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Isolation and optimized production of putative antimicrobial compounds from Egyptian soil isolate *Streptomyces* sp. MS. 10

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Abstract

Background: The rapid spread of antibiotic resistance has increased research interest in the discovery of natural products, mainly from actinomycetes, which have been the primary source of antimicrobial compounds. This study aimed to isolate, characterize, and optimize the production of some of the bioactive compounds from bioactive soil actinomycetes.

Results: One promising soil actinomycete, which was molecularly identified as *Streptomyces* sp. and designated as *Streptomyces* sp. MS. 10, showed broad-spectrum antimicrobial activity, including activity against methicillin-resistant *Staphylococcus aureus*. Thus, it was selected for isolation of its major bioactive compounds. Polymerase chain reaction amplification of the genes responsible for antibiotic biosynthesis showed the presence of genes encoding type I and type II polyketide synthase. Liquid chromatography-mass spectrometry analysis found that the major antimicrobial compounds produced by *Streptomyces* sp. MS. 10 were weakly ionized bioactive secondary metabolites. A large-scale fermentation experiment of *Streptomyces* sp. MS. 10 using pre-optimized culture conditions followed by bioassay-guided chromatographic separation of its secondary metabolites resulted in the isolation of putative bioactive compounds that were identified as fatty acids using proton nuclear magnetic resonance spectroscopy.

Conclusions: Egyptian soil is still a good source for exploring bioactive actinomycetes. Additionally, this study highlighted the importance of combining both physicochemical and genotypic characterization with spectroscopic analysis of the major natural products when isolating bioactive metabolites.

Keywords: Actinomycetes, Antimicrobial natural products, Liquid chromatography-mass spectrometry, Methicillin-resistant *Staphylococcus aureus*, Nuclear magnetic resonance, *Streptomyces*

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1 Background

The substantial increase in the number of multidrug-resistant microorganisms has led to a boost in the field of antibiotic discovery [1–4]. During 1981–2014, 45% of all new antimicrobials approved by the Food and Drug Administration were either of natural origin (natural products or their derivatives) or synthetic drugs mimicking the mode of action or pharmacophores of natural products [5]. The natural products that are derived from microorganisms (bacteria and fungi) and their analogs and derivatives are the most successful defense line against infectious diseases [5, 6].

Species of the *Streptomyces* genus are responsible for producing 75% of the reported metabolites isolated from actinobacteria [7, 8]. Some of these compounds play a critical role in ecological systems by suppressing microbial competitors in their environment to protect their food supply. The production of such bioactive metabolites demonstrates highly organized and coordinated metabolic pathways in *Streptomyces* sp., which allows them to dominate among other ecosystem inhabitants [9, 10]. *Streptomyces* sp. have a high GC content, which is thought to accumulate over the years through selection processes of adaptation to new ecosystems and is believed to grant their dominance in soil niches [11]. Many secondary metabolites produced by *Streptomyces* sp. have been successfully used as antibiotics in the treatment of drug-resistant infections in both humans and animals [12].

This study aimed to use a bioassay-guided approach to isolate naturally occurring antimicrobial products from a crude extract of *Streptomyces* sp. MS. 10 using different chromatographic separation techniques, followed by their chemical characterization using spectroscopic techniques.

2 Methods

2.1 Isolation and antimicrobial activity screening of *Streptomyces* sp. MS. 10

Streptomyces sp. MS. 10 was recovered from a superficial soil layer sample collected from Ihnasia City in Beni-Suef Governorate, Egypt. It was isolated on International Streptomyces Project (ISP) 4 agar using the soil dilution plate technique, as previously described [13, 14].

Antimicrobial activity screening of *Streptomyces* sp. MS. 10 was performed using the cup diffusion method [15] against the following indicator strains: *Sarcina lutea* (environmental sample), *Bacillus subtilis* (environmental sample), *Enterococcus faecalis* (food sample), *Salmonella enterica* (ATCC 35664), *Pseudomonas aeruginosa* (ATCC 9027), *Proteus* sp. (clinical sample), *Escherichia coli* (clinical sample), and methicillin-resistant *Staphylococcus aureus* (MRSA; clinical sample). Briefly, tryptone soya agar plates were surface inoculated with the indicator strains, followed by the use of a sterile borer to make 10-mm cups in the agar plates that were filled with 150 μL of the supernatant from a liquid broth culture of *Streptomyces* sp. MS. 10. The antimicrobial activity against each indicator strain was determined according to the diameter of the inhibition zones around the cups, following incubation for 24 h at the appropriate temperature for growth of each indicator strain. An inhibition zone diameter ≥ 12 mm was recorded as positive for bioactivity.

2.2 Taxonomical identification using 16S rRNA gene sequencing

Genomic DNA of *Streptomyces* sp. MS. 10 was extracted and purified, as previously described [16]. Then, polymerase chain reaction (PCR) amplification of the 16S rRNA gene was performed using forward primer 27F (5′-AGAGTTTG ATCMTGGCTCAG-3′) and reverse primer 1492R (5′-GGTTACCTTGTTACGACTT-3′) [17]. The PCR reaction was performed in a final volume of 50 μL using 10 μL of 5× reaction buffer, 500 ng of genomic DNA, a 10 mM dNTP mixture, 2.5 units of *Taq* DNA polymerase, and 1 μL of both forward and reverse primers. The PCR cycling conditions were as follows: initial denaturation of the DNA template for 3 min at 94 °C; followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 60 s, and extension at 72 °C for 60 s; and a final extension at 72 °C for 5 min.

Agarose gel electrophoresis (1.5%, w/v) was performed for 60 min at 90 V using 5 μL of each PCR product to determine the success of PCR amplification and the purity of the PCR product. A 1-kb DNA ladder was also included to estimate the size of the PCR products [18]. The PCR product of the 16S rRNA gene was then purified using a PCR purification kit before being sequenced at Macrogen Korea using forward primer 785F (5′-GGATTAGATACCCTGGTA-3′) and reverse primer 907R (5′-CCGTCAATTCMTTTRAGTTT-3′) [19].

The megaBLAST tool of the National Center for Biotechnology Information (NCBI) was used to compare the good-quality sequences of *Streptomyces* sp. MS. 10 with the GenBank database to identify the closest related strains that showed a high sequence similarity [20]. Then, we performed multiple sequence alignments of the amplified sequences and those obtained from GenBank, followed by phylogenetic analysis of *Streptomyces* sp. MS. 10 using the MEGA7 software [21].

2.3 Fermentation and extraction of the bioactive compounds

Bacterial fermentation of *Streptomyces* sp. MS. 10 was performed by inoculating tryptone soya broth (TSB) with a single pure colony and incubating for 3 days at 30 °C. Then, ISP4 broth was seeded with 5% bacterial inoculum in TSB and incubated on a rotary shaker at 160 rpm for 7 days at 30 °C. Next, the bacterial broth was filtered through a Whatman No. 1 filter, followed by the addition of ethyl acetate (EtOAc; 1:1 v/v), and the organic-aqueous mixture

was shaken frequently in a separating funnel to aid the optimal extraction of the bioactive metabolites from the ISP4 broth. Finally, the organic layer was separated and collected in another flask, and the EtOAc solvent was evaporated using a rotary evaporator [16].

A small-scale fermentation experiment (1.5 L) was performed using the abovementioned culture conditions and extraction method. Then, a large-scale fermentation experiment (10 L) was performed using the pre-optimized culture conditions, and the bioactive metabolites were extracted using the optimal extraction solvent (1:1 v/v dichloromethane [DCM]), as discussed later.

2.4 High-performance liquid chromatography

The bioactive compounds in the EtOAc crude extract from the small-scale fermentation experiment were chromatographically separated by preparative HPLC on a Dionex Ultimate 3000 HPLC system (Agilent) using a Nucleosil C18 column. The solvent gradient started with 100% H_2O (HPLC grade) and 0% acetonitrile (ACN; HPLC grade) at a flow rate of 3 mL/min, and the percentage of ACN was linearly increased to 100% at 25 min. Final washing was performed for 15 min, using 100% ACN Then, a second preparative HPLC run was performed on fraction 25 (retention time [R_t] = 25 min), which showed the highest antimicrobial activity, using the same protocol as the first HPLC run, except that the solvent gradient started with 30% H_2O and 70% methanol (HPLC grade) and linearly increased to 100% methanol at 25 min, with final washing for 15 min using 100% methanol.

2.5 Characterization of the major bioactive compounds

We tested the effect of different temperatures and degrading enzymes, including protease, amylase, and α-chymotrypsin, on the bioactivity of the supernatant from a liquid broth culture of *Streptomyces* sp. MS. 10 to characterize the major bioactive metabolites. Tubes containing 400 μL of the broth supernatant were incubated for 30 min in water baths that were set to different temperatures (60 °C, 80 °C, and 100 °C) to test the effect of temperature. The effect of 121 °C on the major bioactive compounds was determined by autoclaving for 15 min. To test the effect of degrading enzymes, they were separately added to the broth supernatant in different tubes. Their concentrations were adjusted to obtain a final concentration of 1 mg/ml in a final volume of 200 μL, and then the tubes were incubated at 37 °C for 30 min. Finally, the antimicrobial activity of all the treated broth supernatant samples was assessed using the agar diffusion method and compared with the activity of the original broth (positive control). Negative controls for the degrading enzymes were also included.

2.6 PCR amplification of the genes responsible for antibiotic biosynthesis

The genomic DNA of *Streptomyces* sp. MS. 10 was screened using PCR for the presence of genes responsible for the biosynthesis of natural products, including non-ribosomal peptide synthetase (NRPS), polyketide synthase (PKS) I, PKS II, and glycopeptide monooxygenase B. The following primer pairs were used: NRPS/A3 F (5′-GCST ACSYSATSTACACSTCSGG-3′) and NRPS/A7 R (5′-SASGTCVCCSGTSGCGTAS-3′) to amplify the NRPS gene, with an expected product size of 700 base pairs (bp) [22]; PKS/K1 F (5′-TSAAGTCSAACATCCGBCA-3′) and PKS/M6 R (5′-CGCAGGTTSCSGTACCAGTA-3′) to amplify the PKS I gene, with an expected product size of 1200–1400 bp [22]; ARO-PKS-F (5′-GGCAGCGGITTC GGCGGITTCCAG-3′) and ARO-PKS-R (5′-CGITGTTI ACIGCGTAGAACCAGGCG-3′) to amplify the PKS II gene, with an expected product size of 492-630 bp [23]; and oxyB F (5′-CTGGTCGGCAACCTGATGGAC-3′) and oxyB R (5′-CAGGTACCGGATCAGCTCGTC-3′) to amplify the glycopeptide monooxygenase B gene, with an expected product size of 696 bp [23]. The PCR cycling conditions included initial denaturation for 5 min at 94 °C; followed by 40 successive cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 59 °C, 55 °C, 64 °C, and 60 °C for NRPS, PKS I, PKS II, and glycopeptide monooxygenase primers, respectively; and extension at 72 °C for 2 min. The final extension was at 72 °C for 10 min [24]. The success of PCR amplification of the targeted genes was determined by running the PCR products on a 1.5% (w/v) agarose gel using both 100-bp and 1-kb ladders as size markers [18].

2.7 Optimization of the culture conditions and the extraction solvents

We optimized the culture conditions for *Streptomyces* sp. MS. 10 to determine those necessary for the optimal production of the major antimicrobial compounds by comparing different growth media, carbon sources, and nitrogen sources [16]. The best culture media was selected by comparing the antimicrobial activity of *Streptomyces* sp. MS. 10 inoculated in different culture media, including ISP4, ISP4 supplemented with glucose, and TSB. The fermentation experiments for selecting best culture media were done using a shaker incubator at 160 rpm for 7 days at 30 °C. The best carbon source was determined by replacing the starch in ISP4 broth with other carbon sources, including fructose, glucose, maltose, sucrose, lactose, glycerol, mannitol, and sorbitol, while the best nitrogen source was selected by replacing the ammonium sulfate in ISP4 broth with ammonium citrate, urea, peptone, tryptone, proteose peptone, albumin, casein, casamino acid, and yeast extract. The fermentation experiments for testing optimal carbon and nitrogen sources were done using a shaker incubator at 160 rpm for 11 days at 30 °C with daily sampling starting from day 3.

The extraction conditions were optimized using different organic solvents, including hexane, chloroform, DCM, and EtOAc, at different solvent: broth proportions. The cup diffusion method was used to screen for antimicrobial activity under the different extraction conditions. Extraction success was defined as the absence of activity in the fermentation broth after liquid/liquid extraction, indicating complete extraction of the total metabolites in the bacterial broth.

2.8 Column chromatography

A total of 250 mg of crude extract was obtained from the extraction of the large-scale fermentation broth (10 L) of *Streptomyces* sp. MS. 10 using DCM (1:1, v/v) for extraction. The bioactive secondary metabolites in the crude extract were chromatographically separated using a normal phase packed silica gel column (column chromatography) and gradient elution with DCM and methanol in 1% increments until 100% methanol. The packed silica column was prepared using 12.5 g of silica gel. Briefly, the dry crude extract of *Streptomyces* sp. MS. 10 was solubilized in DCM. Then, 0.5 g of silica was added, and the extract was left to dry to make a dry band, which was added to the top of the packed silica column. Fractions of 10 ml each were collected, with a total of 160 fractions. The similarity of the collected fractions was checked using thin-layer chromatography (TLC) plates with DCM-methanol as the solvent system and p-anisaldehyde as the spray reagent, and the similar fractions were pooled. Additionally, the collected fractions were screened for antimicrobial activity using the cup diffusion method. Fractions showing good purity on the TLC plates and high antimicrobial activity were selected for further analysis using proton nuclear magnetic resonance (^{1}H NMR) spectroscopy in an effort to determine their chemical classes.

2.9 Spectroscopic characterization

The most bioactive fraction from the second HPLC run (fraction 14) underwent analysis using liquid chromatography-mass spectrometry (LC-MS) at the Faculty of Postgraduate Studies of Advanced Science, Beni-Suef University, Egypt, to determine the major ion peaks and their mass-to-charge ratios (*m/z*). LC-MS was performed in both the negative and positive ion modes using electrospray ionization. The bioactive compounds that were isolated from the large-scale fermentation experiment underwent ^{1}H NMR spectroscopy using a 400 MHz NMR system (Bruker) at the Faculty of Pharmacy, Beni-Suef University, Egypt [25]. The NMR solvent was deuterated methanol (MeOD).

3 Results

3.1 Antimicrobial activity screening

Preliminary identification of *Streptomyces* sp. MS. 10 as an actinomycete was made based on its mycelia coloration, pigment production, and morphological characteristics, followed by antimicrobial activity screening showing broad-spectrum activity against all tested indicator strains in this study, including MRSA. Therefore, *Streptomyces* sp. MS. 10 was selected for further isolation and purification of its bioactive metabolites using bioassay-guided chromatographic separation.

3.2 Molecular identification using 16S rRNA gene sequencing

A comparison of the partial 16S rRNA gene sequence of *Streptomyces* sp. MS. 10 with sequences in GenBank showed high similarity (> 99%) with the sequences of many *Streptomyces* species. We performed multiple alignments of these sequences with the partial 16S rRNA gene sequence of *Streptomyces* sp. MS. 10 and constructed a phylogenetic tree using the maximum likelihood method in the MEGA7 software (Fig. 1) [21]. However, because of the high similarity of the 16S rRNA gene sequence of *Streptomyces* sp. MS. 10 with sequences of different species of *Streptomyces*, we could only confirm its genus as *Streptomyces*, and we were unable to determine its species. Thus, isolate MS. 10 was designated as *Streptomyces* sp., and its sequence was submitted to NCBI GenBank under accession number MN148619.

3.3 Chromatographic separation of the bioactive metabolites from the small-scale fermentation experiment

The first HPLC run performed on the crude extract of *Streptomyces* sp. MS. 10 resulted in the collection of 40 fractions, of which fraction 25 showed the best antimicrobial activity. However, it was insufficiently pure for further chemical identification, so it was subjected to a second fractionation run by preparative HPLC. Fraction 14 (R_t = 14 min) eluted from the second HPLC run showed the highest zone of inhibition among all collected fractions. LC-MS analysis of the bioactive fraction 14 showed the presence of many major ion peaks (Fig. 2).

3.4 Characterization of the major bioactive compounds

Characterization of the major bioactive metabolites of *Streptomyces* sp. MS 10 revealed that they were unaffected by proteolytic enzymes (protease K and α- chymotrypsin) and amylase (Table 1). Thermal stability was observed at temperatures of up to 80 °C, and antimicrobial activity was diminished at 100 °C and 121 °C (autoclave) (Table 1).

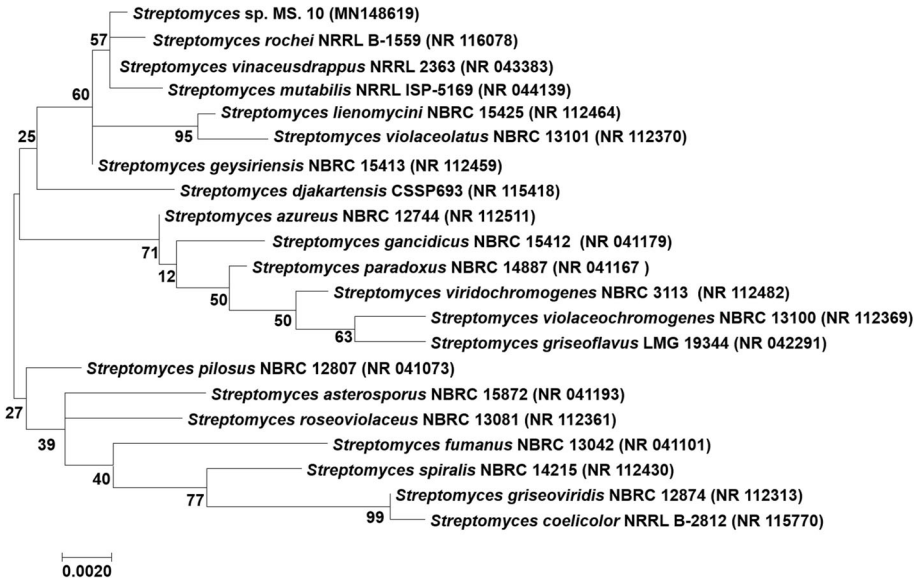

Fig. 1 Phylogenetic tree of *Streptomyces* sp. MS. 10 based on partial 16S rRNA gene sequences using the maximum likelihood method. The evolutionary history was inferred based on the Kimura 2-parameter model [26]. The tree with the highest log-likelihood (– 2875.0856) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The initial tree(s) for the heuristic search were obtained automatically by applying the neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach and then selecting the topology with the most superior log-likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 21 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were 1400 positions in the final dataset. The evolutionary analyses were conducted using the MEGA7 software [21]

3.5 Molecular identification of the genes responsible for antibiotic biosynthesis

PCR amplification of the screened genes revealed the absence of both NRPS and glycopeptide monooxygenase B genes in the genomic DNA of *Streptomyces* sp. MS. 10, whereas genes encoding PKS I and PKS II were detected using agarose gel electrophoresis to compare the size of the PCR products with size markers.

3.6 Optimization of the culture conditions and the extraction solvent

ISP4 broth was shown to be the best culture medium among those tested, leading to the optimal production

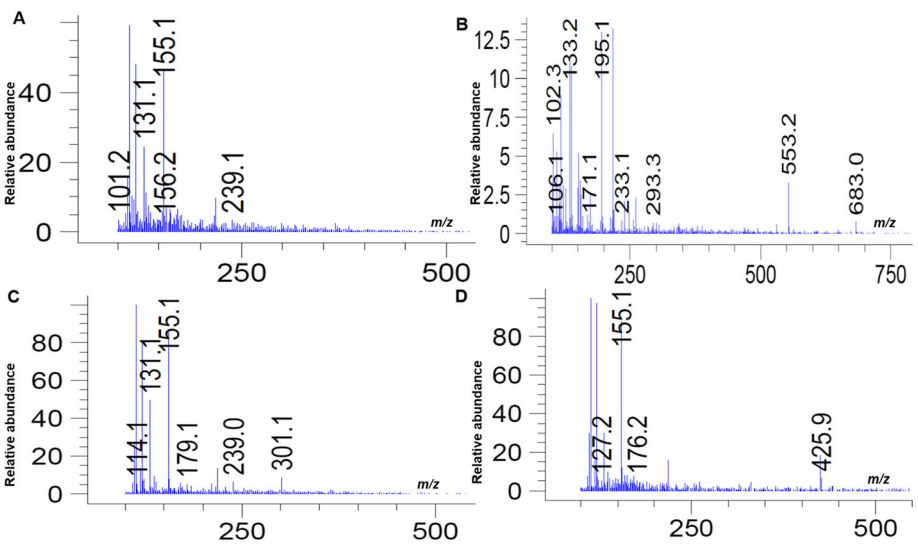

Fig. 2 Mass spectra of different ion peaks of fraction 14 at different R_t showing the detection of components with different *m/z* values. **a** Mass spectrum at R_t = 2.00 min. **b** Mass spectrum at R_t = 2.92 min. **c** Mass spectrum at R_t = 14.75 min. **d** Mass spectrum at R_t = 19.76 min

Table 1 Characterization of the total antimicrobial activity of *Streptomyces* sp. MS. 10

Test condition	Effect on antimicrobial activity
Protease K	No effect
α-chymotrypsin	No effect
Amylase	No effect
60 °C	No effect
80 °C	No effect
100 °C	Diminished activity
Autoclave (121 °C)	Diminished activity

of the antimicrobial agents. This was followed by ISP4 supplemented with glucose, which showed a smaller inhibition zone diameter compared with using ISP4 alone. Replacing starch as the carbon source in ISP4 broth with different carbon sources, including monosaccharides, disaccharides, polysaccharides, and sugar derivatives, revealed maltose (disaccharide) as the optimal carbon source, showing the largest zone of inhibition. Casein was identified as the optimal nitrogen source among the tested nitrogen sources used to replace the ammonium sulfate in ISP4 broth, showing the largest inhibition zone.

Regarding extraction solvent optimization, DCM (1:1, v/v) was the best solvent to extract all antimicrobial agents from the fermentation broth, with no bioactivity remaining in the fermentation broth after the liquid/liquid extraction, and EtOAc in higher solvent: broth proportion (2:1, v/v) showed the same result. Thus, DCM (1:1, v/v) was selected for further extraction of the total metabolites from the bacterial broth of *Streptomyces* sp. MS. 10 due to less solvent proportion required for total extraction of the bioactive compounds

3.7 Chromatographic separation of the bioactive metabolites using column chromatography

Among the 160 fractions collected following column chromatography, fractions 3–90 showed moderate to good antimicrobial activity in terms of inhibition zones, while fractions 7–9 and 21–35 showed the best activity, with the largest zone of inhibition. According to the antimicrobial activity and the TLC results, the highly bioactive fractions 8 and 24 were selected for spectroscopic analysis by ^{1}H NMR spectroscopy.

^{1}H NMR spectroscopy of fraction 8 suggested the presence of a saturated fatty acid (FA) as a major bioactive compound (Fig. 3). This was confirmed by the presence of both α-protons, H_2-2 resonating at δ_H 2.38 ppm and the long chain of methylene group $(CH_2)_n$ resonating at δ_H 1.24-1.43 ppm, while the terminal methyl groups resonating at δ_H 0.93 were also detected (Fig. 3). Moreover, this FA was suggested as a saturated FA because of the absence of any olefinic protons which usually resonate at δ_H 5.00-5.50 ppm. This final conclusion was also supported by elution of this compound by 90% DCM, which was the nonpolar solvent in the solvent gradient.

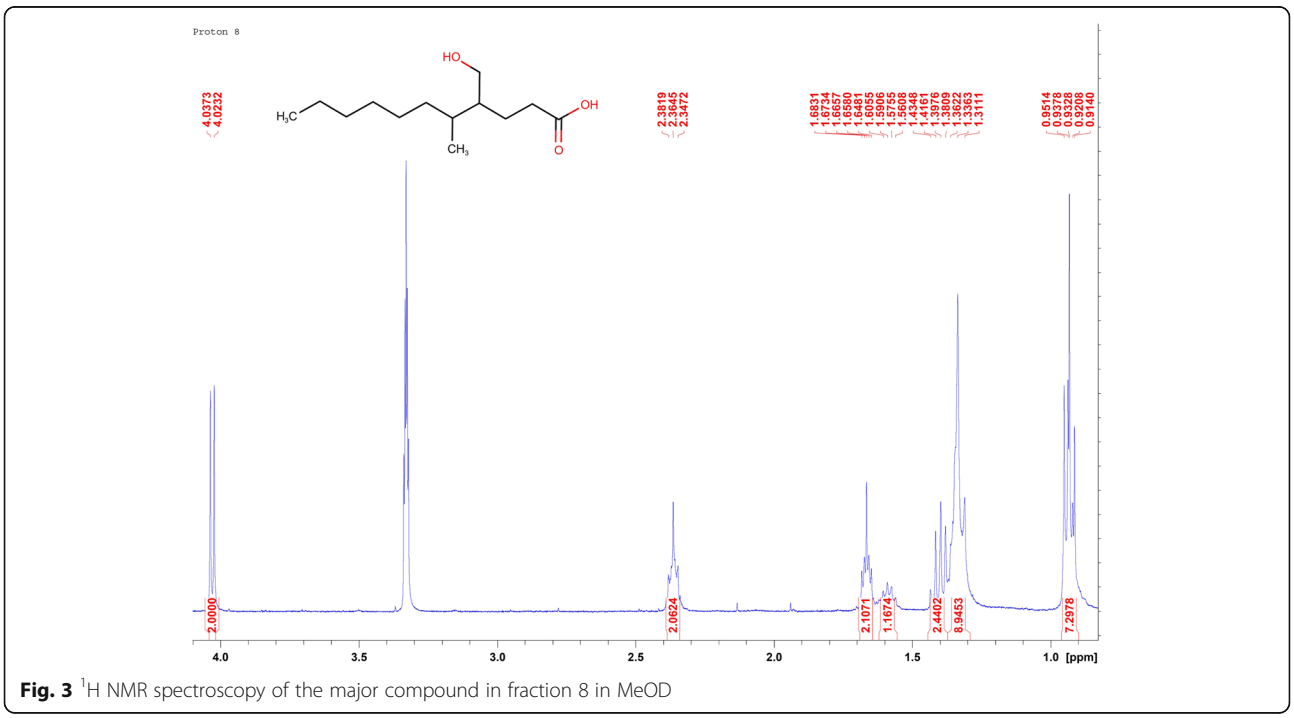

Fig. 3 ^{1}H NMR spectroscopy of the major compound in fraction 8 in MeOD

Proton-proton correlated spectroscopy (^{1}H-^{1}H COSY) and proton-carbon heteronuclear multiple bond correlation (^{1}H-^{13}C HMBC) NMR experiments confirmed the presence of saturated fatty acid as major bioactive compound in fraction 8 (Fig. S1 and Fig. S2). Both α-protons, H_2-2 resonating at δ H 2.38 ppm and β-protons, H_2-3 resonating at δ_H 1.67 ppm were coupled to each other as shown in the ^{1}H-^{1}H COSY spectrum (Fig. 4a and Fig. S1). Notably, the α-protons, H_2-2 and β-protons, H_2-3 showed 2-bond correlation (2*J*) and 3-bond correlation (3*J*) to C-1 resonating at δ_C 173.91 ppm (carboxylic group), respectively (Fig. 4b and Fig. S2). Also, ^{1}H-^{1}H COSY experiment revealed that β-protons, H_2-3 and H-4 resonating at δ_H 1.59 ppm were coupled to each other, and H-4 was coupled to couple of protons resonating at δ_H 4.03 ppm and H-5 resonating at δ_H 1.4 ppm (Fig. 4a and Fig. S1). Interestingly, the two protons (H_2) resonating at δ_H 4.03 ppm were deshielded suggesting that they were on a carbon atom linked to a heteroatom. By investigating the proton-carbon heteronuclear single quantum coherence (^{1}H-^{13}C HSQC) spectrum, it was clear that those deshielded protons were connected to a deshielded carbon resonating at δ_C 66.31 ppm suggesting the presence of a heteroatom linked to this carbon (Fig. S3). This finding suggested the presence of a hydroxy FA as a major bioactive compound in fraction 8. ^{1}H-^{13}C HMBC showed many interesting connectivity including connectivity between these deshielded protons and the C-1 (carboxylic group) and C-4 resonating at δ_C 38.82 ppm (Fig. 4b and Fig. S2).

Another branching of this FA was suggested by detecting three protons (methyl group) resonating at δH 0.93 ppm coupled to H-5 as shown in ^{1}H-^{1}H COSY experiment (Fig. 4a and Fig. S1). ^{1}H-^{13}C HMBC confirmed this suggestion by revealing the presence of 3-bond correlation (3*J*) between the methyl H_3 and C-4 and between H-5 and the hydroxylated carbon atom (Fig. 4b and Fig. S2). All carbons-protons assignments were done by ^{1}H-^{13}C HSQC (Fig. S3). Finally, this compound was elucidated as a mono-hydroxylated saturated FA by comparison with literature [27, 28]. Determination of the length of the methylene group chain in this FA was challenging due to absence of its exact mass, so it was extracted from its ^{1}H NMR spectrum suggesting that the molecular formula of this FA was $C_{13}H_{26}O_3$. However, this molecular formula cannot be confirmed without determining the exact mass. Therefore, this FA final structure was not confirmed, and it was shown as a proposed structure (Fig. 5).

The identification of the major bioactive compound in fraction 24 using ^{1}H NMR spectroscopy was more difficult than fraction 8 due to the presence of some impurities and one or more minor compound(s) (Fig. S4).

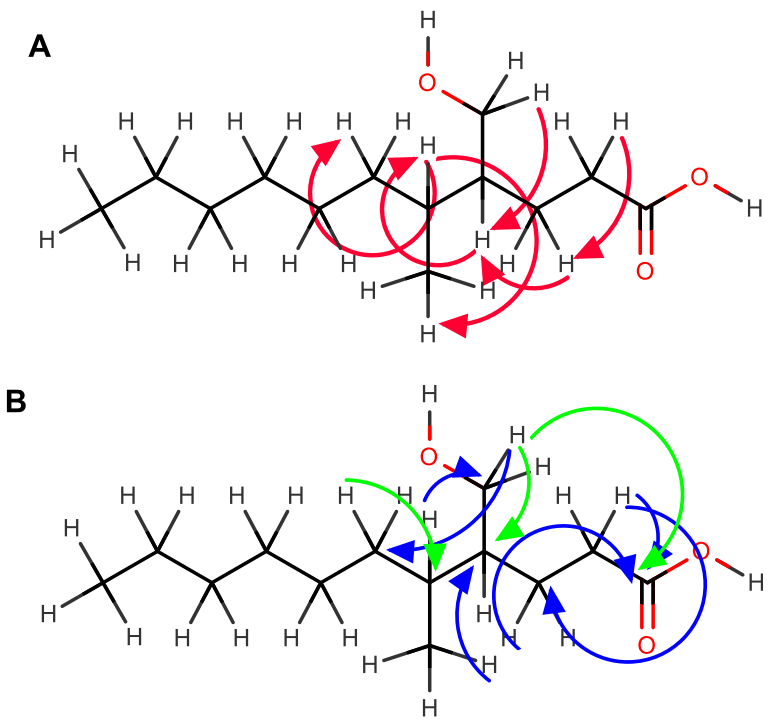

Fig. 4 Proton-proton and proton-carbon coupling in substructures of the major compound in fraction 8. **a** Proton- proton coupling as extracted from ^{1}H-^{1}H COSY spectrum (Fig. S1). **b** Proton-carbon connectivity in substructure of compound 1 as extracted from ^{1}H-^{13}C HMBC spectrum (Fig. S2)

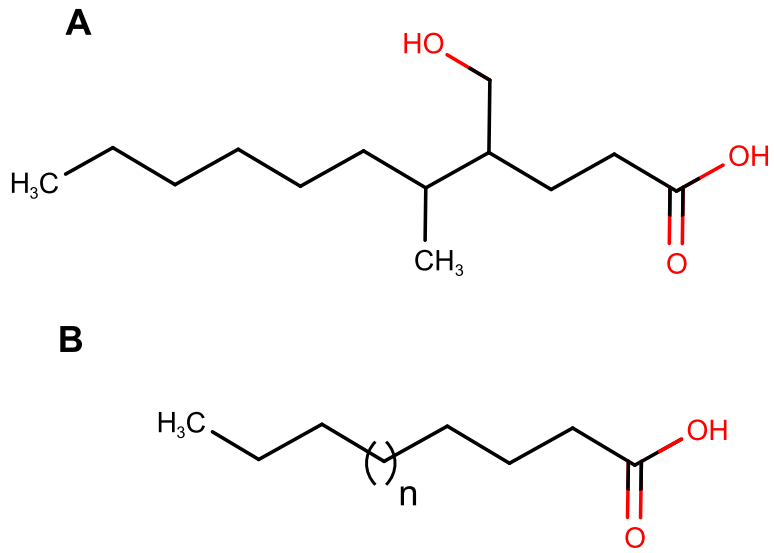

Fig. 5 Proposed chemical structures of the major bioactive compounds from large-scale of *Streptomyces* sp. MS. 10. **a** Proposed chemical structure of the major bioactive compound in fraction 8. **b** Proposed chemical structure of the major bioactive compound in fraction 24

Similar to the major compound in fraction 8, the presence of a saturated FA as a major compound in fraction 24 was proposed due to presence of both α-protons of the FA, resonating at δ_H 2.29 ppm, and the long chain of the methylene group, resonating at δ_H 1.35–1.45 ppm, while the terminal methyl group(s), resonating at δ_H 0.90, were also detected (Fig. 6). Also, similar to the FA in fraction 8, the absence of proton chemical shifts in the olefinic region suggested the saturation of this FA.

^{1}H-^{1}H COSY and ^{1}H-^{13}C HMBC experiments confirmed the presence of saturated FA as major bioactive compound in fraction 8 (Fig. S5 and Fig. S6), while all carbons-protons assignments were done by ^{1}H-^{13}C HSQC experiment (Fig. S7). Although this major compound was elucidated as saturated FA (Fig. 5), the length of its methylene group chain was not determined exactly due to absence of its exact mass and presence of other impurities (Fig. S4). Finally, the presence of this long-

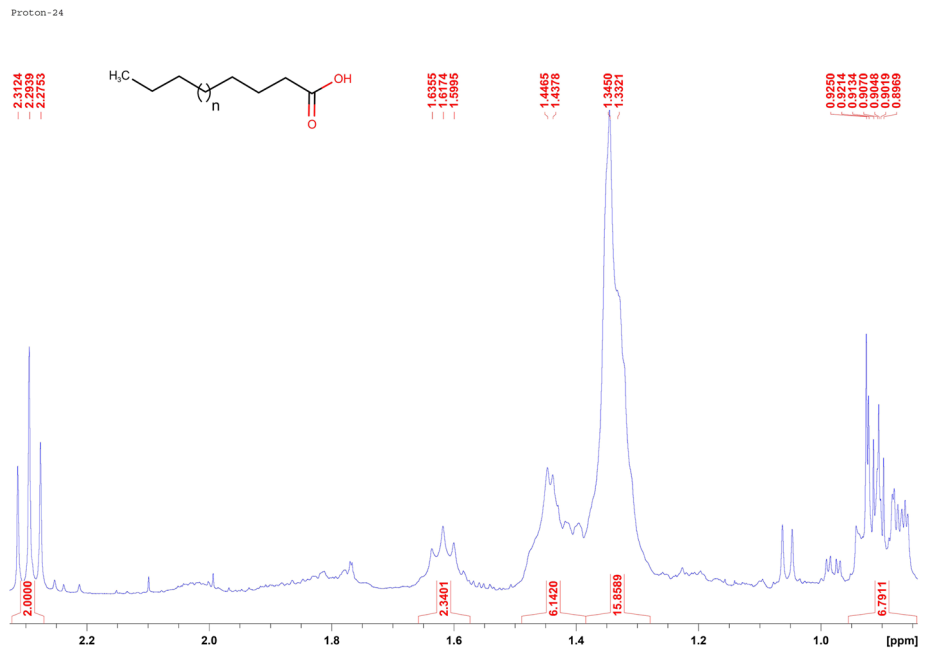

Fig. 6 ^{1}H NMR spectroscopy of the major compound in fraction 24 in MeOD

chain saturated FA was confirmed by comparing with literature [29].

On the other hand, the presence of a sugar/peptide moiety, which might be part of a glycolipid/lipoprotein as a minor compound in fraction 24, was indicated from the small integration of some protons that resonating at δ_H3.00–5.00 ppm (Fig. S4). Similarly, an aromatic compound was also detected as a minor compound in fraction 24 because the integration of the protons resonating at δ_H 6.28–7.75 ppm was minimal compared with those of the major saturated FA. The two minor compounds were suggested to be either an FA derivative (glycolipid/lipoprotein) with another minor aromatic compound or only one minor compound containing both the sugar/peptide moiety and the aromatic moiety, which might be a glycolipid/lipoprotein linked to an aromatic moiety.

4 Discussion

We isolated an actinomycete (*Streptomyces* sp. MS. 10) with broad-spectrum antimicrobial activity and chemically isolated bioactive compounds from its crude extract, thereby achieving our study aim. The isolation of a broad-spectrum actinomycete from a soil sample confirmed the richness of the soil niche with bioactive actinomycetes [16].

Streptomyces sp. MS. 10 was molecularly identified as *Streptomyces* sp. due to the high similarity of its 16S rRNA gene sequence with different species of *Streptomyces*, confirming the findings of an old report that 16S rRNA sequencing was insufficient to assign the phylogeny of the closely related species [30]. On the other hand, another study conducted in 2004 reported that 16S rRNA gene sequencing could be useful for phylogenetic identification of some strains at the species level but not others [31].

After HPLC fractionation of the crude extract from the small-scale fermentation experiment of *Streptomyces* sp. MS. 10, LC-MS analysis of the bioactive fraction 14 showed many major ion peaks, although it was expected that only one or very few major ion peaks would be detected in its mass spectrum. This suggested low ionization of the major compound(s) in this fraction, which was restated by highly ionized minor compounds also appearing as major peaks (Fig. 2). A similar strategy to isolate and identify natural microbial products using HPLC and LC-MS was followed earlier [16]; however, they used NMR spectroscopy to completely identify the isolated bioactive compounds, which was not applicable in our study due to the low dry weight of the isolated compounds. Therefore, we optimized the culture conditions for the production of the bioactive metabolites and subsequently upscaled the *Streptomyces* sp. MS. 10 fermentation experiment to produce higher amounts of the targeted antimicrobial compounds prior to their chromatographic separation using column chromatography.

The optimization results showed that ISP4 broth was the best culture medium among the tested media for the optimal production of antimicrobial agents by *Streptomyces* sp. MS. 10. Similarly, ISP4 broth supplemented with 0.4% yeast extract has been used for the production of many bioactive compounds by *Streptomyces* sp. [32]. Notably, another study compared the use of ISP4 broth for antibiotic production against ISP2 broth, starch-nitrate broth, and tryptone-yeast extract-glucose broth, revealing that ISP4 broth was the best fermentation broth among the tested culture media [33]. Replacing starch as carbon source and ammonium sulfate as the nitrogen source in ISP4 broth with different carbon and nitrogen sources, respectively, showed the importance of these feeding experiments because the bioactivity of *Streptomyces* sp. MS. 10 in terms of the size of the zone of inhibition was greatly affected, as mentioned earlier. A study has reported that the maximum expression of some antibiotics was achieved using glucose (monosaccharide) and lactose (disaccharide) as carbon sources [34], while another study showed that ammonium nitrate and soya meal were the best nitrogen sources in terms of antimicrobial activity [35], but this did not include the testing of casein. Optimization of the best solvent for extracting the major bioactive metabolites in the current study revealed that DCM (1:1, v/v) was the best to extract the major antimicrobial agents. Another study that used different solvents (1:1, v/v), including EtOAc, methanol, and chloroform, to extract the bioactive compounds and compared the zones of inhibition of the solvent extracts revealed that EtOAc was the best among the tested solvents [36], which partially matches our findings that EtOAc was the second-best solvent after DCM, which was not tested in this previous study.

Characterization of the major bioactive compounds was previously described in old reports using different methods of characterization [35, 37, 38]. The use of different methods could help to more comprehensively identify the nature of the major bioactive compounds for subsequent chemical isolation. The stability of the major bioactive metabolites of *Streptomyces* sp. MS. 10 against different enzymes and at temperatures of up to 80 °C demonstrated that its major bioactive compounds were neither peptides nor starchy compounds. This was supported by the detection of genes encoding PKS I and PKS II, suggesting the presence of polyketide secondary metabolites

among the natural products yielded by *Streptomyces* sp. MS. 10 [39], while the absence of the NRPS gene suggested that peptides were not the main bioactive compounds. Interestingly, both PKS I and PKS II have been detected in *Streptomyces* sp. [39, 40], while in another study, only PKS II was detected in the genomic DNA of *Streptomyces* sp. AGM12-1 [16].

After scaling-up *Streptomyces* sp. MS. 10 fermentation to 10 L using the pre-optimized culture and extraction conditions, we followed an earlier strategy of separation and characterization of the bioactive compounds using column chromatography followed by spectroscopic analysis [35]. Interestingly, the collected fractions that showed the highest antimicrobial activity were predicted to be fatty compounds or compounds with lipid moieties because they were eluted with solvent proportions of more than 90:10 (DCM: methanol). This was confirmed by structural elucidation of the isolated bioactive compounds using ^{1}H NMR spectroscopy, which showed the presence of two FAs as the major bioactive compounds with a minor FA derivative. Indeed, the absence of an effect of protease enzymes on the total antimicrobial activity of *Streptomyces* sp. MS. 10 and the failure to detect the NRPS gene using PCR amplification supported the idea that peptides were not the major bioactive compounds in its crude extract. Thus, it was supposed that the detected minor FA derivative was a glycolipid rather than a lipoprotein. It was also suggested that this supposed glycolipid had a small sugar moiety because amylase did not affect the total antimicrobial activity of *Streptomyces* sp. MS. 10 and because of the small integration of the protons of this moiety in the ^{1}H NMR spectrum.

The presence of FAs and their derivatives as major bioactive compounds in the crude extract of *Streptomyces* sp. MS. 10 may explain the reason behind the difficulty in determining the ion peaks of the major bioactive compounds from the small-scale fermentation experiment using LC-MS because many of the FAs do not ionize well using electrospray ionization prior to their mass detection. Therefore, other minor compounds, which could possibly be highly ionized, appeared as major compounds besides the true major compounds. Different FAs and their derivatives, including monohydroxy FAs [41], polyhydroxy FAs [42], and keto acids [43], have been isolated from *Streptomyces*.

Furthermore, FAs have been reported to have antibacterial activity, including activity against *Staphylococcus epidermidis* and *E. coli* [44], as well as activity against *S. aureus* [45]. Interestingly, two hydroxylated FAs similar to the major compound in fraction 8 showed antibacterial activity against *Bacillus subtilis*, *Micrococcus luteus*, and *Staphylococcus aureus* although these FAs were isolated from plant origin [27]. Additionally, highlighting the importance of the FAs produced by actinobacteria, this group of compounds were mostly identified as constituents of more complex compounds in actinomycetes, such as the lipopeptide antibiotic complex A21978 C, which is produced by *Streptomyces roseosporus* [46], and amphomycin, another lipopeptide antibiotic [47]. Furthermore, approximately 50% of the simple microbial glycolipids are produced by actinobacteria [48], and some glycolipids show antimicrobial activity [49].

5 Conclusions

The present study suggested the importance of the bioassay-guided approach in the chromatographic separation of major bioactive metabolites in crude microbial extracts. Furthermore, we proved that the soil niche is still a good source of actinomycetes that produce bioactive secondary metabolites. Moreover, PCR screening of the genes responsible for antibiotic biosynthesis and the physicochemical characterization of the total antimicrobial activity could help to predict, target, and subsequently, isolate the major bioactive compounds from crude microbial extracts.

6 Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s43088-021-00099-7.

Additional file 1: Figure S1. ^{1}H-^{1}H COSY spectrum for the major compound in fraction 8, measured in MeOD. **Figure S2.** ^{1}H-^{13}C HMBC spectrum for the major compound in fraction 8, measured in MeOD. **Figure S3.** ^{1}H-^{13}C HSQC spectrum for the major compound in fraction 8, measured in MeOD. **Figure S4.** Full ^{1}H NMR spectrum of fraction 24, measured in MeOD. **Figure S5.** ^{1}H-^{1}H COSY spectrum for the major compounds in fraction 24, measured in MeOD. **Figure S6.** ^{1}H-^{13}C HMBC spectrum for the major compound in fraction 24, measured in MeOD. **Figure S7.** ^{1}H-^{13}C HSQC spectrum for the major compounds in fraction 24, measured in MeOD.

Abbreviations

^{1}H NMR: Proton nuclear magnetic resonance; ACN: Acetonitrile; Bp: Base pair; COSY: Correlated spectroscopy; DCM: Dichloromethane; EtOAc: Ethyl acetate; FA: Fatty acid; HMBC: Heteronuclear multiple bond correlation; HPLC: High-performance liquid chromatography; HSQC: Heteronuclear single quantum coherence; ISP: International Streptomyces Project; LC-MS: Liquid chromatography-mass spectrometry; MRSA: Methicillin-resistant *Staphylococcus aureus*; MeOD: Deuterated methanol; *m/z*: Mass-to-charge ratio; NCBI: National Center for Biotechnology Information; NRPS: Non-ribosomal peptide synthetase; PCR: Polymerase chain reaction; PKS: Polyketide synthase; R_t: Retention time; TLC: Thin-layer chromatography; TSB: Tryptone soya broth

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Authors' contributions

All microbiology experiments were designed by AOE and revised by AES, WB, and SA. All chromatographic separation experiments and spectroscopic analysis in the study were designed by ASM. Isolates collection and purification, 16S rRNA sequences analysis, and LC-MS sample submission

were carried out by MS and AOE, while the biological activity, the chromatographic separation work, and the NMR data processing were performed by MS. All project's results were interpenetrated by MS and AOE. This research project was supervised by AES, SA, ASM, WB, and AOE. The initial draft of this manuscript was written by MS and revised by SA, ASM, WB, AOE, and AES prior to its submission. The author(s) read and approved the final manuscript.

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Availability of data and materials
The 16S rRNA gene sequence of *Streptomyces* sp. MS. 10 was submitted to NCBI Genbank under accession number MN148619.

Ethics approval and consent to participate
This article does not contain any experiment done on either humans or animals.

Consent for publication
Not applicable.

Competing interests
The authors declare no conflict of interest.

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