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Antimycobacterial activity of the secondary metabolite fraction derived from endophytic bacterium *Bacillus velezensis* strain DJ4 isolated from *Archidendron pauciflorum*

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ABSTRACT: The discovery and development of new antituberculosis medications effective against multidrug-resistant *Mycobacterium* strains are necessary to reduce the mortality in tuberculosis (TB) cases. To accelerate the discovery of anti-TB drugs, *Mycobacterium smegmatis* was commonly used as a surrogate bacterium in the preliminary screening of antituberculosis compounds. This study aimed to fractionate the secondary metabolites of the endophytic bacterium *Bacillus velezensis* strain DJ4 isolated from *Archidendron pauciflorum*, to evaluate its antimycobacterial activity against *M. smegmatis*, and to identify the secondary metabolite profile of its active fraction. Fifty-six fractions were successfully obtained through thin-layer chromatography (TLC) and column chromatography techniques. Among all fractions, ten fractions showed remarkable antimycobacterial activity against *M. smegmatis* with minimum inhibitory concentration (MIC) values ranging from 85–1,325 µg/ml. Fraction F53 exhibited the strongest antimycobacterial activity with the lowest MIC of 85 µg/ml. This fraction also inhibited the biofilm formation of *M. smegmatis* at 1× MIC and ½× MIC by 62,3%, and 52,1%, respectively. Observation using a scanning electron microscope (SEM) showed that the biofilm matrix of *M. smegmatis* treated with fraction F53 was thinner, less compact, and separated into small pieces compared to the negative control. Furthermore, liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis revealed that this corresponding fraction contained five proposed compounds, including 2-(p-anisyl)-5-methyl-1-hexene, nigakilactone H, tetratriacontanamine, and two other unidentified compounds.

KEYWORDS: Antimycobacterial; *Archidendron pauciflorum*; fractionation; MIC; *Mycobacterium smegmatis*.

1. INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* that causes high mortality worldwide. WHO reported in 2022 that there were 7.5 million new cases of TB and 1.3 million deaths caused by the infection [1]. TB is generally treated using first-line antibiotics, such as isoniazid, rifampicin, pyrazinamide, and ethambutol. However, the presence of *Mycobacterium* strains that are resistant to these antibiotics hinders common TB treatment [2, 3]. Research and development of novel anti-TB are also hampered by the slow growth of *M. tuberculosis* in laboratory culture conditions and must be handled under a high biosafety level and strict containment laboratory [4]. Therefore, the use of a surrogate microorganism, namely *Mycobacterium smegmatis*, can be used in the preliminary study for discovery of anti-TB compounds. This bacterium is commonly used in anti-TB compound research because it is nonpathogenic, fast-growth, and has the similar sensitivity to anti-TB drugs as *M. tuberculosis* [5].

The emergence of bacterial resistance to antibiotics can also be supported by the formation of a biofilm structure. This structure is a complex matrix consisting of extracellular polysaccharides, proteins, lipids, nucleic acids (DNA/RNA), and other biomolecules that cause bacterial cells to form strong bonds on biotic or abiotic surfaces [6]. The biofilm structure acts as a physical and physiological barrier that can inhibit the

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penetration of antibiotics into bacterial cells. Around 99% of microorganisms are able to form biofilm, including *M. tuberculosis* and *M. smegmatis* [7]. The ability of biofilm to support bacterial resistance to antibiotics is influenced by one of the cellular communication mechanisms called quorum sensing. This mechanism regulates the synthesis of exopolysaccharide (EPS) polymers in obtaining nutrients in hostile environmental conditions, as well as the enzymatic system and gene expression that are related to virulence factors [8, 9]. Therefore, the search for antibacterial compounds that are effective in inhibiting the growth and biofilm formation of target bacteria is crucial to increase the effectiveness of treating infectious diseases.

Endophytic microorganisms are potential sources of various bioactive compounds, especially antibacterial substances. These microorganisms colonise the internal tissues of host plants without causing disease [10]. Approximately 46% of endophytic microorganisms isolated from Asian medicinal plants were able to produce antimicrobial compounds [11]. *Bacillus velezensis* strain DJ4 is an endophytic bacterium that has been isolated from dogfruit (*Archidendron pauciflorum*) leaves and has been known to have antibacterial activity. Previous studies have reported that the crude extract of secondary metabolites produced by this bacterium possessed strong antibacterial activity against four strains of multidrug-resistant pathogenic bacteria, including *Escherichia coli* strain M4, *Bacillus subtilis* strain M18, *Pseudomonas aeruginosa* strain M19, and *Klebsiella pneumoniae* strain M19 [12], and *M. smegmatis* [13]. However, further purification and identification of antibacterial compounds, especially against *M. smegmatis*, have not been investigated. This study aimed to fractionate the secondary metabolites of the endophytic bacterium *Bacillus velezensis* strain DJ4 isolated from *A. pauciflorum*, to evaluate its antimycobacterial activity against *M. smegmatis*, and to identify the secondary metabolite profile of its active fraction.

2. RESULTS

2.1. Secondary metabolite fractions of *Bacillus velezensis* strain DJ4

Fifty-six fractions were successfully obtained from the fractionation of crude extract of *B. velezensis* strain DJ4 isolate using column chromatography. The TLC profiles showed that most of the secondary metabolite fractions generally had small spots with a blackish gray color in UV light λ 254 nm (Figure 1). On the other hand, most of the fractions exhibited red and light blue spots when observed under UV light λ 365 nm (Figure 2).

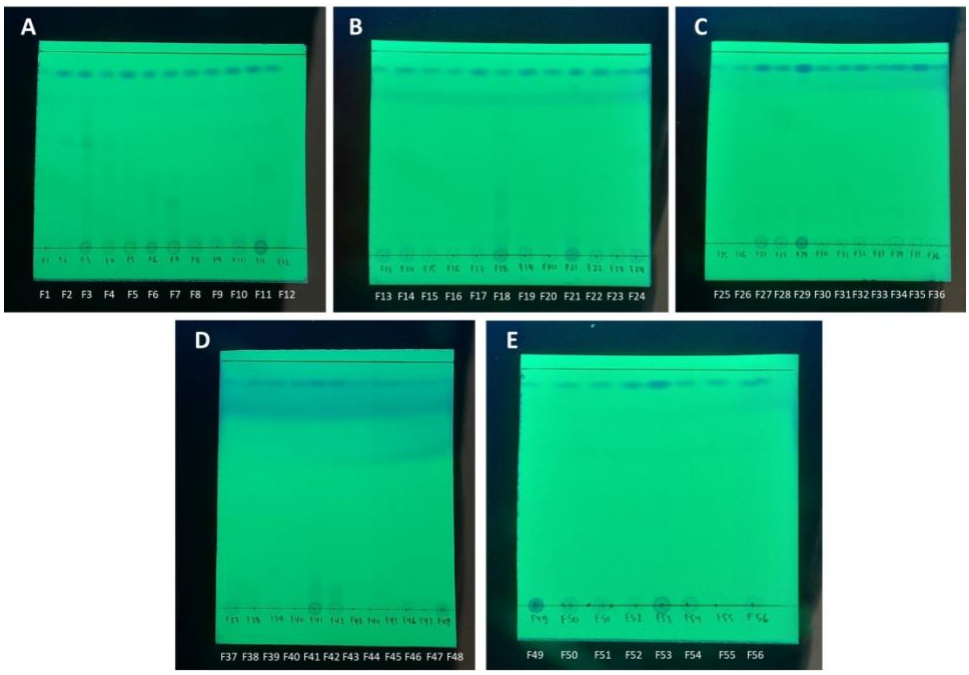

Figure 1. Thin layer chromatography (TLC) profile of 56 secondary metabolite fractions of *Bacillus velezensis* strain DJ4 using hexane: ethyl acetate (7:3 v/v) as mobile phase observed under UV light at λ = 254 nm. Fractions (A) F1–F12, (B) F13–F24, (C) F25– F36, (D) F37–F48, and (E) F49–F56.

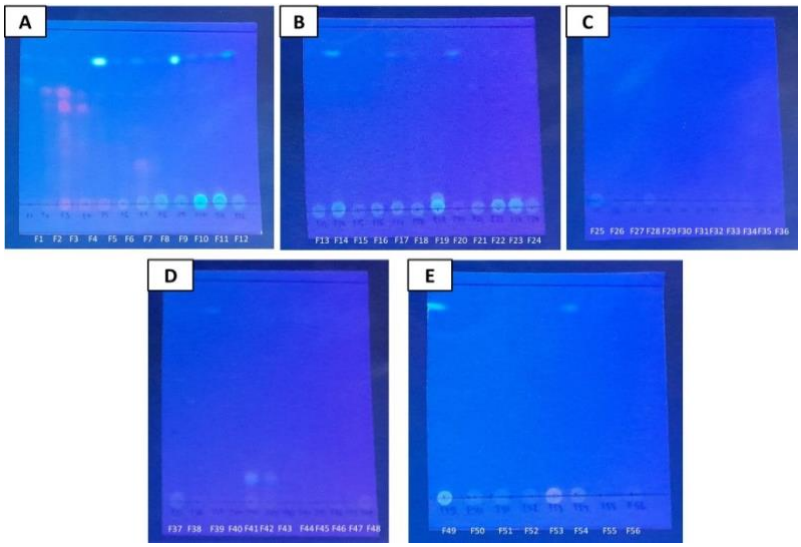

Figure 2. TLC profile of 56 secondary metabolite fractions of *B. velezensis* strain DJ4 using hexane:ethyl acetate (7:3 v/v) as mobile phase observed under UV light at λ = 365 nm. Fractions (A) F1–F12, (B) F13–F24, (C) F25– F36, (D) F37–F48, and (E) F49–F56.

2.2. Antibacterial activity of secondary metabolite fractions of *B. velezensis* strain DJ4

The minimum inhibitory concentration (MIC) of all secondary metabolite fractions was determined. Among the 56 fractions obtained, 10 fractions had antibacterial activity against *Mycobacterium smegmatis* with varying in MICs and effectivities (Table 1). Fraction F53 had the lowest MIC compared to other fractions, which was 85 µg/mL, and was considered as very strong antibacterial activity. Six fractions were classified as having moderate antibacterial activity (MIC range: 500–1000 µg/mL), while one fraction was classified as weak (MIC range: 1000–2000 µg/mL). Forty-seven other fractions were classified as inactive because they had MIC values of more than 2000 µg/mL. Despite having a remarkable antibacterial activity, rifampicin as a positive control still had a lower MIC value than fraction F53 of 0.78 µg/mL considered as very strong antibacterial activity. Fraction F53 was then selected further to evaluate its bioactivity and identify its compound profile.

TLC separation using hexane:ethyl acetate (7:3 v/v) mobile phase showed that fraction F53 had three spots with varying R_f values when observed under UV light λ 254 nm and 365 nm (Figure 3). The first to third spots had R_f values of 0, 0.89, and 0.96, respectively.

2.3. Antibiofilm activity of the selected active fraction of *B. velezensis* strain DJ4

The antibiofilm activity of a fraction with the strongest antibacterial activity was investigated. Fraction F53 was able to inhibit the formation of *M. smegmatis* biofilm. This was indicated by the percentage of inhibition that increased along with the increase of fraction concentration (Table 2). Fraction F53 with a concentration of ½× MIC was able to inhibit biofilm formation by more than 50%. The percentage of inhibition increased to 62.3% in the treatment of 1× MIC concentration.

Table 1. MIC value of active fractions of *B. velezensis* strain DJ4 against *M. smegmatis* ATCC 700084

Fraction code	MIC (µg/mL)	Antibacterial effectivity
F6	618.75	Moderate
F7	940	Moderate
F41	620	Moderate
F43	1325	Weak
F49	498.75	Strong
F51	745	Moderate
F52	1152.5	Weak
F53	85	Very strong
F54	928.75	Moderate
F55	855	Moderate
Rifampicin	0.78	Very strong

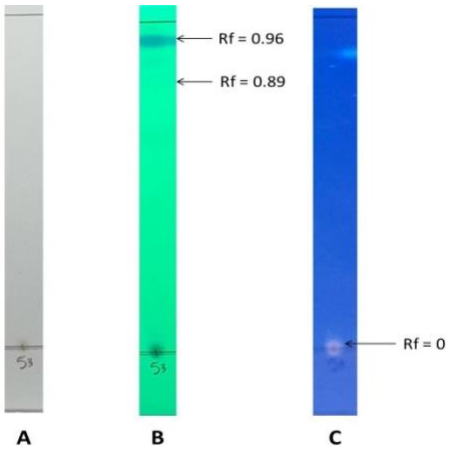

Figure 3. TLC profile of fraction F53 using the mobile phase hexane:ethyl acetate (7:3 v/v) observed under (A) visible light, UV light (B) λ 254 nm, and (C) λ 365 nm.

Table 2. Percentage of inhibition of biofilm formation by fraction F53 against *M. smegmatis*

Treatment	Inhibition of biofilm formation (%)*
1× MIC	62,3±1,4[a]
½× MIC	52,1±1,3[b]
Methanol	0±0[c]

*Data are presented as mean ± standard deviation from triplicate experiments. Different superscript letters in the same column indicate statistically significant differences ($p < 0.05$).

2.4. The effects of the selected active fraction of *B. velezensis* strain DJ4 on *M. smegmatis* biofilm structure

The antibiofilm activity of the active fraction was also evaluated by observing changes in the biofilm structure of *M. smegmatis* using scanning electron microscope (SEM). The biofilm matrix of negative controls (media control and methanol control) had thicker layers compared to the one treated with the F53 fraction at a concentration of 1× MIC (Figure 4). In comparison to the controls, the biofilm structure of *M. smegmatis* treated with the F53 fraction appeared thinner, less compact, and separated into smaller parts.

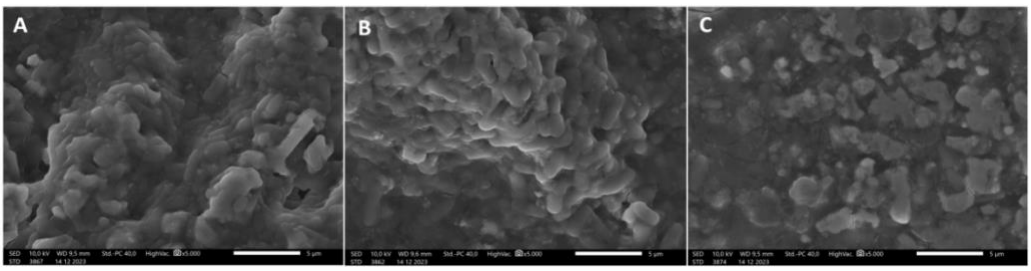

Figure 4. Comparison of *M. smegmatis* biofilm structures between (A) media control with (B) methanol control and (C) F53 fraction treatment of *B. velezensis* strain DJ4.

2.5. Chemical profile of the selected active fraction of *B. velezensis* strain DJ4

LC-MS/MS analysis of fraction F53 showed five detected compound peaks with different retention times (Figure 5). Three of the five peaks have been identified as several different compounds, including 2-(p-anisyl)-5-methyl-1-hexene with a retention time (RT) of 4.67 minutes, nigakilactone H with a retention time of 9.84 minutes, and tetratriacontanamine with a retention time of 10.9 minutes, while the other two compounds have not been identified (Table 3).

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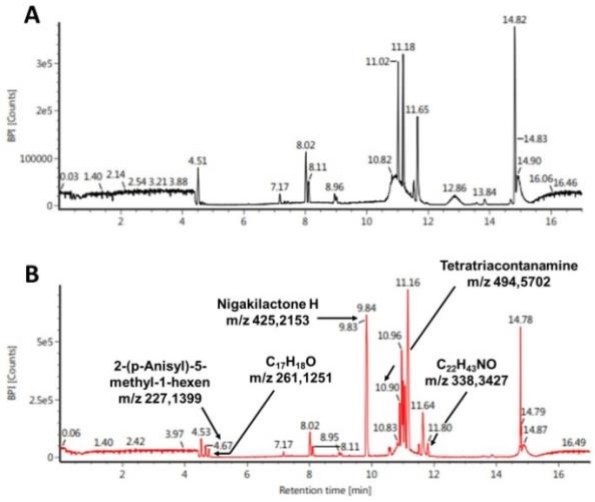

Figure 5. LC-MS/MS chromatograms of (A) blank and (B) F53 fraction of *B. velezensis* strain DJ4 isolate

Table 3. Chemical profile of bioactive compounds in fraction F53 of crude extract of *B. velezensis* strain DJ4 isolate based on LC-MS/MS analysis

Proposed compound	Chemical formula	RT (min)	Bioactivity	Reference
2-(p-anisyl)-5-methyl-1-hexene	$C_{14}H_{20}O$	4,67	-	-
Unknown	$C_{17}H_{18}O$	4,78	-	-
Nigakilactone H	$C_{22}H_{32}O_8$	9,84	Cytotoxic to MCF-7 and MDA-MB-231 cell lines	[14]
Tetratriacontanamine	$C_{34}H_{71}N$	10,90	-	-
Unknown	$C_{22}H_{43}NO$	11,80	-	-

3. DISCUSSION

Fractionation of bacterial secondary metabolites is an important step in characterising compounds and investigating their antibacterial activity. Fifty-six fractions were successfully obtained from the fractionation of crude extract of *B. velezensis* strain DJ4 isolate using thin layer chromatography (TLC) and column chromatography. These fractions were obtained from the results of combining multiple eluates according to the separation pattern on TLC plates. Evaluation of the TLC separation pattern is a preliminary method used to identify similarities in compound composition between fractions based on their polarity [15]. When two or more fractions have the same spot pattern and retention factor (R_f) value, the fractions likely contain a chemical composition with similar polarity [16]. Furthermore, 10 of the 56 fractions obtained possessed antibacterial activity with varying MICs and effectivity against *M. smegmatis*. Antibacterial activity is classified as very strong if it has an MIC value < 100 µg/mL, strong if the MIC value is in the range of 100–500 µg/mL, moderate if the MIC is in the range of 500–1000 µg/mL, weak if the MIC value is between 1000–2000 µg/mL, and inactive if the MIC value is > 2000 µg/mL [17]. Based on these criteria, fraction F53 was significantly active against *M. smegmatis* with the lowest MIC value of 85 µg/mL. Compared to the previous study, fraction F53 demonstrated antibacterial efficacy in the same category as the crude extract of *B. velezensis* strain DJ4 against *M. smegmatis* (MIC of 31.25 µg/mL), which was also classified as very strong antibacterial activity [13]. On the other hand, rifampicin had a much lower MIC value of 0.78 µg/mL compared to the fraction F53. The use of rifampicin as a positive control in this study is in accordance with its application as one of the first-line treatments for tuberculosis and its broad spectrum against some mycobacterial strains [4, 18]. Furthermore, this fraction also tends to have a higher MIC value compared to the fraction F23 from *Loigolactobacillus coryniformis* BCH-4, which is able to inhibit the growth of *Escherichia coli*, *Bacillus cereus*, and *Staphylococcus aureus*, with MICs of 15.6 µg/mL, 3.9 µg/mL, and 31.2 µg/mL, respectively [19].

The TLC profile of fraction F53 displayed three spots with different R_f values under visible light and UV light at 254 nm and 365 nm. These different migration rates in the mobile phase of hexane:ethyl acetate (7:3 v/v) imply that this fraction may contain multiple compounds with different polarities. The difference

in R_f values for each spot within the fraction is attributed to the compound's polarity. Polar compounds tend to interact with the silica gel, causing a temporary halt in the elution process, resulting in the mobile phase moving without producing spots on the TLC plate, and thus leading to smaller R_f values [20]. Conversely, the elution process of nonpolar compounds are more swiftly through the stationary phase, resulting in larger R_f values. According to these results, it is evident that further purification of the fraction F53 is necessary to isolate a pure compound.

The ability of *M. smegmatis* to form biofilms can cause this bacterium to become more resistant to antibiotics [7]. The potency of antibacterial agents in interfering biofilm formation can be a promising prospect for the development of infectious disease treatment. The effect of the fraction F53 at a concentration of ½× MIC has inhibited more than 50% of *M. smegmatis* biofilm formation. The percentage of inhibition increased to 62.3% in the fraction F53 treatment with a concentration of 1× MIC. This indicates that the selected fraction worked in a dose-dependent manner. The higher concentration of the fraction tested, the higher the effectiveness in inhibiting *M. smegmatis*. Bacterial secondary metabolites can inhibit biofilm formation through various mechanisms, including inhibiting quorum sensing signals, preventing cell adhesion, and inhibiting extracellular polymer synthesis [21].

LC-MS/MS analysis of fraction F53 showed five detected peaks with different retention times. Three peaks were identified as 2-(p-anisyl)-5-methyl-1-hexene, nigakilactone H, and tetratriacontanamine, respectively. However, these three proposed compounds have never been reported to have antibacterial activity. Nigakilactone H exhibited a higher peak compared to other proposed compounds, suggesting that this compound is likely the most dominant in the fraction. Previous studies have reported that nigakilactone H isolated from the *Hibiscus sabdariffa* calyx fraction had cytotoxic properties to human breast cancer cell lines (MDA-MB-231) [14]. This compound belongs to the triterpenoid group, which plays a role in regulating Bax and Bcl-2 proteins in inducing apoptosis [22]. Further investigation is needed to prove the cytotoxicity of this compound to bacterial cells. On the other hand, the other two compounds could not be identified using this method, suggesting limitations in the LC-MS/MS database in identifying these compounds. This also presents an opportunity for discovering new types of compounds from endophytic bacteria.

4. CONCLUSION

A total of 10 out of the 56 secondary metabolite fractions obtained from *B. velezensis* strain DJ4 crude extract have antibacterial activity against *M. smegmatis* ATCC 700084 with various MICs and effectivities. Fraction F53 was considered as the most potential fraction because it has an MIC less than 100 µg/ml, indicating very strong antimycobacterial activity. At a concentration of 1× MIC, this fraction was able to inhibit the formation of *M. smegmatis* biofilm by more than 60%. This fraction contains five proposed compounds, namely 2-(p-anisyl)-5-methyl-1-hexene, nigakilactone H, tetratriacontanamine, and two other unknown compounds.

5. MATERIALS AND METHODS

5.1. Source of bacterial strains

Bacillus velezensis strain DJ4, a collection of the Laboratory of Microbiology, Department of Biology, IPB University, was isolated from leaves of *Archidendron pauciflorum* in previous studies [12, 13]. The 16S rRNA gene sequence and complete genome sequence of the bacterial isolate can be accessed at https://www.ncbi.nlm.nih.gov/ with accession numbers PP178167.1 and CP144358.1, respectively. The tested bacterium used in this study was *Mycobacterium smegmatis* ATCC 700084, obtained from the Laboratory of Microbiology, Research Centre of Pharmaceutical Ingredients and Traditional Medicine, National Research and Innovation Agency (BRIN).

5.2. Bacterial culture and extraction of its secondary metabolites

B. velezensis strain DJ4 was cultured in 2 L nutrient broth (NB) and incubated at ±28 °C with agitation 120 rpm for 3 days. The culture was then added with 2 L of ethyl acetate, and shaken continously for 20 minutes. Subsequently, the upper layer was evaporated at 50 °C using rotary evaporation. The obtained crude extract was then dissolved in 1 mL of methanol with gradual addition and then sonicated using a sonicator for 3 minutes and centrifuged at a speed of 10,000 x g for 1 minute. The supernatant and pellet were transferred into separate vials, and dried in an oven at 50 °C for 24 hours [12].

5.3. Fractionation of the bacterial crude extract

5.3.1. Thin layer chromatography (TLC)

Briefly, 2 mg of crude extract of *B. velezensis* strain DJ4 was dissolved in methanol. The crude extract suspension was then applied on a TLC plate covered in silica gel 60 F_{254} (Merck, Darmstadt, Germany) using a capillary tube and left to dry. Subsequently, the TLC plate was placed in a chamber containing a mobile phase system of hexane and ethyl acetate in various proportions (10:0, 7:3, 3:7, and 0:10 v/v). The results of the separation of compounds were observed under visible light and ultraviolet (UV) light at 254 nm and 365 nm. The retention factor (R_f) value of each spot was calculated with the following formula:

$$R_f = \frac{\text{Distance travelled by spot (cm)}}{\text{Distance travelled by mobile phase (cm)}}$$

The proportion of mobile phases that provide the best compound separation was indicated by the major number of spots on the surface of TLC plate, and then used for column chromatography [23].

5.3.2. Column chromatography

Fractionation of the bacterial crude extract was carried out using column chromatography according to Chepkorir et al. [24]. Column packing was performed by mixing 45 g of silica gel 60 (0.063–0.2 mesh size) with hexane (10:0 v/v) and stirring to form a homogeneous suspension-like slurry. The silica gel slurry was then poured into a glass column containing a small amount of cotton and sand. Silica gel was also gradually added to the bacterial crude extract and mixed until it formed a fine green powder. The sample mixture was subjected to the column and eluted using mobile phase combinations of hexane:ethyl acetate and methanol, which created an increasing polarity gradient (10:0, 9:1, 7:3, 5:5, 3:7, 1:9, 0:10 v/v). The eluates produced from each mobile phase combination were collected and evaporated using a rotary evaporator at 50 °C. Subsequently, the eluates were evaluated with TLC (eluted with hexane:ethyl acetate (7:3 v/v)) to identify similarities between their separation spots. Eluates with a similar TLC profile were pooled together in the same vial.

5.4. Determination of the minimum inhibitory concentration (MIC)

MIC values of secondary metabolite fractions were determined using a standard micro broth-dilution assay [25]. The *M. smegmatis* bacterial suspension that had been incubated for 24 hours was diluted with 0.85% NaCl solution to achieve a concentration of 10^4 CFU/mL. A total of 20 µL of the fraction was then added to a 96-well plate containing 180 µL of sterile Mueller-Hinton Broth (MHB) (Himedia) until the final volume reached 200 µL in row A. Serial dilution was carried out from row A to H, followed by adding 100 µL of bacterial suspension to each well, resulting in a final concentration of bacterial cells 5×10^3 CFU/mL. Rifampicin (concentration range: 0.78-100 µg/mL) and methanol were used as positive and negative controls, respectively. The plate was then agitated at a speed of 150 rpm and 37 °C for 24 hours. MIC was determined as the lowest fraction concentration that could inhibit the growth of *M. smegmatis*, indicated by the clear medium in wells. This assay was conducted with three replications.

5.5. Determination of antibiofilm activity

Antibiofilm activity of selected active fractions was performed by crystal violet staining in 96-well plate according to CLSI with slight modifications [25]. A total of 100 µL of *M. smegmatis* suspension with a concentration of 10^4 CFU/mL was inoculated into Brain Heart Infusion (BHI) supplemented with 0.25% glucose. Selected active fractions with different concentrations (1× MIC and ½× MIC) were added to the 96-well plate until the final volume reached 200 µL in each well, then agitated at 150 rpm and 37°C for 24 hours. After the incubation period, the media and bacterial cells were removed twice using 0.85% NaCl solution followed by the addition of 200 µL of 0.1% crystal violet to each well. The plate was incubated at 37 °C for 30 minutes and washed using 0.85% NaCl solution. The biofilm stained by crystal violet was then dissolved in 200 µL of 99% DMSO and its absorbance was measured using an ELISA microplate reader (Thermo Scientific Varioskan Flash, Massachusetts, USA) at a wavelength of 595 nm. *M. smegmatis* culture without selected active fractions treatment was used as a control. Methanol was also used as a negative control with a final concentration in the well of 3.5% v/v. The percentage of inhibition of biofilm formation was determined using the following formula:

$$\text{Inhibition of biofilm formation (\%)} = \frac{OD_{\text{negative control}} - OD_{\text{treatment}}}{OD_{\text{negative control}}} \times 100$$

5.6. Scanning electron microscope (SEM) observation of biofilm formation

The *M. smegmatis* bacterial suspension that had been incubated for 24 hours was diluted with 0.85% NaCl solution to achieve a concentration of 10^4 CFU/mL. The bacterial suspension was inoculated into BHI supplemented with 0.25% glucose in a 96-well plate, then the selected active fraction was added with a concentration according to the determined MIC value. The preparation for observing the biofilm formation was prepared by dissolving the biofilm treated with the active fraction using sterile distilled water, then applied it on a single polished silicon wafer plate (Sigma-Aldrich, Germany). Each specimen was then observed using a scanning electron microscope (SEM) (JSM-IT200, JEOL, Tokyo, Japan) with a secondary electron emission mode and accelerating voltage of 10 kV. Images were taken at a magnification of 5000x [26].

5.7. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

The bioactive compounds in selected active fractions were identified using liquid chromatography-tandem mass spectrometry (LC-MS/MS) Xevo G2-XS QTof (quadrupole time-of-flight) (Waters, USA) through an electron spray interface (ESI). Separation conditions were conducted using a liquid chromatography system with the column temperature maintained at 40 °C. The sample was eluted gradually using the mobile phase contained of 0.1% formic acid in water (A) and acetonitrile (B) for 17 min with a flow rate of 0.3 mL/min. Mass spectrometric data were acquired in positive ion mode with the MS precursor m/z range of 100–1200. The source temperature was set to 120 °C, and the capillary voltage was 2 kV. The composition and mass of each fragment and precursor ion were determined using the data available in the UNIFI software database.

5.8. Statistical analysis

The mean values along with standard deviation (±SD) of biofilm formation inhibition data were analysed using the one-way analysis of variance (ANOVA) with Duncan test for further analysis. The analyses were carried using the Statistical Package for the Social Sciences (SPSS) software (Version 20.0, SPSS Inc., Chicago, USA), with $p < 0.05$ considered statistically significant [27].

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Conflict of interest statement: The authors declared no conflict of interest in the manuscript.

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