL (MDR-M.smeg). These results indicate that although KRST 05-8 exhibited potent activity against the drug-sensitive strain, its activity was

TABLE 1. Inhibitory activity of selected actinobacterial isolates (>85% inhibition against WT-M.smeg)

Isolate code	WT-M.smeg	INH^R-M.smeg	IF^R-M.smeg	MDR-M.smeg
KRST 01-3	100	0	0	0
KRST 02-10	87.48	0	0	0
KRST 02-20	98.81	100	99.62	99.44
KRST 03-10	100	99.26	97.47	98.09
KRST 04-5	89.54	8.82	0	21.21
KRST 05-8	100	99.59	98.97	98.29

considerably reduced against the resistant *M. smegmatis*. In contrast, the extract of KRST 03-10 demonstrated stronger and more consistent antimycobacterial activity against both the wild-type and drug-resistant strains, highlighting its potential as a promising source of bioactive compounds effective against resistant *Mycobacterium*.

IDENTIFICATION OF ISOLATES KRST 03-10 AND KRST 05-08 BASED ON 16S RRNA GENE SEQUENCES

The 16S rRNA gene sequence analysis showed that the most potent isolates (KRST 03-10 and KRST 05-08) were identified as members of the genus *Streptomyces*, which is widely recognized as a producer of antibiotics. Isolate KRST 03-10 shared 98.91% sequence similarity with *Streptomyces cinereoruber* NBRC 12756, whereas KRST 05-08 showed 99.93% similarity with *Streptomyces pratensis* ch24 (Figure 3). Previous studies have reported that *S. cinereoruber* exhibits antibacterial activity (Nayaka et al. 2020; Upadhyay et al. 2020). Specifically, *S. cinereoruber* sp. MM1AG isolated from Chilika Lake produced a partially purified compound with an HPLC peak similar to that of streptomycin (Upadhyay et al. 2020). Similarly, *S. pratensis* has been reported to exhibit antibacterial activity against *Streptococcus epidermidis, Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa* (Barghouthi et al. 2017). Moreover, genome analysis of *S. pratensis* has identified *bldB* gene homologs, which are essential for spore formation and antibiotic production (Doroghazi & Buckley 2014).

VOLATILE COMPOUND PROFILING OF KRST 03-10 EXTRACT USING GC-MS

Both volatile and non-volatile compounds from isolate KRST 03-10 were identified using GC-MS and LC-HRMS. Volatile compounds were detected using GC-MS, whereas non-volatile compounds were characterized by LC-HRMS. The GC-MS analysis showed 19 volatile compounds in the ethyl acetate extract of isolate KRST

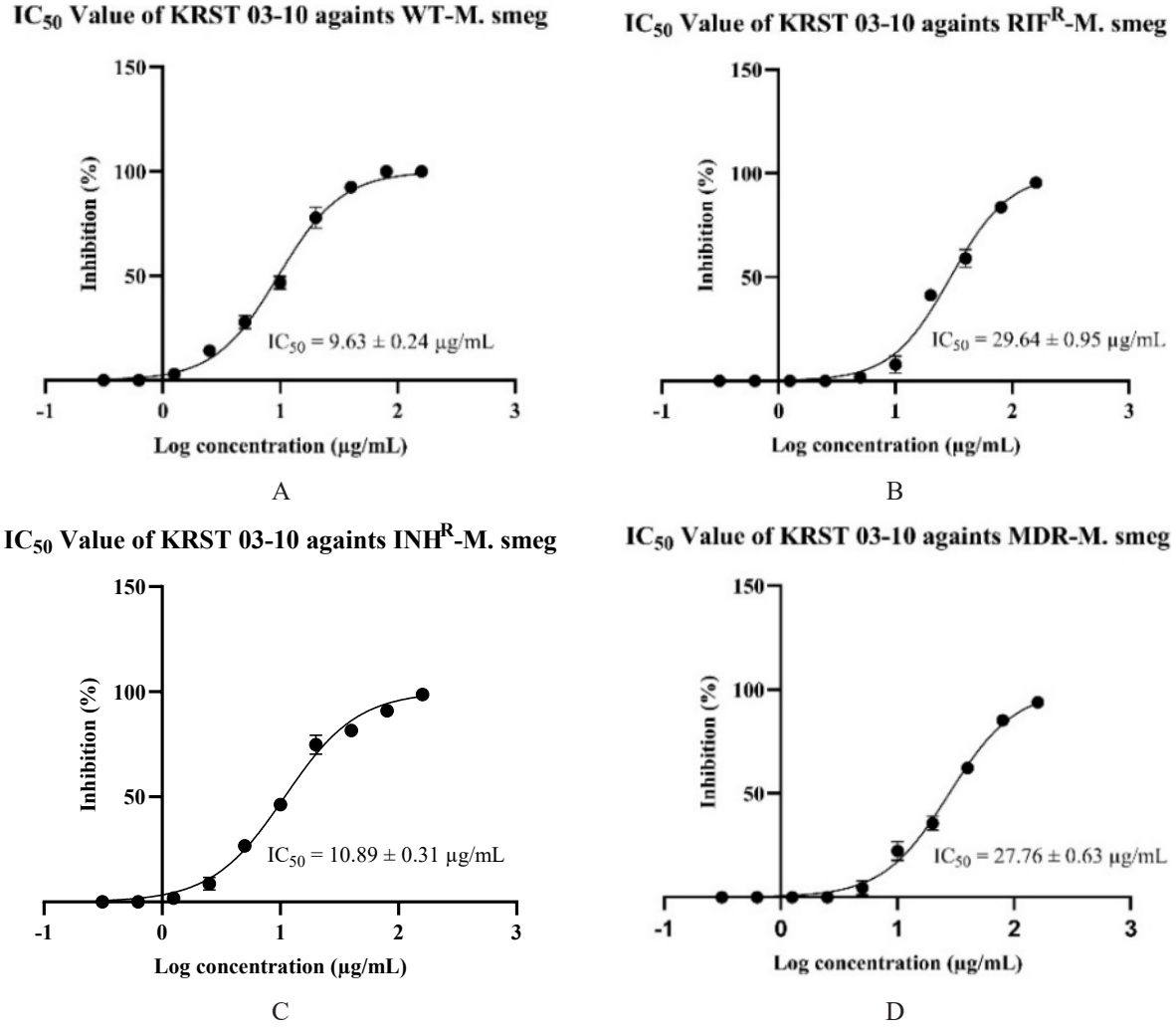


FIGURE 2. Determination of IC_{50} values of the ethyl acetate extract of isolate KRST 03-10 against *M. smegmatis* strains. (A) WT-M.smeg, (B) RIF^R-M.smeg, (C) INH^R-M.smeg and (D) MDR-M.smeg

03-10, with similarity indices ranging from 75% to 98% (Table 2). These compounds were classified into several chemical groups, including esters (hexadecanoic acid methyl ester and 11-octadecenoic acid, methyl ester), fatty acids (n-hexadecanoic acid), hydrocarbons (heneicosane, 1-octadecene, 1-heptadecene, 1-pentadecene, pentacosane, and 1-tricosene), and alcohols (octacosanol). The most abundant compounds were [1,4,7] trioxonane (28.38% area), carbamic acid, N, N-dimethyl-, 6-chlorohexyl ester (21.90% area), and ethanediamide (10.21% area). Notably, heneicosane was detected at multiple retention times (21.26-23.11 minutes) (Table 2).

Several volatile compounds identified in the extract of KRST 03-10 have been previously reported to possess antimicrobial or related bioactivities. Notably, 2,4-di-tert-butylphenol has demonstrated antituberculosis activity (Kaari et al. 2023). Other compounds, such as n-hexadecanoic acid (Purushothaman, Vishnuram & Ramanathan 2014), methyl hexadecanoate (methyl palmitate) (Patil & Jadhav 2021; Shaaban, Ghaly & Fahmi 2021), 1-pentadecene (Kumari, Menghani & Mithal 2019), heneicosane (Vanitha et al. 2020), 1-tricosene (Mohamed Gameil et al. 2019), butylated hydroxytoluene (Dai et al. 2023), and octacosanol (Kumari, Menghani & Mithal 2019; Wang et al. 2012) are also known for their antimicrobial, antioxidant, or anticancer properties. The diversity of volatile metabolites detected suggests that both major and minor components may contribute synergistically to the overall antimicrobial potential of the KRST 03-10 extract.

NON-VOLATILE COMPOUND PROFILING OF KRST 03-10 EXTRACT USING LC-HRMS

LC-HRMS analysis of the ethyl acetate extract of isolate KRST 03-10 detected of 350 compounds (Figure 4). Among these, 25 compounds were assigned known molecular formulas and structures, although their specific names remain unidentified. The major compound, NP-011220 ($C_{11}H_{18}N_2O_2$), exhibited the highest peak area (6.74%). Other significant metabolites detected in the ethyl acetate extract of isolate KRST 03-10 included NP-020439, NP-013736, L-α-palmitin, nocardamine, erucamide, 2-anisic acid, 1-stearoylglycerol, and cyclo(phenylalanyl-prolyl) (Table 3). The structures of the major non-volatile compounds identified through LC-HRMS analysis are presented in Figure 5.

Several compounds were detected in the ethyl acetate extract of strain KRST 03-10, which have been previously reported to possess antimicrobial and antioxidant properties. One of these, L-α-palmitin, was detected as a dominant compound in the LC-HRMS profile. This compound has been reported from the fungus *Diaporthe* sp. SmAk1, where it exhibited antibacterial and antioxidant activities (Efendi et al. 2025). Similarly, palmitic acid, which also possesses antioxidant potential, was reported to be produced by *Syzygium litorella* (Hidajati et al. 2018). Another metabolite, nocardamine, was detected in the KRST 03-10 extract and has been previously reported to exhibit weak antimicrobial activity against *Enterococcus faecium* and *Bacillus subtilis* (Kalinovskaya et al. 2011).

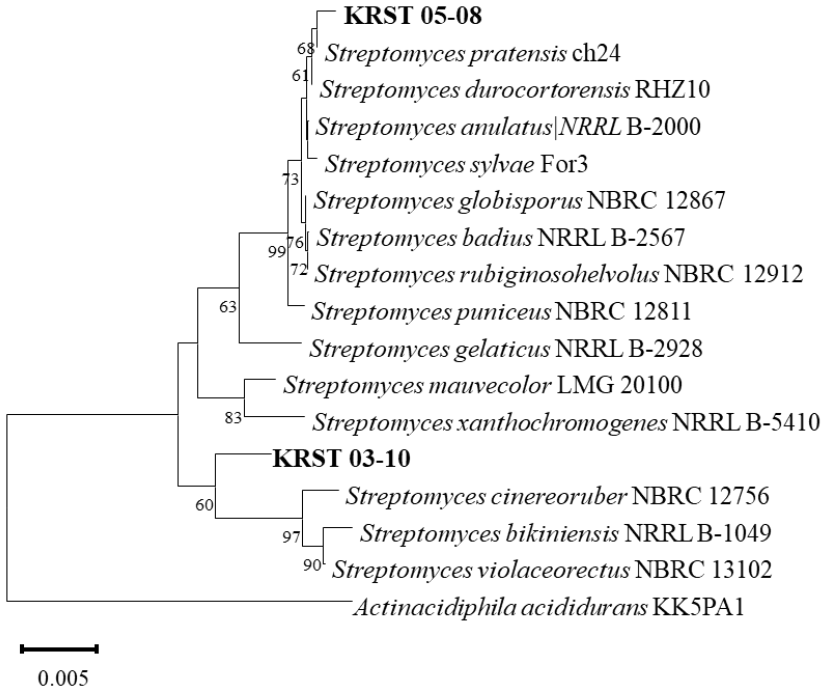


FIGURE 3. Phylogenetic tree based on 16S rRNA gene sequences of KRST 03-10 and KRST 05-08, constructed using the Neighbour-Joining method. Bootstrap values were calculated from 1,000 replicates. *Actinocidiphila acidurans* was used as an outgroup

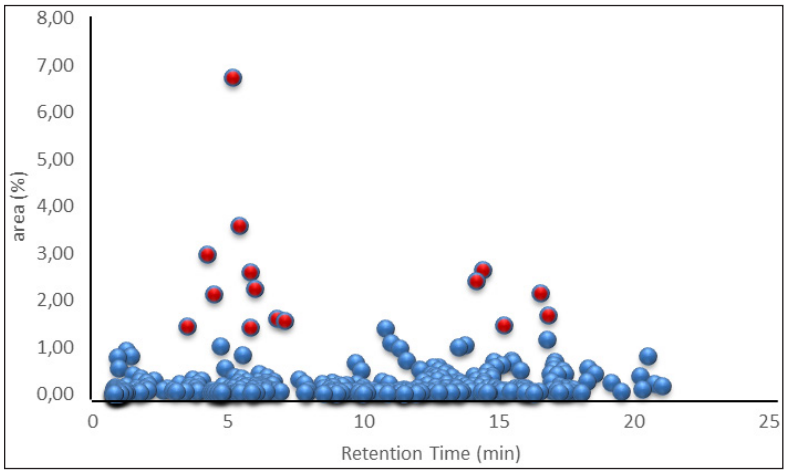
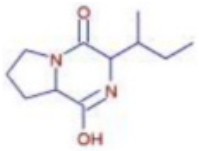
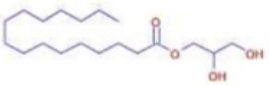


FIGURE 4. Visualization of compound in KRST 03-10 extract based on LC-HRMS data, with the 15 dominant compounds (≥1.42% of the total peak area) highlighted in red

TABLE 2. Profile of major volatile compounds in KRST 03-10 extracts analysed by GC-MS

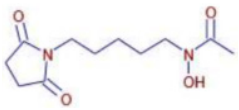
No	Compound name	Formula	RT (min)	Area (%)	Similarity (%)	Biological function
1	[1,4,7] Trioxonane	$C_6H_{12}O_3$	2,25	28,38	82	-
2	Carbamic acid, N, N-dimethyl-, 6-chlorohexyl ester	$C_9H_{18}ClNO_2$	2,32	21,9	75	Insecticidal (Melnikov 1971)
3	Ethanediamide	$C_2H_6N_2O$	3,20	10,21	77	Cholinesterase inhibition (Koca et al. 2015)
4	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	14,34	5,11	95	Antituberculosis and antifungal (Kaari et al. 2023)
5	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	17,71	3,38	90	Antibacterial and antioxidant (Purushothaman, Vishnuram & Ramanathan 2014
6	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	17,36	3,03	97	Antifungal (Patil & Jadhav 2021; Shaaban, Ghaly & Fahmi 2021)
7	11-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	18,51	2,91	95	-
8	1-Octadecene	$C_{18}H_{38}$	17,80	2,81	96	Biocontrol (Kumari, Menghani & Mithal 2019; Mangalgikar et al. 2023)
9	Heneicosane	$C_{21}H_{44}$	22,43	2,47	97	Antimicrobial (Vanitha et al. 2020)
10	Heneicosane	$C_{21}H_{44}$	21,82	2,28	97	Antimicrobial (Vanitha et al. 2020)
11	Heneicosane	$C_{21}H_{44}$	23,11	2,18	96	Antimicrobial (Vanitha et al. 2020)
12	Heneicosane	$C_{21}H_{44}$	21,27	1,94	97	Antimicrobial (Vanitha et al. 2020)
13	1-Heptadecene	$C_{17}H_{34}$	16,44	1,76	97	-
14	1-Pentadecene	$C_{15}H_{30}$	14,96	1,68	98	Antimicrobial, antioxidant (Kumari, Menghani & Mithal 2019)
15	Pentacosane	$C_{25}H_{52}$	23,90	1,53	96	-
16	1-Tricosene	$C_{23}H_{46}$	19,04	1,32	97	Anticancer (Mohamed Gameil et al. 2019)
17	Butylated Hydroxytoluene	$C_{15}H_{24}O$	14,30	1,11	96	Antioxidant (Dai et al. 2023)
18	Octacosanol	$C_{24}H_{48}$	20,19	1,09	97	Antioxidant, anti-parkinsonian (Kumari, Menghani & Mithal 2019; Wang et al. 2012)
19	Heneicosane	$C_{21}H_{44}$	20,75	0,84	97	Antimicrobial (Vanitha et al. 2020)

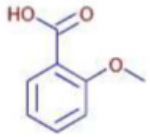

NP-011220


L-α-palmitin

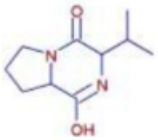

Nocardamin


Erucamide


NP-020439


2-Anisic acid


Stearoylglycerol


NP-013736

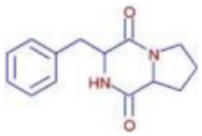

Cyclo(phenylalanyl-prolyl)

FIGURE 5. Structure of major non-volatile compounds in KRST 03-10 extracts analyzed by LC-HRMS

TABLE 3. Profile of major volatile compounds in KRST 03-10 extracts analyzed by LC-HRMS

No	Compound name	Formula	RT (min)	Area (%)	Similarity (%)	Biological function
1	NP-011220	$C_{11}H_{18}N_2O_2$	5,18	6,74	98,5	-
2	NP-011220	$C_{11}H_{18}N_2O_2$	5,42	3,59	96,3	-
3	NP-020439	$C_{11}H_{18}N_2O_4$	4,25	2,97	96,6	-
4	L-α-palmitin	$C_{19}H_{38}O_4$	14,45	2,65	-	Antibacterial and antioxidant (Efendi et al. 2025)
5	Nocardamin	$C_{27}H_{48}N_6O_9$	5,85	2,60	97	Antimicrobial (Kalinovskaya et al. 2011; Park et al. 2017)
6	L-α-palmitin	$C_{19}H_{38}O_4$	14,19	2,40	-	Antibacterial and antioxidant (Efendi et al. 2025)
7	Nocardamin	$C_{27}H_{48}N_6O_9$	6,02	2,23	96,7	Antimicrobial (Kalinovskaya et al. 2011; Park et al. 2017)
8	Erucamide	$C_{22}H_{43}NO$	16,58	2,14	70,5	Antidepressant and anxiolytic (Li et al. 2017)
9	NP-020439	$C_{11}H_{18}N_2O_4$	4,50	2,11	94,9	-
10	Erucamide	$C_{22}H_{43}NO$	16,86	1,67	81	Antidepressant and anxiolytic (Li et al. 2017)
11	2-Anisic acid	$C_8H_8O_3$	6,81	1,60	98,7	Antimicrobial and antioxidant (Wang et al. 2018)
12	2-Anisic acid	$C_8H_8O_3$	7,10	1,55	98,8	Antimicrobial and antioxidant (Wang et al. 2018)
13	1-Stearoylglycerol	$C_{21}H_{42}O_4$	15,22	1,47	97	-
14	NP-013736	$C_{10}H_{16}N_2O_2$	3,53	1,44	98,6	-
15	Cyclo(phenylalanyl-prolyl)	$C_{14}H_{16}N_2O_2$	5,86	1,42	97,5	Antifungal, antitumor (Hamza, Clark & Murphy 2018; Jha et al. 2024; Kim et al. 2022)

It has also been described as a siderophore produced by *Streptomyces albus* J1074, playing an essential role in iron acquisition (Park et al. 2017). This compound is considered crucial for bacterial survival under iron-limited conditions, enhancing competitiveness against other microorganisms.

In addition, 2-anisic acid was identified in the extract and has been reported to possess both antimicrobial and antioxidant activities. Two derivatives of this compound, rhizopycnis acid A and B, were previously isolated from the endophytic fungus *Rhizopycnis vagum*, exhibiting antibacterial activity against six bacterial species (*B. subtilis*, *Staphylococcus hemolyticus*, *Agrobacterium tumefaciens*, *Pseudomonas lachrymans*, *Ralstonia solanacearum*, and *Xanthomonas vesicatoria*) with IC_{50} values ranging from 16.1 to 81.3 µg/mL (Wang et al. 2018). Another compound, cyclo(L-phenylalanyl-L-prolyl), was also detected and has been reported from various microorganisms. It has been described as a quorum-sensing inhibitor and exhibits antifungal and antitumor properties (Hamza, Clark & Murphy 2018; Jha et al. 2024; Kim et al. 2022).

The identification of bioactive metabolites such as L-α-palmitin, nocardamine, 2-anisic acid, and cyclo(phenylalanyl-prolyl) indicates that isolate KRST 03-10 represents a promising source of anti-Mycobacterium agents. The metabolite profiling of isolate KRST 03-10 using GC–MS and LC–HRMS thus confirms that this isolate possesses strong potential as a source of antimicrobial compounds. The discovery of an anti-*Mycobacterium*-producing strain from a karst cave environment supports the notion that unique habitats can drive microbial evolution and promote the biosynthesis of unique bioactive metabolites (Tiwari & Gupta 2013).

CONCLUSIONS

Karst caves on Sumba Island represent an unexplored habitat that harbors diverse actinobacteria with promising antimycobacterial potential. Among the isolates tested, *Streptomyces cinereoruber* KRST 03-10 exhibited the strongest inhibitory activity against both drug-sensitive and drug-resistant *M. smegmatis*, with IC_{50} values confirming its high potency, particularly against resistant strains. The GC–MS profiling of the KRST 03-10 extract showed 19 volatile compounds, dominated by [1,4,7] trioxonane, carbamic acid N, N-dimethyl-,6-chlorohexyl ester, and ethanediamide. Several of these, such as 2,4-di-tert-butylphenol, n-hexadecanoic acid, methyl hexadecanoate, and heneicosane, have been previously reported to possess antimicrobial or antioxidant activities, suggesting their possible contribution to the observed antimycobacterial effect. Furthermore, LC–HRMS analysis identified 350 non-volatile compounds in the ethyl acetate extract of KRST 03-10, including L-α-palmitin, nocardamine, 2-anisic acid, and cyclo(phenylalanyl-prolyl), which are known bioactive metabolites. The detection of both volatile and non-volatile bioactive compounds suggests that the antimycobacterial effect of the KRST 03-10 extract may result from the combined activity of several metabolites. These results highlight the potential of actinobacteria from karst cave environments as promising sources of novel bioactive metabolites with antimycobacterial activity. Further purification, structural characterization, and comprehensive biological evaluation are required to verify their specific functions and therapeutic potential.

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