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RESEARCH

Antimicrobial activities and beta-lactamase inhibitory property of actinomycetes from Atlas forest soils in Northeastern Algeria

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Abstract

Actinomycetes bacteria are an inexhaustible natural source of secondary metabolites with diverse antimicrobial activities. In the current study, screening based on antimicrobial activity of the cell-free supernatant (CFS) of 23 actinomycetes isolates from Atlas forest soils in Northeastern Algeria was performed against ten human bacterial pathogens and *Candida albicans* ATCC 10231 strain. Among them, three isolates AM138DZ, AM141DZ and AM183DZ showed antagonistic effects towards indicator pathogens. The isolate AM183AZ exhibited strong activities against *Staphylococcus aureus* ATCC 6538, *C. albicans* ATCC 10231, clinical strains *Esherichia coli* BLSE and *Salmonella* spp. BLSE. The isolates AM141AZ and AM138AZ displayed high antimicrobial activity towards *C. albicans* 10231. Interestingly, the CFS of AM138DZ and AM141DZ combined with amoxicillin inhibited β-lactamase activity from *Klebsiella pneumoniae* ATCC 700603, an extended-spectrum β-lactamase producing strain. The ethyl acetate extracts of the three isolates displayed a large spectrum of antimicrobial activity against all pathogens tested. Analysis of their contents by UPLC-ESI-MS/MS revealed the identification of 16 to 18 compounds, among them 5 flavonoids, 6 organic acids and 2 phenols. Overall, flavonoids (such as rutin, chrysin, quercetin and catechin) were the major compounds detected in the three extracts. Depending on the isolate, other notable compounds were detected at significant levels: β-caroten, salicin and salycilic acid. Molecular identification of the three isolates using 16S rRNA sequence homology suggested that these isolates should be assigned as *Streptomyces flavogriseus* AM138DZ, *Streptomyces felleus* AM141DZ, and *Streptomyces rubiginosohelvolus* AM183DZ strains. Our study provides a promising natural alternative source of antimicrobials and β-lactamase inhibitors in particular.

Keywords Actinomycetes · Antimicrobials · B-lactamase inhibitors · Soil

Introduction

The alarming rise and pervasiveness of antimicrobial resistance (AMR) has become a substantial threat to the current therapeutic word and to the global public health, leading to higher morbidity and mortality rates in humans. According to a comprehensive analysis of 204 countries and territories, published in *The Lancet*, it was estimated that 4.95 million fatalities were associated with bacterial AMR in 2019, of which 1.27 million were attributable to bacterial AMR (Antimicrobial Resistance Collaborators 2022). Hence, there is a dire need to develop potential and viable antimicrobial strategies to minimize AMR. This situation has compelled scientists and encouraged pharmaceutical companies, which have abandoned their antibiotic research and development programmes for many years, to renew search for novel antimicrobials (Dutescu and Hillier 2021).

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Natural bio-resources are reputed to produce secondary metabolites, which have significantly contributed to drug discovery and pharmacotherapy, especially for infectious diseases and cancer. Microorganisms are of considerable importance as a promising and inexhaustible source of a great variety and huge number of bioactive compounds (Pham et al. 2019; Schneider 2021). In this regard, actinomycetes, widely recognized for their metabolic versatility, are of a great importance as producers of structurally and functionally diverse bioactive compounds that cover more than one-fourth of the natural products from microbial origin (Ossai et al. 2022). In particular, they are considered as the richest source of the naturally occurring antimicrobial agents and produce approximately 55% of all established antibiotics (Ngamcharungchit et al. 2023). Around 80% of all actinobactirial antibiotics, such as cephalosporins, carbapenems, monobactams, β-lactamase-inhibitors (clavulanic acid and olivanic acid), glycopeptides, aminoglycosides, macrolides, tetracyclines, and rifamycins, were identified from *Streptomyces* which still continue to be the biggest producer of the world's clinical antibiotics and a valuable resource for new bioactive compounds (De Simeis and Serra 2021). Recent advancements in genome sequencing and bioinformatics analysis revealed that actinomycetes genomes possess a large number of unexplored silent secondary metabolite biosynthetic gene clusters (Lee et al. 2020).

Actinomycetota (formerly known as of Actinobacteria), one of the largest bacteria phyla, are Gram-positive bacteria with high guanine-cytosine (G + C) content in their genomes (Barka et al. 2015; Oren and Garrity 2021). *Streptomyces* represent the largest genus of this phylum. They exhibit a variety of morphological features, differing mainly with respect to the absence or presence of a substrate mycelium or aerial mycelium, the appearance and color of the mycelium, the production of melanoid pigments, the surface characteristics and shape of their spores (Barka et al. 2015). Actinomycetes, widely distributed in various terrestrial and aquatic ecosystems around the globe including soil, plants, animals, marine, freshwater and extreme environments, are typically dominant bacteria in soil, where they are present at densities on the range of 10^6 to 10^9 cells per gram of soil (Ngamcharungchit et al. 2023; Meenakshi et al. 2024). Soil ecosystems are important reservoirs of bacterial diversity (Anthony et al. 2023). Meta-analysis and Omics technologies, which have increasingly been used in recent years, allowed the evaluation of soil microbial phylogenetic diversity and functional information (Bertola et al. 2021; Labouyrie et al. 2023). Nevertheless, this ecosystem remains largely unexplored and unexploited source for actinomycetes with the potential to produce biologically active secondary metabolites.

Algeria has a multitude of natural ecosystems with a particular typology and ecology in the world, in terms of biodiversity and functional role. However, these very original and diverse habitats are still uncharted to evaluate their biological resources especially in terms of microbial diversity, and in particular only limited information on the autochthonous actinomycetale microbiota is available. While several studies dealing with the isolation and metabolic profile investigation of actinobacteria from Saharan soils in Algeria, few ones investigated biologically active actinobacteria from Algerian forest soils (Djinni et al. 2019; Ayari et al. 2012, 2016). In this context, the aims of the present work were to explore the diversity of actinobacteria from Algerian Atlas forest soil in the region of Souk Ahras, north-east Algeria and to investigate their antimicrobial potential.

Materials and methods

Sample collection

Soil samples were collected, between 2020 and 2021, from three different sites of Ain El Morra of the Atlas Mountains, Ouillen Commune in the province of Souk-Ahras, northeastern Algeria (36°20'44"N, 8°08'45"E). Soils were sampled at 20 cm depth and transferred to sterile flask sealed tightly (Messaoudi et al. 2015). All samples were transported to the laboratory in ice box.

Isolation of actinomycetes

Soil samples were air-dried at room temperature and sieved to remove stones and plant debris. The isolation of actinomycetes was carried out according to the dilution plate method as previously described by (Messaoudi et al. 2015). Firstly, 10 gr soil samples were pre-treated with 1 gr of calcium carbonate for 7 to 9 days at 40 C° in order to stimulate the development of actinomycetes belonging to different genera and to reduce the fungal flora. One gram of pre-treated samples was then suspended in 9 mL of sterile physiological saline solution (9 g/L NaCl). Afterwards, serial 10-fold dilutions were prepared for each suspension (10^{-1} to 10^{-4}). Aliquots (0.4 mL) of each dilution were spread onto selective media (pH 7.2) prepared as previously described: International Streptomyces Project ISP2 (Pridham et al. 1956), ISP4 (Küster 1959), GAUSS (Gauss 1958), and CZAPEK (Czapek 1902). Plates were incubated at 28 ± 2 °C for 7–21 days. Colonies showing typical actinomycetes morphologies were repeatedly subcultured on ISP2 media to obtain clonal isolates. Pure colonies of actinomycetes were stored in slanted ISP2 agar tubes.

Morphological characterization

Actinomycetes isolates were inoculated on six different agar media (ISP1, ISP3, ISP4, ISP5, ISP6 and ISP7) at 28 °C ± 2 for 7 days to access the morphological characteristics as described previously (Shirling and Gottlieb 1966). The colonies were observed under a high-power magnifying lens. They were examined with respect to color, form, aerial spore mass, substrate mycelia, and the pigment production (e.g. secondary metabolites). The isolates were subjected to Gram staining by conventional method to identify Gram-positive bacteria.

Evaluation of antimicrobial activities

Fermentation

Actinomycetes isolates, which showed high production of secondary metabolites on ISP2 compared to other media, were selected for fermentation by the submerged state culture according to the method of Badji et al. (2006) with few modifications. A scrape of a colony's isolate was inoculated into 50 mL of ISP2 broth (pH 7.2) and incubated for 3 days at 28 °C ± 2 with 180 rpm shaker speed (Edmund Buhler GmbH, Germany). One mL of the bacterial pre-culture was then poured in 100 mL of ISP2 broth and incubated for 7 days at 28 °C ± 2 with shaking speed of 200 rpm. After complete fermentation, cell-free supernatant (CFS) was collected by centrifugation (45000 xg, 4 °C, 40 min) (SIGMA 3-16KL, Germany) and stored at -20 °C for further application.

Screening for antimicrobial activity

The antibacterial activity of actinomycetes isolates was determined against indicator pathogenic bacteria and fungi. Strains of *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas aeruginosa* ATCC 9027, extended-spectrum β-lactamase (ESBL)-producing *K. pneumoniae* ATCC 700603, *Klebsiella pneumoniae* ATCC 13883, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633, *Listeria innocua* CLIP ATCC 74915, and *Candida albicans* ATCC 10231 were obtained from the American Type Culture Collection, and clinical strains of *E. coli* BLSE and *Salmonella* spp. BLSE were isolates at the Biological Laboratory of Ibn Roched Hospital (Souk-Ahras, Algeria). The antimicrobial activity of the actinomycetes isolates was performed according to the agar well diffusion method as previously described by Tung et al. (2022). The indicator strains were cultured in nutrient agar medium (HiMedia, India) at 37 °C for 18 h then enriched in sterile physiological saline solution (0.9% NaCl) and adjusted to 0.5 McFarland standard. Then, they were streaked on Mueller-Hinton (MH) agar plate. Afterwards, wells of 8 mm were bored keeping a distance between them of 25 mm, and 70 µL of each CFS from actinomycetes isolate was poured into wells. The plates were placed at 4 °C for 2 h to allow diffusion of the CFS, then incubated at 37 °C for 18 h. Antibacterial activity against the indicator pathogens was ascertained by measuring the size of the inhibition zone (IZ) around the well.

Extraction of antimicrobial metabolites

CFS of actinomycetes isolate was subjected to solvent extraction for recovery of antimicrobial metabolites using two different organic solvents ethyl acetate and n-hexane (Badji et al. 2006). In brief, the CFS was extracted with the organic solvents at ration of 1:1 and vigorously shaken twice for 30 min. The mixture was left undisturbed for 1 h to get separated phases. The organic phase (e.g. the upper layer), was collected and the solvent was removed using a rotary evaporator at 40 °C for 15 min. The residue obtained was weighted and dissolved either in DMSO or in methanol. Meanwhile, the aqueous phase was kept dried at 45 °C for 4 days. The residue obtained was also weighted and dissolved either in DMSO or in methanol.

The three resulting extracts (e.g. ethyl acetate, n-hexane and aqueous extracts) were assessed for antimicrobial activity as described above. The best one was selected for further assays. DMSO was placed in the wells as negative control.

Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of the organic extract was determined by the broth microdilution method in MH broth (Tung et al. 2022) against the indicator pathogens using 96-well microplates. Briefly, the ethyl acetate extracts were serially diluted with DMSO to reach concentrations ranging from 0.005 mg/mL to 5 mg/mL. Afterward, 100 µL of each concentration was added to 100 µL of MH broth inoculated with 100 µL of a standardized suspension of the tested pathogen. Wells without extract was served as negative control. Plates were then incubated at 37 °C for 48 h. The MIC was determined as the lowest concentration of extract that inhibited the growth of the test microorganism.

Stability study of antimicrobial metabolites

Temperature and pH stability assay

Temperature and pH stability of the antimicrobial metabolites in the actinomycetes ethyl acetate extract was performed according to the procedure of Badji et al. (2006) with slight modifications. Firstly, the sample was aliquoted

into five test tubes. Then, two of them were exposed to various temperatures (4 °C for one week, and 110 °C for 30 min) and cooled to room temperature for 10 min. The remaining three aliquots were adjusted to pHs of 3, 7 and 11 (using 0.1 M HCl or 0.1 M NaOH) and then they were neutralized. Afterwards, the treated extracts were evaluated for antimicrobial activity against indicator pathogens using the agar well diffusion assay as described above.

Synergistic assay for β-lactamase Inhibition activity

In order to check the β-lactamase inhibitory effect of the CFS, synergistic assay against the ESBL-producing *K. pneumoniae* ATCC 700603 strain was performed according to the disk diffusion susceptibility method. The indicator strain was cultured in nutrient agar medium at 37 °C for 18 h, then enriched in sterile physiological saline solution (0.9% NaCl) and adjusted to 0.5 McFarland standard. An inoculum of the bacterial suspension was seeded on MH agar plates and left to dry at room temperature for 5 min. Thereafter, discs of β-lactam antibiotic amoxicillin (30 µg) (Bio-Rad, Hercules, CA, USA) was soaked in CFS until saturated (referred here as combined disc). The discs were air dried at room temperature for 20 min to allow the bioactive molecules adsorb to the discs under a microbiological hood. Disc of amoxicillin (20 µg)/clavulanic acid (10 µg) (Bio-Rad, Hercules, CA, USA) was used as positive controls. The impregnated discs were then carefully placed onto the inoculated agar plates. The synergistic assay was also performed on organic extracts according to the agar well diffusion method as described above. Aliquots of 50 µL extract (10 mg/mL) and 50 µL amoxicillin (25 µg/mL) mixture, and 10 µL of amoxicillin (25 µg/mL) were added to respective wells. DMSO was added as negative control, and combination of clavulanic acid/amoxicillin was included as positive controls. The plates were incubated at 37 °C for 18 h. Each experiment was performed in duplicate.

The diameter of the inhibition zone (IZ) around each combined disc was measured and compared to that of the antibiotic alone disc (Arora and Nandi 2017).

Total polyphenol content determination

The total polyphenol content in the organic extract was determined by spectrophotometry using the colorimetric method with Folin-Ciocalteu reagent as described by Singleton and Rossi (1965) with some modifications. Briefly, 200 µL of each extract at a concentration of 1 mg/mL dissolved in methanol was mixed with 1 mL of 10% Folin-Ciocalteu reagent. Subsequently, 800 µL of 7.5% Na_2CO_3 was added and the total volume was made up to 2 mL. The mixture was shaken and left for 30 min at room temperature. Then, the absorbance was measured at 765 nm against a blank without extract. A calibration curve was prepared simultaneously under the same conditions using gallic acid at various concentrations (0 to 200 µg/mL). The outcome data were expressed as milligrams of gallic acid equivalents per gram of dry extract weight (mg GAE/g DW).

Total flavonoid content determination

The flavonoid contents of organic and aqueous extracts were measured as described by Zhishen et al. (1999) with some modifications. An aliquot of 400 µL of each extract at a concentration of 1 mg/mL dissolved in pure methanol was mixed with 120 µL of 5% $NaNO_2$. A volume of 120 µL of 10% $AlCl_3$ was added 5 min later, and the solution was vigorously mixed. After 6 min, 800 µL of 1 M NaOH was added to the mixture and the total volume was made up to 1440 µL. Absorbance was immediately read at 510 nm against a blank without extract. Quercetin (0 to 500 µg/mL) was used as the standard for a calibration curve. The flavonoid content of the extract was expressed as milligrams of quercetin equivalents per gram of dry extract weight (mg QE/g DW).

Ultra-performance liquid chromatography-electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS) analysis

Ethyl acetate extracts of actinomycetes isolates were further explored for bioactive compounds through LC-MS/MS method. Firstly, they were dissolved in methanol (LC grade), filtered through a 0.2 µm syringe filter and then analyzed. The analysis was performed using UPLC-ESI-MS-MS Shimadzu 8040 Ultra-High sensitivity with UFMS technology equipped with binary bump Nexera XR LC-20AD. Separation was achieved with an Ultra-force C18 column (I.D. 2.1 mm x 150 mm, 3 µm particle size; Restek) at 25 °C oven temperature. The chromatographic separation was carried out using a mixture of 30% (water, 0.1% formic acid) as mobile phase A and 70% methanol as mobile phase B. The duration of gradient elution was applied as: 0 min to 1 min A 85%; 1 min to 10 min A 5%; 10 min to 18 min A 5%; 18 min to 23 min A 85%. The flow rate was set at 0.2 mL/min. The injection volume was 5 µL, passed through a Millex-LCR (PTFE) filter with 0.22 µm pore sizes. The separation was performed at room temperature, while the run lasted for 60 min. The ESI conditions employed in the MS/MS were as follows: CID gas, 230 KPs; conversion dynode, −6.00 Kv◦C; DL temperature, 250 °C; nebulizing gas flow, 3.00 L/min; heat block, 400 °C; drying gas flow, 10 L/min. The ion trap mass spectrometer was operated in both negative and positive ions with multiple reactions monitoring (MRM)

mode. Compounds were accurately identified according to their typical fragments and were further validated by comparison with standards stored in the established database.

Biochemical characterization and molecular identification

Biochemical characterization

Biochemical characterization of isolates was investigated using 50CHB and ZYM strips tests in accordance with the manufacturer's instructions (bioMérieux, SA, Marcy-l'Etoile, France).

PCR amplification and cloning of 16S rRNA gene

The polymerase chain reaction (PCR) amplification of the 16S rRNA gene was carried out using two universal primers designed from the conserved zones within the rRNA operon of *E. coli* (Gürtler and Stanisich 1996). The forward primer (27F) 5'-AGAGTTTGATCCTGGCTCAG-3' extended from base position 8 to 27; the reverse one (1525R) 5'-AAGGAGGTGATCCAAGCC-3' extended from base position 1541 to 1525. The genomic DNA was purified using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA). PCR reaction was performed in a final volume of 100 µL using 2 μL DNA (~ 300 ng), 10 pM per primer, 10 pM dNTPs, 25 mM $MgCl_2$ and (2 U) Taq polymerase using Thechne thermal cycler. Cycling conditions were as follows: an initial step denaturation of 5 min at 94 °C, 40 cycles of denaturation at 94 °C for 45 s, annealing at 60 °C for 90 s, and extension at 72 °C for 60 s extension; and a final extension step at 72 °C for 10 min. The amplified ~ 1.5 kb PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA). The *E. coli* DH5α (F^-*supE44 Φ80 δlacZ ΔM15 Δ(lacZYA-argF) U169 endA1 recA1 hsdR17* (r_k^-, m_k^+) *deoR thi-1* λ^-*gyrA96 relA1*) (Invitrogen, Carlsbad, CA, USA) was used as a host strain. All recombinant clones of *E. coli* were grown in Luria-Bertani broth media with the addition of ampicillin (100 µg/mL), isopropyl-thio-β-D-galactopyranoside (IPTG) (0.67 mM) and X-gal (360 µg/mL) for screening. DNA electrophoresis, DNA purification, restriction, ligation, and transformation were all performed according to the method previously described elsewhere (Sambrook et al. 1989).

DNA sequencing and molecular phylogenetic analysis

The nucleotide sequences of the cloned 16S rRNA genes were performed at least three times on both strands using BigDye Terminator cycle sequencing ready reaction kits and the automated DNA sequencer ABI PRISM® 3100-Avant Genetic Analyser (Applied Biosystems, Foster City, CA, USA). All sequencing data were assembled using the STADEN (version 4.5; http://www.mrclmb.cam.ac.uk/pubseq) and DNASTAR (DNASTAR Inc., Madison, WI, US) software packages. Multiple nucleotide sequence alignment was performed using the BioEdit version 7.0.2 software program. The nucleotide sequences data were analyzed using the Softberry Gene Finding tool (http://linux1.softberry.com/berry.phtml).

The sequences obtained were compared with sequences present in the public sequence databases and with the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/), a web-based tool for the identification of prokaryotes based on 16S rRNA gene sequences from type strains. Additionally, the 16S rRNA sequence analysis was performed using the Sequence Match software of the Ribosomal Database Project II (http://rdp.cme.msu.edu/html) and BLAST search against sequences in the GenBank non-redundant nucleotide database (http://www.ncbi.nlm.nih.gov). Sequences were also analyzed using ARB software package (http://www.arb-home.de). All sequences having more than 1200 nucleotides were imported into ARB database and automatically aligned with the existing 16S rDNA sequences. The resulting alignments were manually checked and corrected when necessary. Phylogenetic and molecular evolutionary analyses were conducted via the molecular evolutionary genetics analysis (MEGA) software version 4.1 (http://www.megasoftware.net.). Distances and clustering were calculated using the neighbor-joining DNA distance algorithm and the Jukes-Cantor correction model available in the ARB database. Bootstrap analysis was used to assess the tree topology of the neighbor-joining data by performing 100 re-samplings.

Results

Identification and morphological characterization of actinomycetes

Different selective culture media were used leading to the isolation of 36 presumed actinomycetes strains from the three soil samples. Their growth rate varied considerably from high to moderate and slow, depending on the isolate and the medium. The cultural and physiological features of the isolates were determined according to the International Streptomyces Project (ISP2). They were described based on colonial morphology onto ISP2 medium, appearance of aerial hyphae, substrate mycelia, pigment production, and spore morphology. The isolates, which were positive in Gram staining, exhibited variable morphological and

microscopic characteristics (Fig. 1). As outlined in Table 1, the colonies were circular-shaped, lobulated or irregular. They showed well-developed aerial mycelium that appeared powdery (8 isolates; 22.2%), smooth (1; 2.7%), granular (23; 63.8%), floccose (4; 11.1%). Also, the isolates produced colorful colonies with gray (20; 55.5%), white (8; 22.2%), grayish-white (3; 8.3%), whitish gray (2; 5.5%), greenish blue (1; 2.8%), reddish brown (1; 2.8%) and beige (1; 2.8%). Distinctive colors of the substrate mycelium were recorded: brown (16; 44.4%), yellowish brown (7; 19.4%), beige (5; 13.8%), yellow orange (2; 5.6%), brownish gray (1; 2.7%) orange (1; 2.7%), whitish gray (1; 2.7%), gray (1; 2.7%), yellow (1; 2.7%) and reddish-brown (1; 2.7%).

Morphological spore characteristics are important criteria for actinomycetes identification. Thirty three isolates produced spores arranged singly (9 isolates), in short to long chains 23, or as free spores/chains (1). The morphology of the spore chains, observed under light microscopy, varied depending on the isolate and was categorized as: spiral looped and hooked (2 isolates; 5.6%), hooked and looped (6; 16.6%), straight (8; 22.2%), both straight and hooked and looped (7; 19.44) and filaments (16; 44.4%).

Pigment production, another key feature for actinomycetes characterization, was also considered. Seven isolates produced variable shades of diffusible pigments in the surrounding medium. Interestingly, 6 isolates were melanoid-pigment producers. On the other hand, neither isolate was able to produce exudates on the surface of the colony.

Among the 36 presumptive actinomycetes isolates, 23 with characteristic features, such as melanoid pigment production, the aerial mycelium appearance, and the colors of the mycelium were selected for antimicrobial assay.

Antimicrobial activity

The 23 selected isolates were assessed for antimicrobial activities against 11 pathogenic strains. The CFSs of 3 actinomycetes isolates AM138DZ, AM141DZ, and AM183DZ showed varying spectrum and degrees of antagonistic effects toward the indicator pathogens (Fig. 2), whereas no inhibition of pathogen growth was detected by the remaining 20 CFSs. The isolate AM183DZ exhibited strong activity (IZs ranging from 16.5 mm to 22 mm) against *E. coli* BLSE, *Salmonella* spp. BLSE, *S. aureus* ATCC 6538 and *C. albicans* ATCC 10231 strains. Also, the isolate AM183DZ showed a weak activity against *P. aeruginosa* ATCC 27853 (IZ 10 mm). On the other hand, the isolates AM141DZ and AM138DZ exhibited strong antimicrobial activity against only *C. albicans* ATCC 10231 (IZs 18 mm ± 1 and 22 mm ± 1, respectively) and weak activity against *E. coli* (IZs 9.5 mm ± 0.5 and 10 mm ± 1, respectively) while they failed to inhibit the growth of *P. aeruginosa* ATCC 9027, *K. pneumonia* ATCC 700603, *E. coli* ATCC 25922, *K. pneumoniae* ATCC 13883, *B. subtilis* ATCC 6633 and *L. innocua* CLIP 74915.

Interestingly, our results on the antimicrobial activity of organic extracts of the three isolates revealed that ethyl acetate extracts displayed the largest spectrum of inhibitory activity including all tested pathogens as compared to n-hexane and water extracts, which showed antagonistic effect against two or five pathogens, respectively, depending on the actinomycetes isolate (data not shown). As shown in Fig. 3, strong inhibitory effect (IZs ≥ 16 mm) of the three ethyl acetate extracts was observed on *Salmonella* spp. BLSE and *E. coli* ATCC 25922. High antagonistic effect towards *K. pneumoniae* ATCC 700603 and *L. innocua* CLIP 74915 (IZs 16 mm ± 1 and 19 mm ± 1 respectively) was noted for the ethyl acetate extract of AM183DZ. Also, the growth of *L. innocua* CLIP 74915 strain was significantly inhibited (IZ 15 mm ± 1) by the ethyl acetate extract of AM141DZ. Compared to the CFSs, the ethyl acetate extracts of the three isolates showed enhanced antibacterial activity, with a few exceptions, but had lower antagonistic effect against *C. albicans* ATCC 10231.

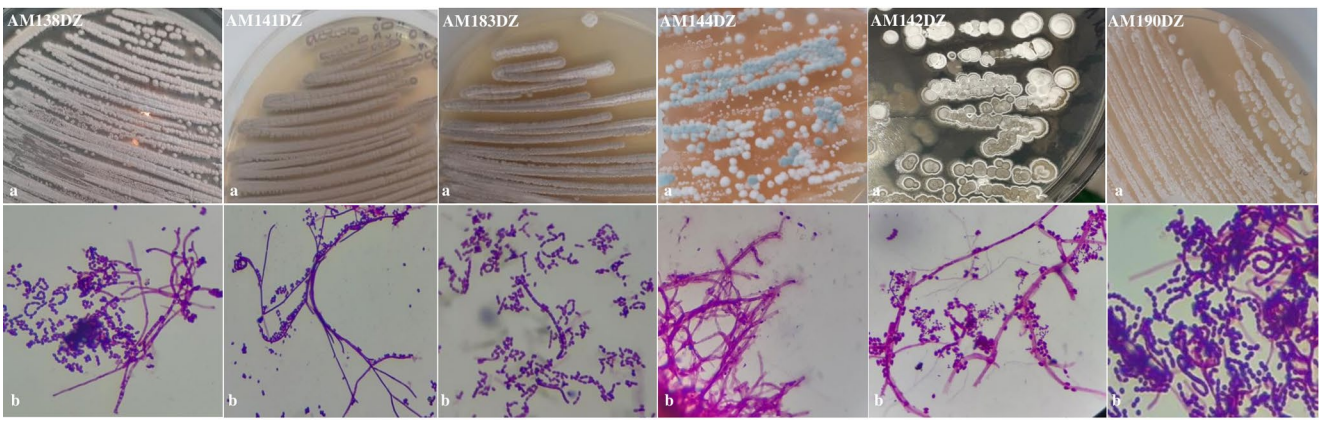

Fig. 1 Colony morphology (**a**) and microscopic observation (**b**) of some actynomycetes isolates AM138DZ, AM141DZ, AM183DZ, AM144DZ, AM142DZ, and AM190DZ

Table 1 Morphological characteristics of the 36 actinomycetes isolates after incubation on ISP2 for 7 days at 28±2 °C

Isolates	Colony morphology on ISP 2 agar medium			Microscopic morphology	
	AM	SM	Pigments	Filaments	Spores
AM168DZ	Gray	Brown	-	+	LHSSC
AM170DZ	Gray	Brownish gray	-	-	HLSC
AM171DZ	Gray	Gray	-	-	HLSC
AM172DZ	Gray	Brown	Light brown	-	HLSC
AM173DZ	Gray	Brown	Light brown	-	SSC / HLSC
AM174DZ	White	Yellowish orange	Beige	-	SSC / HLSC
AM175DZ	Grayish white	Brown	Reddish brown	-	SSC / HLSC
AM176DZ	Grayish white	Yellowish brown	-	-	HLSC
AM177DZ	Gray	Brown	Yellowish brown	-	SSC / HLSC
AM183DZ	Whitish gray	Whitish gray	-	-	SSC / HLSC
AM184DZ	Gray	Brown	-	-	SSC / HLSC
AM185DZ	Gray	Brown	-	-	SSC / HLSC
AM186DZ	Gray	Yellowish brown	-	+	LHSSC
AM137DZ	White	Beige	-	+	SSC
AM138DZ	Gray	Brown	-	+	SSC
AM139DZ	Gray	Brown		+	SSC
AM140DZ	Grayish white	Yellowish brown	-	+	Free spores
AM141DZ	Gray	Brown	-	+	HLSC
AM142DZ	White	Beige	-	+	Free spores
AM144DZ	Blue green	Orange	Yellowish Orange	+	Free spores
AM145DZ	White	Beige	-	-	SSC
AM146DZ	Gray	Brown	-	-	HLSC
AM147DZ	Gray	Brown	-	-	SSC
AM148DZ	Gray	Brown	-	+	Free spores
AM152DZ	Gray	Yellowish brown	-	+	-
AM153DZ	Gray	Brown	-	-	SSC
AM154DZ	Gray	Brown	-	-	SSC
AM155DZ	White	Yellowish orange	-	-	Free spores
AM86DZ	White	Yellow	-	-	Free spores
AM87DZ	Whitish gray	Yellowish brown	-	-	Free spores
AM89DZ	Gray	Yellowish brown	-	+	-
AM90DZ	White	Beige	-	+	Free spores/SSC
AM91DZ	Reddish brown	Reddish brown	Brown	+	Free spores
AM92DZ	White	Yellowish brown	-	+	-
AM93DZ	Beige	Beige	-	+	Free spores
AM94DZ	Gray	Brown	-	-	SSC

AM: Aerial mycelium, SM: Substrate mycelium, +: present, -: absent, LHSSC: Looped and hooked spiral spore chains, HLSC: Hooked and looped spore chains, SSC: Straight spore chains

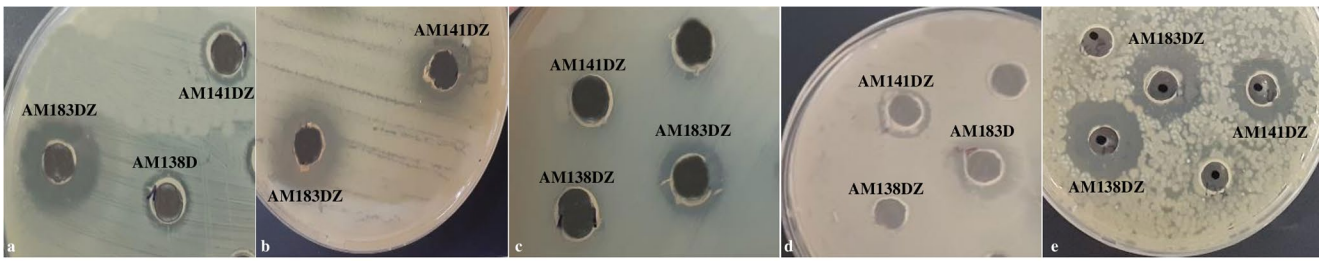

Fig. 2 Antimicrobial activity of the CFS of the three actinomycetes AM183DZ, AM141DZ, and AM183DZ using the agar well diffusion method against (**a**) *E. coli* BLSE (AM183DZ IZ of 18.5 mm±1, AM141DZ 9.5 mm±0.5, and AM138DZ 10 mm±1); (**b**) *Salmonella* spp. BLSE (AM183DZ 20 mm±0 and AM141DZ 15 mm±0); (**c**) *P. aeruginosa* ATCC 27853 (AM183DZ 10 mm±0); (**d**) *S. aureus* ATCC 6538 (AM183DZ 16.5 mm±0.5 and AM141DZ 14.5 mm±0.5); (**e**) *C. albicans* ATCC 10231 (AM183DZ 22 mm±1; AM138DZ 22 mm±1 and AM141DZ 18 mm±1)

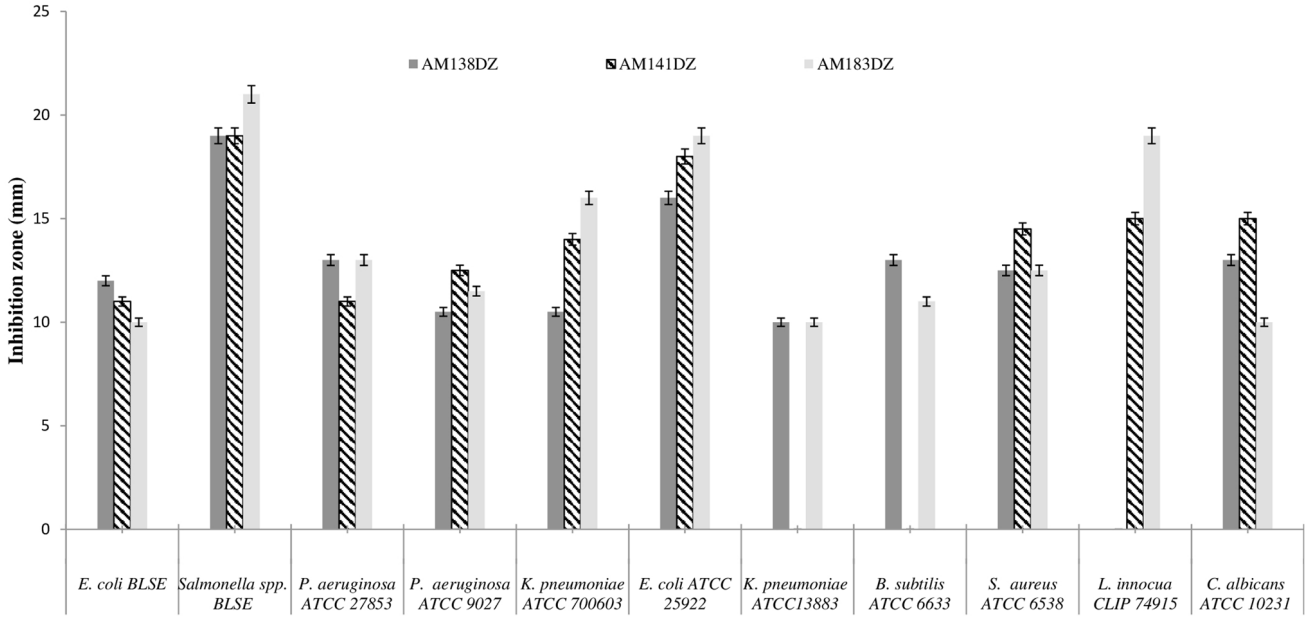

Fig. 3 Antibacterial activity of the ethyl acetate extracts from actinomycetes AM138DZ, AM141DZ and AM186DZ isolates against 11 human pathogens. Error bars represent the standard deviation (SD) of two independent replicates ±.S.D, n=2

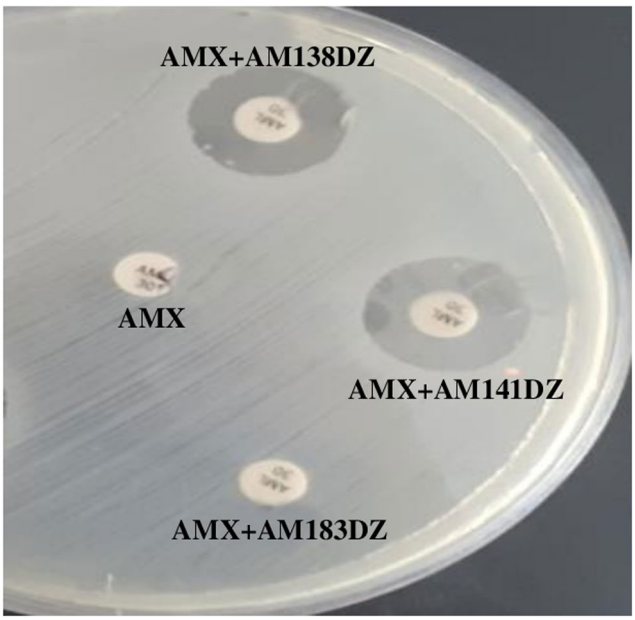

Fig. 4 βlactamase inhibitory property of the CFS of actinomycetes AM138DZ and AM141DZ isolates towards the ESBL-producing *K. pneumoniae* ATCC 700603 strain, as determined by synergistic assay performed with amoxicillin (AMX) (IZ of 0 mm), AMX+CFS of AM138DZ (IZ 13.5 mm±1.5), AMX+CFS of AM141DZ (IZ 15.5 mm±1.5) and AMX+CFS of 183DZ (IZ 0 mm)

β-lactamase inhibitory activity

In a next step, the 23 isolates were subjected to a secondary screening for their ability to produce β-lactamase inhibitors against the ESBL-producing *K. pneumoniae* ATCC 700603 strain, which exhibited resistance to amoxicillin. As demonstrated by disc diffusion method, among the 23 actinomycetes isolates, results of synergistic assay showed a clear zone around the disc of amoxicillin combined with CFS of only two isolates AM138DZ (13.5 mm±1.5) and AM141DZ (15.5 mm±0.5) against *K. pneumoniae* ATCC 700603 compared to the disk of amoxicillin alone (Fig. 4). This test indicated that the CFSs of the two isolates exhibited β-lactamase inhibitory activities from *K. pneumoniae* ATCC 700603 thus impeding amoxicillin hydrolysis by the enzyme. On the other hand, it is important to note, however, that neither of the organic extracts of AM138DZ and AM141DZ isolates displayed β-lactamase inhibitory activity.

Determination of the minimum inhibitory concentration

MICs of the organic crude extracts of the actinomycetes isolates AM138DZ, AM141DZ and AM183DZ against 11 pathogenic strains were in the range of 0.1 mg/mL −0.5 mg/mL, 0.05 mg/mL −1 mg/mL, and 0.025 mg/mL −0.5 mg/mL, respectively (Table 2). The three isolates possess varying degrees of antagonistic effects depending on the pathogen strain. Overall, the organic crude extract of actinomycetes AM183DZ showed remarkably high antibacterial activity (MICs, ranging from 0.025 mg/mL to 0.05 mg/mL) towards *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922, *S. aureus* ATCC 6538, and *L. innocua* CLIP 74915; however, it exhibited the lowest potency (MIC of 0.5 mg/

Table 2 The minimum inhibitory concentration (MIC) of the ethyl acetate extracts from actinomycetes isolates AM138DZ, AM141DZ and AM183DZ against human pathogens

Strains	MIC of ethyl acetate extracts (mg/mL)		
	AM138DZ	AM141DZ	AM183DZ
P. aeruginosa ATCC 27853	0.1	0.05	0.05
K. pneumoniae ATCC 13,883	0.1	0.1	0.25
E. coli ATCC 25922	0.5	0.25	0.025
P. aeruginosa ATCC 9027	0.5	0.5	0.5
K. pneumoniae ATCC 700603	0.25	0.25	0.25
E. coli BLSE	0.1	0.1	0.1
Salmonella spp. BLSE	0.5	0.25	0.25
S. aureus 6538	0.1	0.25	0.05
B. subtilis 6633	0.1	0.1	0.5
L. innocua CLIP 74915	0.1	1	0.05
C. albicans 10231	0.1	0.25	0.25

mL) against *P. aeruginosa* ATCC 9027 and *B. subtilis* ATCC 6633. The strongest antimicrobial property (MIC of 0.05 mg/mL) was observed towards *P. aeruginosa* ATCC 27853 for AM141DZ. The three actinomycetes isolates showed good and similar potency against *K. pneumoniae* ATCC 700603, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 9027. Also, they exhibited low MICs (ranging from 0.1 mg/mL to 0.25 mg/mL) towards *K. pneumoniae* ATCC 13883 and *C. albicans* ATCC 10231.

pH and temperature stability of the antimicrobial activity

The pH and temperature stability of the antibacterial activity of the ethyl acetate extracts of AM138DZ, AM141DZ and AM183DZ against the indicator strains are outlined in Table 3. The antimicrobial compounds of AM141DZ isolate were stable at pHs 11, 7 and 3 against all or the majority of the pathogens. At pH 11, the activities of AM138DZ and AM183DZ were preserved towards all indicator strains, but completely disappeared at pH 7 against most of the pathogens. Overall, temperature treatment at 110 °C–4 °C did not affect the antimicrobial potency of the three ethyl acetate extracts, with the exception of a complete loss of activity at either or both temperatures against *K. pneumoniae* ATCC 13883, and *C. albicans* ATCC 10231.

Total phenolic and flavonoid contents

As summarized in Table 4, the ethyl acetate extracts of isolates AM138DZ and AM183DZ had the highest total phenolic contents (34.380 mg GAE/g DW ± 2.685 and 30.456 mg GAE/g DW ± 0.358 respectively). Concerning flavonoids contents, overall, the richest extracts were detected for the isolate AM141DZ (43.615 mg QE/DW ± 1.360) followed by AM183DZ (43.423 mg QE/DW ± 0.544).

UPLC-ESI-MS/MS analysis

As illustrated in Fig. 5, the UPLC-ESI-MS/MS spectra of the ethyl acetate extracts of the three actinomycetes isolates revealed the presence of variable secondary metabolites in variable amounts. Table 5 provides the retention times, mass to charge ratios (m/z), and chemical formula of compounds proposed. The outcomes of the UPLC-ESI-MS/MS analysis of AM138DZ, AM141DZ and AM183DZ ethyl acetate extracts, revealed the identification of 18, 18 and 16 compounds, respectively. Six organic acids were consistently identified in the three extracts: peak 1 (fragment ion at m/z 442.1000 [M + H]+), peak 2 (m/z 177.1500 [M + H]+), peak 7 (m/z 457.1000 [M + H]+), peak 8 (m/z 142.8000 [M + H]+), peak 13 (m/z153.2000[M + H]+) and peak 20 (137.0000 [M-H]-) were tentatively assigned to folic acid, ascorbic acid, oleanolic acid, kojik acid, vanillin, and to salycilic acid, respectively. Caffeic acid (179.2000 [M-H]-) was found only in the ethyl acetate extract of AM141DZ.

Major compounds of flavonoids detected in the three extracts were as following: peaks 6 (with fragment ion at m/z 611.2000 [M + H]+), 9 (254.8000 [M + H]+), 11 (302.4000 [M + H]+), and 14 (291.1000 [M + H]+) were tentatively assigned to rutin, chrysin, quercetin and catechin, respectively. The peak 12 (m/z 432.9000 [M + H]+) was tentatively identified as the flavonoid vitexin that was detected only in the ethyl acetate extract of AM138DZ isolate.

The phenolic compound thymol (at m/z 151.1000 [M + H]+), curcumin (m/z 368.9000 [M + H]+) and vanillic acid (m/z167.2000 [M-H]-) were present in the three extracts. Other notable specific compounds were identified in the three extracts such as 8-hydroxyquinoline (m/z 146.2000 [M + H]+), which is classified as a quinolone, beta carotene (m/z 536.9000 [M + H]+), belongs to the family of terpenoids joining the class of carotenoids, and salicin, an alcoholic β-glucoside (m/z 287.0000 [M + H]+).

Based on peak areas, which is related to the molar concentration of the given compound in the extract, overall, flavonoids are major compounds for the three isolates (Fig. 6). The concentration of catechin was markedly higher in the three extracts (peak areas> 1000000), followed by rutin and chrysin in the isolates AM183DZ and AM141DZ, respectively. Compared to chrysin, quercetin was more abundant in the extracts of AM138DZ and AM183DZ. Among non flavonoids, the following compounds were detected at significant levels (peak areas > 100000), β-caroten (in AM138DZ and AM183DZ), salicin (AM183DZ) and salycilic acid (AM141DZ).

Table 3 pH and temperature stability of the antibacterial activity of the ethyl acetate extracts from actinomycetes AM138DZ, AM141DZ and AM186DZ isolates against human pathogens (inhibition zone in mm)

Isolates		*K. pneumoniae* ATCC 700603	*P. aeruginosa* ATCC 27853	*K. pneumoniae* ATCC 13883	*E. coli ATCC25922*	*P. aeruginosa* ATCC 9027	*E. coli* BLSE	*Salmonella spp. BLSE*	*S. aureus* ATCC 6538	*B. subtilis* ATCC6633	*L. innocua* CLIP 74915	*C. albicans* ATCC 10231
AM138 DZ	Without treatment	10.5±0.5	13±0	10±1	16±1	10.5±0.5	12±0	19±1	12.5±1.5	13±1	13.5±1.5	13±1
	pH=11	10.5±0.5	13±1	10±0	9±0	12±1	14±2	11±1	14±1	12.5±0.5	15.5±1.5	12.5±0.5
	pH=3	10±0	11.5±1.5	10±0	10.5±1.5	11.5±0.5	10±0	0	22.5±2.5	12.5±0.5	13±1	0
	pH=7	0	0	0	0	10±0	11.5±0.5	14±0	11±0	0	0	11±1
	T° 110	11±1	12±2	0	11.5±0.5	15±1	10.5±0.5	11.5±0.5	10±1	15.5±0.5	15±0	9±0.5
	T° 4	11.5±0.5	14±0	0	11±0	14±0	12±0	12±0	14±1	14±0	11±0	0
AM141DZ	Without treatment	14±2	11±0	11.5±1.5	18±0	12.5±1	11±0	19±1	14.5±1.5	12.5±0.5	15±1	15±1
	pH=11	10.5±0.5	12.5±1.5	10±0	9.5±0.5	11.5±0.5	13±1	12±2	18.5±1.5	12.5±0.5	14±2	9.5±0.5
	pH=3	0	10.5±1.5	10±0	9.5±0.5	10.5±0.5	9.5±0.5	0	16±4	12±0.5	13.5±1.5	11±0
	pH=7	12±0	0	0	9.5±0.5	10±0	10±0	18±0	10±0	0	14±0	13±1
	T° 110	11.5±1.5	11.5±1.5	10±0	12±1	15.5±0.5	11±0	11.5±0.5	9.5±0.5	15±1	12±0	0
	T° 4	10.5±0.5	13±0	0	11±0	15±0	11±0	10±0	15±1	14±0	12±0	11±0
AM183DZ	Without treatment	16±1	13±0	10±1	19±0	11±0.5	10±0	21±1	12.5±1.5	11±0	19±1	10±0
	pH=11	10.5±0.5	10.5±0.5	10±0	9.5±0.5	12.5±0.5	13±1	11±1	17±1.5	13.5±0.5	13.5±1.5	14±1
	pH=3	9±0	12±1	10±0	9±0	0	10.5±0.5	0	20±0	10.5±0.5	14.5±1.5	0
	pH=7	10.5±0.5	0	0	11.5±0.5	10±0	11±0	16±0	0	0	0	13.5±0.5
	T° 110	11±1	11±1	0	11.5±0.5	15.5±0.5	11±0	10.5±0.5	9±0	16±2	14.5±0.5	11.5±0.5
	T° 4	11.5±0.5	12±0	10±0	10±0	16±0	10±0	10±0	20±0	12±0	0	11±0

Values represent means of duplicate experiments±.S.D, $n=2$

Table 4 Total phenolic content and total flavonoid content in the Ethyl acetate extracts from actinomycetes AM138DZ, AM141DZ and AM186DZ isolates

Contents	Ethyl acetate extract's concentration		
	AM138DZ	AM141DZ	AM183DZ
Total phenolic content (mg GAE/g DW)	34.380 ± 2.685	23.810 ± 0.269	30.456 ± 0.358
Total flavonoid content (mg QE/g DW)	39.962 ± 1.632	43.615 ± 1.360	43.423 ± 0.544

Values represent means of duplicate experiments±.S.D, $n = 2$

Biochemical characterization and molecular identification

Biochemical characterization of the three biologically active actinomycetes isolates AM138DZ, AM141DZ and AM183DZ showed that, based on results of the API 50CH test, they were able to utilize a variety of carbon sources, including galactose, sucrose, maltose, cellobiose, fucose, raffinose, D-xylose, L-arabinose, and D-ribose. However, they could not assimilate lactose, starch, L-rhamnose, erythritol, adonitol, and inositol. The API ZYM test revealed that they exhibited alkaline phosphatase, esterase lipase (C8), leucine arylamidase, and valine arylamidase activities, but they lacked lipase (C14), trypsin, α-chymotrypsin, N-acetyl-β-lucosamidase, β-glucuronidase, α-mannosidase and α-fucosidase activities.

Phylogenetic tree (Fig. 7) based on the 16S rRNA gene homology sequence analysis showed that the three actinomycetes isolates clustered with members of the genus *Streptomyces*, the nearest neighbour being (i) *Streptomyces flavogriseus* strain CBS 101.34^T (GenBank accession no.: AJ494864) (98.52% sequence similarity) for strain AM138DZ, *Streptomyces felleus* strain DSM 40,130^T (GenBank accession no.: Z76681) (98.71% sequence similarity) for strain AM141DZ, and *Streptomyces rubiginosohelvolus* strain NBRC 12,912^T (GenBank accession no.: AB184240) (97.17% sequence similarity) for strain AM183DZ. Thus, the results obtained strongly suggested that these isolates should be assigned as *Streptomyces flavogriseus* strain AM138DZ, *Streptomyces felleus* strain AM141DZ, and *Streptomyces rubiginosohelvolus* strain AM183DZ.

Nucleotide sequences accession numbers

The nucleotide sequences data of 16 S rRNA genes 1517 bp of strain AM138DZ, 1479 bp of strain AM141DZ, and 1489 bp of strain AM183DZ reported in this paper has been submitted to the GenBank/ENL/DDPJ databases under accession no.s of OQ976969, OQ976970, and OQ976971, respectively.

Discussion

Actinomycetes are a natural huge resource of antimicrobials in particular (De Simeis and Serra 2021; Meenakshi et al. 2024). In the present study, among 36 presumptive actinomycetes, isolated from Atlas forest soils in Northeastern Algeria, 23 isolates with characteristic features were selected for further evaluation of their antibacterial property. Only three isolates displayed antimicrobial activity against strains of human pathogens. They were identified as *Streptomyces flavogriseus* AM138DZ, *Streptomyces felleus* AM141DZ, and *Streptomyces rubiginosohelvolus* AM183DZ strains. Our findings are in agreement with previous studies and confirm that *Streptomyces* genus is the most common soil actinomycetes (Sapkota et al. 2020; Nazari et al. 2022). According to Sapkota et al. 2020, p. 41 actinomycetes strains isolated from 11 soil samples collected from different locations in Nepal were identified as *Streptomyces* spp. (70.7%), *Nocardia* spp. (19.5%), and *Micromonospora* spp. (9.5%). Basilio et al. (2003) isolated a total of 609 of actinomycetes collected from 17 soil samples in diverse areas of Philippines (316 strains), Costa Rica (127 strains), Sri Lanka (84 strains), Mexico (42 strains), Switzerland (22 strains) and Spain (18 strains), among them 376 species were attributed to *Streptomycetaceae* family followed by *Nocardiaceae* (116 species), *Micromonosporaceae* (80 species), *Pseudonocardiaceae* (12 species) and other families (23 species). In a Sudanese study, 173 *Streptomyces* isolates were recovered from 17 sites representing three different terrestrial ecoregions of Sudan and South Sudan (Hamid et al. 2020).

In recent years, there has been a rising interest in the exploration of uncharted ecosystems (such as forest, sebkha, estuary, marine and arid ecosystem) in Algeria for the isolation of new actinobacteria species with the potential to produce biologically active compounds. Meliani et al. (2022) isolated five actinomycetes strains (4 *Streptomyces* sp. and one *Nocardia* sp.) from sediment samples of Chellif estuary in the North-West of Algeria. According to this study, all strains exhibited antibacterial activities at various degrees (IZ values ranging from 0 to 12 mm) against *E. coli* ATCC 25922, *S. aureus* ATCC 6538, *S. typhimurium* ATCC 9289, *B. subtilis* ATCC 6633 and *P. aeruginosa* ATCC 2785. Menasria et al. (2022), reported the isolation of 31 halophilic actinomycetes from hypersaline soils of Algerian inland Wetland Ecosystems "Sebkhas-Chotts" located in arid and hot hyperarid lands (Northeastern of Algeria), among them 18 strains displayed significant activity against *S. aureus* ATCC25923, *Micrococcus luteus* DSM1970, *B. cereus* PCM480 and *B. subtilis* ATCC6633. However, none of the isolates showed activity against *P. aeruginosa* ATCC27853 and *E. coli* ATCC25922. The Algerian desert,

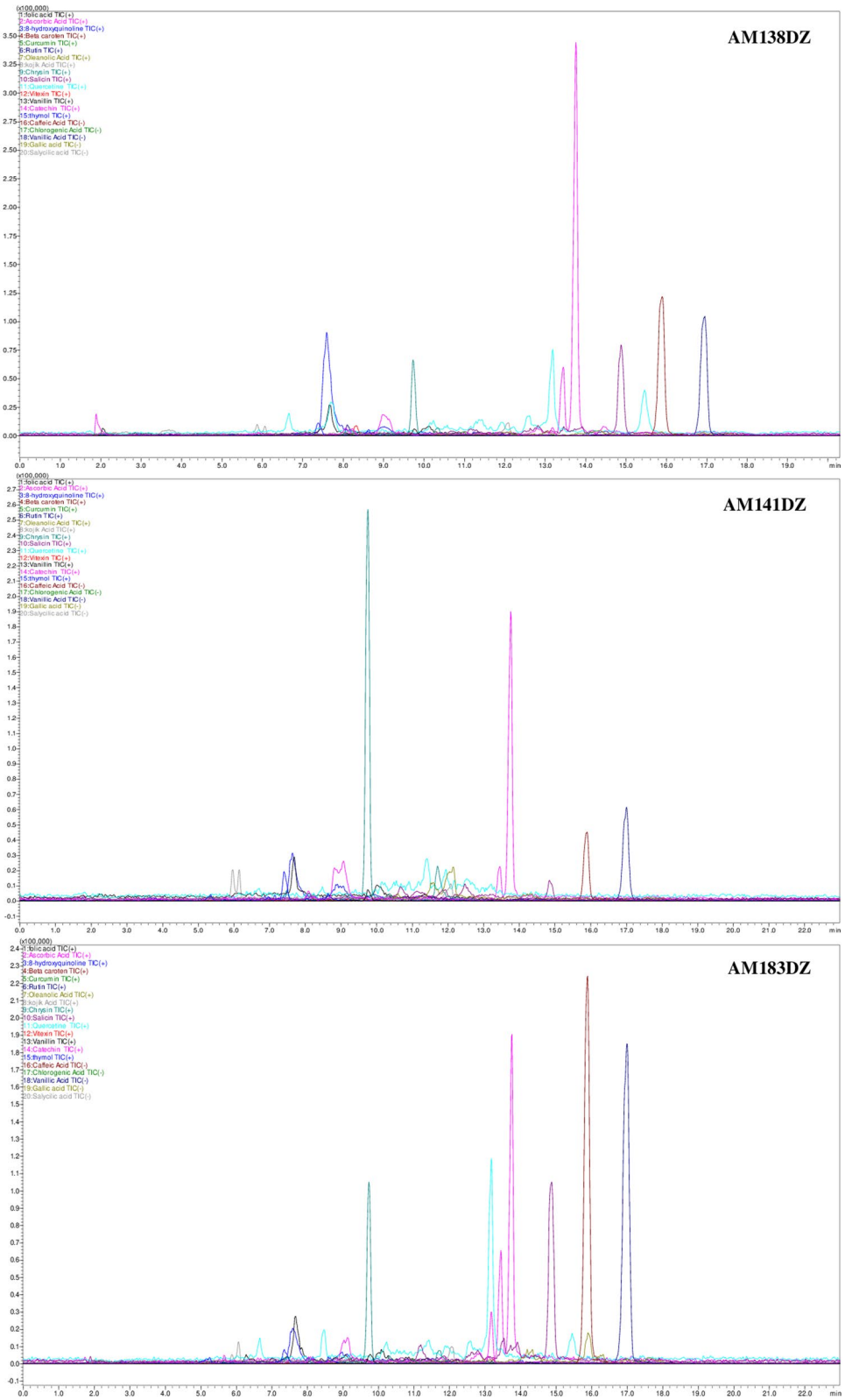

Fig. 5 The UPLC-ESI-MS/MS chromatograms of the ethyl acetate extracts of the three actinomycetes isolates AM138DZ, AM141DZ, and AM183DZ. Standard metabolites and their corresponding peak number are listed on the left of each panel

Table 5 Tentative identification of secondary metabolites in the ethyl acetate extracts from actinomycetes AM138DZ, AM141DZ and AM186DZ isolates through UPLC-ESI MS/MS

Peaks	Compound name	Molecular formula	Molecular weight (g/mole)	ESI charge (+/-)	m/z	AM138DZ	AM141DZ	AM183DZ
						Retention time (min)	Retention time (min)	Retention time (min)
1	Folic acid	$C_{19}H_{19}N_7O_6$	441	(+)	442.1000 > 182.0000	9.767	9.763	9.769
2	Ascorbic acid	$C_6H_8O_6$	176.12	(+)	177.1500 > 90.9500	13.188	8.823	9.041
3	8-hydroxyquinoline	C_9H_7NO	145.16	(+)	146.2000 > 101.0000	7.401	7.427	7.399
4	β-caroten	$C_{40}H_{56}$	536.87	(+)	536.9000 > 148.0000	15.913	15.914	15.860
5	Curcumin	$C_{21}H_{20}O_6$	368.4	(+)	368.9000 > 177.0500	11.002	10.966	10.965
6	Rutin	$C_{27}H_{30}O_{16}$	610.5	(+)	611.2000 > 356.2000	16.934	17.014	16.995
7	Oleanolic acid	$C_{30}H_{48}O_3$	456.7	(+)	457.1000 > 411.2500	14.285	14.302	14.321
8	Kojik acid	$C_6H_6O_4$	142.11	(+)	142.8000 > 69.0000	12.092	12.084	12.065
9	Chrysin	$C_{15}H_{10}O_4$	254.24	(+)	254.8000 > 153.0500	9.741	9.761	9.744
10	Salicin	$C_{13}H_{18}O_7$	286.28	(+)	287.0000 > 133.0500	13.474	14.861	14.869
11	Quercetine	$C_{15}H_{10}O7$	302.23	(+)	302.4000 > 285.1000	13.192	6.715	13.186
12	Vitexin	$C_{21}H_{20}O_{10}$	432.4	(+)	432.9000 > 313.1500	8.334	0.000	0.000
13	Vanillin	$C_8H_8O_3$	152.15	(+)	153.2000 > 93.0000	7.682	10.120	10.139
14	Catechin	$C_{15}H_{14}O_6$	290.27	(+)	291.1000 > 179.1000	13.769	13.769	13.759
15	Thymol	$C_{10}H_{14}O$	150.22	(+)	151.1000 > 91.1500	8.981	8.908	9.123
16	Caffeic acid	$C_9H_8O_4$	180.16	(-)	179.2000 > 135.0000	0.000	7.041	0.000
17	Chlorogenic acid	$C_{16}H_{18}O_9$	354.31	(-)	353.1000 > 191.1000	6.523	0.000	0.000
18	Vanillic acid	$C_8H_8O_4$	168.15	(-)	167.2000 > 121.1000	8.109	8.138	8.134
19	Gallic acid	$C_4H_4O_4$	170.12	(-)	169.2000 > 125.0500	0.000	3.762	0.000
20	Salycilic acid	$C_7H_6O_3$	138.12	(-)	137.0000 > 93.0000	5.879	5.971	6.067

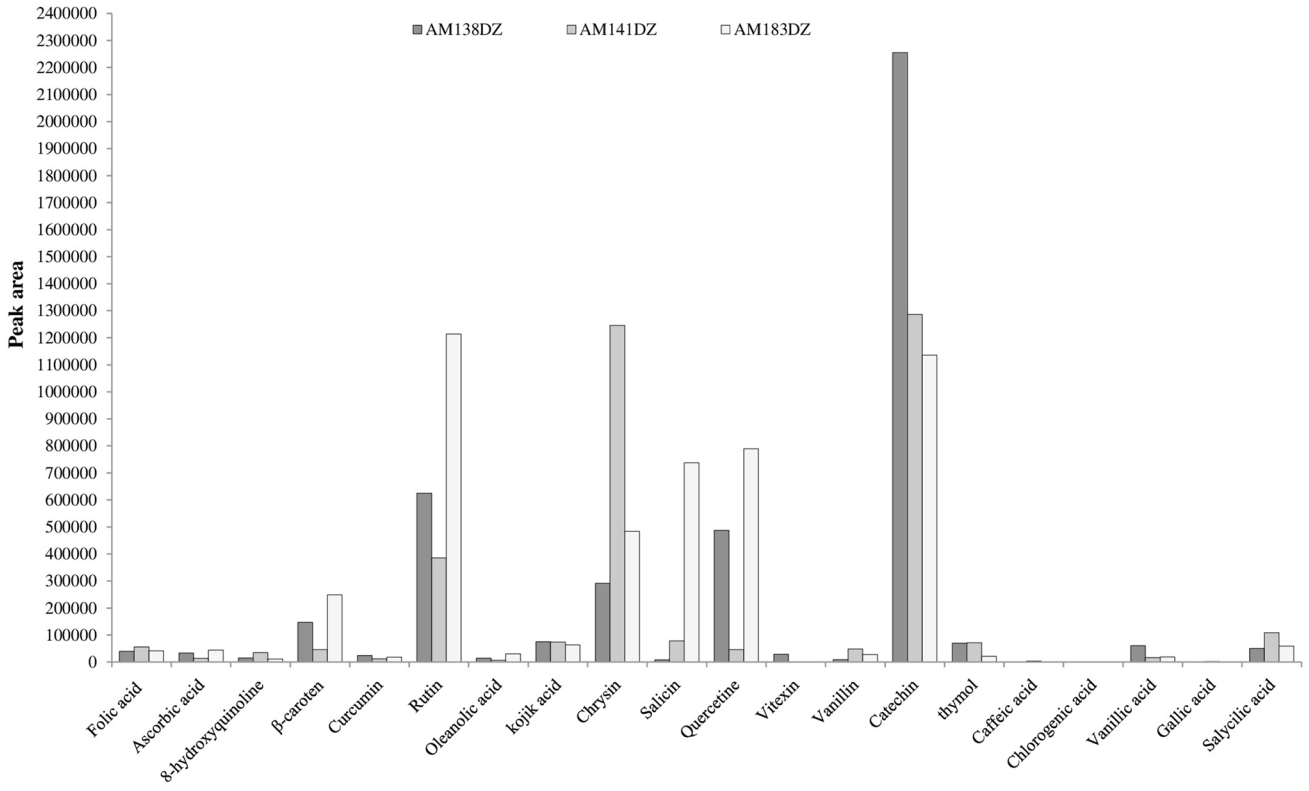

Fig. 6 Histogram of secondary metabolites in the ethyl acetate extracts from actinomycetes AM138DZ, AM141DZ and AM186DZ isolates through UPLC-ESI MS/MS

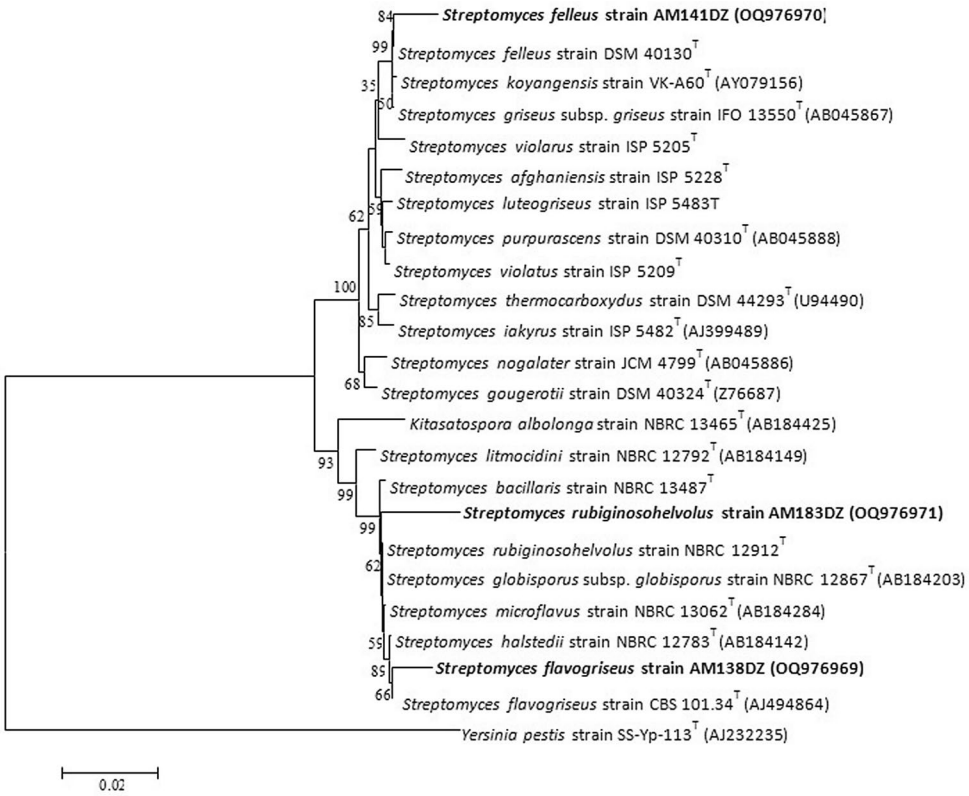

Fig. 7 Phylogenetic tree showing the interrelationships of AM138DZ, AM141DZ, and AM183DZ isolates and closely related previously described species inferred from 16S rRNA gene sequences of the genus *Streptomyces*. The tree was constructed using the neighbour-joining (NJ) method contained in the MEGA software, based on a comparison of approximately 1200 bp, and plotted using NJplot. Numbers at nodes or branch points (>50%) indicate support for the internal branches within the tree obtained by bootstrap analysis (percentages of 1000 bootstraps). *Yersinia pestis* strain SS-Yp-113^T (GenBank accession no.: AJ232235) is the outgroup. Bar 0.02% sequence divergence

which covers more than 80% of the country's total area, is still a huge unexplored potential of novel bacterial resources. Thirty-two actinomycetes, isolated from Saharan soils in the south of Algeria, were classified into 5 genera *Streptomyces* (65.63%), followed by *Nocardia* (9.38%), *Streptosporangium* (9.38%), *Nocardiopsis* (9.38%) and *Actinomadura* (6.25%) (Khirennas et al. 2023). The study reported that 75.00%, 65.63%, and 78.13% of the isolates were active toward Gram-positive bacteria (*S. aureus* ATCC 6538, *B. subtilis* ATCC 6633), Gram-negative bacteria (*E. coli* ATCC 8739, *S. typhimurium* ATCC 13331), and *C. albicans* ATCC 10231 strains, respectively (Khirennas et al. 2023). Harir et al. (2017) reported that among 32 actinomycetes isolated from 6 Algerian Sahara soil samples, three strains identified as *Streptomyces violaceoruber*, *Streptomyces albus* and *Streptomyces badius* showed antimicrobial activities. Their crude extracts exhibited moderate to high antimicrobial activity against most of the tested bacterial pathogens such as strains of *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus*, and *Enterococus faecalis*, and *C. albicans* strain (IZs ranging from 11 to 35 mm).

In the current study, the ethyl acetate extracts of the three isolates exhibited varying spectrum and degree of antagonistic effects toward human pathogens tested. Analysis of their contents revealed flavonoids richness in particular. These compounds are versatile natural products ubiquitously present in plants (Dias et al. 2021; Liga et al. 2023). They perform many functions like plant growth and development, plant defense against biotic and abiotic factors, plant pollination, and mediating plant-microbe interactions. In addition, flavonoids exhibit a multitude of biological activities such as antioxidant, anti-inflammatory, anticancer, antimicrobial, hepatoprotective, antidiabetic, cardioprotective and neuroprotective effects (Dias et al. 2021; Liga et al. 2023). Recently, a wide attention has been given on microorganisms as potential sources of novel flavonoids. The presence of these compounds in actinomycetes has previously been reported (Wang et al. 2020; Almuhayawi et al. 2021). Almost all flavonoids derived from actinomycetes are produced by *Streptomyces* sp. such as simple flavonoids rhamnazin and cirsimaritin which exhibited antifungal activities, flavonoid glycoside quercetin-3-*O*-β-L-rhamnopyranosyl-(1–6)-β-D-glucopyranoside which showed potent antitumor activity, and lavandulylated flavonoids which showed potent antimicrobial activities (El-Gendy et al. 2008; Balachandran et al. 2014; Cao et al. 2019; Wang et al. 2020; Hoa et al. 2020). From the present study, catechin, chrysin and rutin, which were major flavonoid compounds identified, may be mainly responsible for the antimicrobial activities of the three isolates. However, the detection of other components, such as quercetin and vitexin at a relatively low concentration, does not exclude their involvement in the inhibitory effects on pathogen growth. Indeed, Veiko et al. (2023) reported that quercetin had the greatest anti-*S. aureus* activity compared

to catechin and naringenin. They stated that the antibacterial activities of the flavonoids resulted from a disorder in the structural organization of bacterial cell membrane. A recent study explored the effect on biological activity of different functional groups added on the position 7 in chrysin (Omonga et al. 2021). The authors showed that chrysin exhibited either very weak or no antibacterial activity against tested strains of *E. coli*, *P. aeruginosa*, *S. aureus*, *Enterobacter faecalis*, *K. pneumoniae*, *P. fluorescens* and *C. albicans*. However, in respect to the parent molecule, the addition of a carbonyl group or two nitro groups resulted in a considerable improvement of the antimicrobial activity of chrysin derivatives (Omonga et al. 2021). A previous investigation of rutin described its great antibacterial activity against strains of methicillin-resistant *S. aureus* (MRSA), *P. aeruginosa*, and *A. baumannii* (Ivanov et al. 2022). The same study proved rutin's ability to eradicate *P. aeruginosa* and MRSA urinary catheter biofilms for 73.7% and 74.2%, respectively (Ivanov et al. 2022). Vitexin, which was detected at a relatively low concentration in the present work, has been previously investigated for its antibiofilm activity and bactericidal effect against *S. aureus* (Das et al. 2022). The authors reported that vitexin exhibited high inhibitory activity against *S. aureus*, reduced cell membrane permeability and altered cell surface hydrophobicity to obstruct biofilm formation of the pathogen.

The broad range of biological activities displayed by flavonoids underscores their promising potential in therapeutic application and health promotion (Ferraz et al. 2020; Hasnat et al. 2024). Several studies reported that the intake of flavonoid-containing dietary supplements was associated with the prevention and/or the treatment of multiple diseases. The systematic review and meta-analysis conducted by Yao et al. (2022) provided initial evidence that flavonoid-containing supplements may be efficacious and safe in preventing acute respiratory tract infections. Also, the results presented in a systematic review showed that flavonoids (such as quercetin, hesperidin, epicatechin, anthocyanin, and genistein), when used alone as supplements, significantly enhance several metabolic parameters (such as blood pressure, lipid profile, and blood glucose), and hence reduce the risk of metabolic syndrome (Gouveia et al. 2022).

Combination therapy, involving flavonoids and clinical antibiotics, provides a promising strategy to combat antimicrobial resistance and to develop potent therapeutic. In this context, Almuhanna et al. (2024) reported that rutin and quercetin enhanced the efficacy of gentamicin in controlling bacterial infection and promoting wound healing when used on wounds infected with multidrug-resistant *P. aeruginosa* strain in diabetic mice. According to the study of Yuan et al. (2024), flavonoids exhibited extensive synergistic effects when combined with clinical antibiotics, and 25% of combinations restored antibiotic efficacy against *S. aureus* and 50% against *E. coli*.

Despite the remarkable activities of flavonoids, the large-scale application of flavonoids is still limited due to their poor bioavailability (Chen et al. 2022). To overcome this challenge, nanotechnology-based approaches have been applied to develop protective delivery systems of flavonoids (such as nanoparticles, liposomes, nanoemulsion, nanohydrogel, silver nanoparticles, and mesoporous silica nanoparticles) and to optimize their therapeutic potential (Macêdo et al. 2024). According to the study conducted by Aldawsari et al. (2024), rutin-loaded transethosomal gel showed high stability, an enhanced drug permeation through the rat skin, and exhibited promising antibacterial activity, particularly against *S. aureus*. This investigation highlights the significant therapeutic possibilities of rutin in a transethosomal gel formulation for treating dermatological diseases. In vivo mouse experiments revealed that the relative oral bioavailability of casein-based chrysin nanoparticles was approximately 2 times higher than that of the free chrysin suspension (Tang et al. 2024). In addition, these nanoparticles efficiently attenuated pulmonary infections caused by *Acinetobacter baumannii* when compared to free chrysin suspension. Several studies, using in vitro or in vivo experiments, reported an improvement of therapeutic potential (such antibacterial, anticancer, and anti-inflammatory properties) of quercetin encapsulated in chitosan nanoparticles or in combination with silver nanoparticles when compared to unencapsulated quercetin (Lawson 2023; Sharma et al. 2023).

Antibacterial activity of non flavonoid compounds described herein (beta-caroten, salycilic acid and salicin, in particular) has been reported previously. According to the study of Adamczak et al. 2019; among 19 investigated phytochemicals, salicylic acid showed the highest biological effect against tested strains of *E. coli*, *P. aeruginosa*, *E. faecalis*, and *S. aureus*. Salicin, the precursor of salycilic acid, showed an inhibitory effect on *S. aureus* coagulase activity and reduced the content of lung bacteria in a pneumonia model of mice (Jiang et al. 2023).

β-lactamase inhibitors have emerged to potentiate the activity of β-lactam antibiotic against beta lactamase-producing bacteria. To date the clinically used β-lactamase inhibitors are: clavulanic acid, sulbactam, tazobactam, avibactam, and relabactam. In light of the rapid evolution of β-lactamases (such as ESBL, metallo-β-lactamases (MBLs), OXA-type β-lactamases) and the development of resistance to β-lactam/β-lactamase inhibitor combinations among Gram-negative bacteria in particular, there is a serious need to research for new β-lactamase inhibitors (Fröhlich et al. 2021; Rajer et al. 2022). In this context, our preliminary findings showed that AM138DZ and

AM141DZ isolates exhibited an interesting β-lactamase inhibitory activity towards the ESBL-producing *K. pneumoniae* ATCC 700603 strain. *Streptomyces* genus is an exceptional and promising natural source of β-lactamase inhibitors. Clavulanic acid, the first and one of the favorite β-lactamase inhibitor used in clinical practice to date, was originally identified in *S. clavuligerus* and later in other *Streptomyces* species (such as *Streptomyces jumonjinensis* and *Streptomyces katsurahamanus*) (Viana Marques et al. 2018; Simeis and Serra 2021). It is noteworthy that β-lactamase inhibitory activity detected in the CFSs, but not in the ethyl acetate extracts, of AM138DZ and AM141DZ. Based on this observation, we hypothesized that the inhibitor metabolites may be different from clavulanic acid which is stable in ethyl acetate. Although several possibilities exist for combination therapies, combining β-lactam antibiotic with adjuvant molecules remains unexplored strategy to manage bacterial resistance. The use of β-lactamase inhibitors is considered one of the most successful approaches for restoring β-lactam efficacy. The key to the success of β-lactam/β-lactamase inhibitor combinations is their unique dual mechanism of action associating the in vivo antibacterial activity of one β-lactam antibiotic and inhibition of a wide range of β-lactamases (Si et al. 2023). Currently, only a few limited cases of β-lactam/β-lactamase inhibitor combinations are under investigation in clinical trials (such as enmetazobactam, funobactam, vaborbactam, taniborbactam, zidebactam, durlobactam, nacubactam, and xeruborbactam) (Arer and Kar 2023; Zhang et al. 2024). Inhibitors used in clinical can inactivate predominantly serine-β-lactamases but have not been efficient against most OXA-type carbapenemases and MBLs, which pose a real threat to human health because they hydrolyze almost all β-lactam antibiotics (Wong and van Duin 2017; Principe et al. 2022). Although, several MBL inhibitor candidates have been introduced in recent years, designing potent inhibitors that are efficient against all MBL subclasses is still extremely challenging. Taniborbactam, which represents the current hope against the critical priority pathogens provides a great therapeutic potential when combined with cefepime (phase 3 clinical trials) because of its broad spectrum of activity against a wide range of serine-β-lactamases, as well as relevant MBLs (Hamrick et al. 2020; Zhang et al. 2024).

Conclusion

In this paper we report on the diversity of secondary metabolites and their antimicrobial activity from three *Streptomyces* strains: *Streptomyces flavogriseus* AM138DZ, *Streptomyces felleus* AM141DZ, and *Streptomyces rubiginosohelvolus* AM183DZ isolated from Atlas Algerian forest soils. *Streptomyces flavogriseus* AM138DZ, *Streptomyces felleus* AM141DZ strains exhibited also a remarkable β-lactamase inhibitory activity against ESBL-producing *K. pneumoniae* ATCC 700603 strain. This study, which represents a first step in our work,: (i) provides evidence that soil is still a rich resource of *Streptomyces* sp. with the potential to produce bio-active secondary metabolites of great medical interest, (ii) introduced a promising natural alternative source of antimicrobials and β-lactamase inhibitors in particular. Further in-depth molecular investigations are necessary to perform in order: (i) to purify and characterize the activities detected; (ii) to investigate the spectrum of activities of the β-lactamase inhibitors against various β-lactamases; and (iii) to analyze, in silico, the molecular interactions between β-lactamase inhibitor and β-lactamase.

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Author contributions IG designed the study, performed experiments, and drafted the manuscript. NE-HD contributed to the analysis and interpretation of LCMS-MS data. ANM contributed to methodology design and investigation process. AA and SR supervised the work, revised and edited the manuscript. All authors read and approved the final manuscript.

Data availability The nucleotide sequences data of 16 S rRNA genes 1517 bp of strain AM138DZ, 1479 bp of strain AM141DZ, and 1489 bp of strain AM183DZ reported in this paper has been submitted to the GenBank/ENL/DDPJ databases under accession no.s of OQ976969, OQ976970, and OQ976971, respectively.

Declarations

Ethical approval Not applicable.

Competing interests The authors declare no competing interests.

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