ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2022; 27(5): 3713-3721
doi: 10.25083/rbl/27.5/3713.3721

Received for publication, November, 12, 2022
Accepted, February, 16, 2023

Original article

Streptomyces lasiicapitis KSA18 isolated from Saharan soil in Algeria effective against most bacteria and fungi

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Abstract

Saharan soil still represents a formidable source of microorganisms that produce bioactive substances. Twenty-three Actinobacteria strains were isolated from sand samples collected from saharian field in Algeria. Isolates were evaluated for antimicrobial activity. After primary screening, 79% of the isolates showed antimicrobial activity. KSA18 strains were selected for their intense activity. Bioactive metabolite was extracted with ethyl acetate and tested against pathogens microorganisms using the disk diffusion method. The crude extract was partially purified by column chromatography and evaluated for antimicrobial activity. Fraction A1 showed good activity against Staphylococcus aureus (3.25 mg/mL) and Listeria monocytogenes (4 mg/mL). This strain has been identified as Streptomyces lasiicapitis by 16S rRNA sequencing. GC-MS analysis of crude extract showed the presence of about 17 different volatile compounds). Some of them could be directly responsible for antibacterial or antifungal activity. The most important compounds are Phenol, 2,4-Bis(1,1-Dimethylethyl), 3-Isobutylhexahydropyrrolo[1,2-A] Pyrazine-1,4-Dione, 2-methyloctacosane and dibutyl phthalate.

However, this study requires other approaches to identify the bioactive molecules present in the extract.

Keywords Saharian soil, Actinobacteria, Algeria, antimicrobial activity, *Streptomyces lasiicapitis*, GC-MS

To cite this article: MOKHTAR BENREGUIEG. *Streptomyces lasiicapitis* KSA18 isolated from Saharan soil in Algeria effective against most bacteria and fungi. *Rom Biotechnol Lett.* 2022; 27(5): 3713-3721 DOI: 10.25083/rbl/27.5/3713.3721

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Introduction

Abraham and Chain described in 1945 a substance produced by a Colibacillus which completely inhibited penicillin and called it penicillinase. In 1945 Fleming warned the population, in a New York Times article dated June 26, 1945, that abusive use of penicillin could lead to the emergence and spread of resistant bacteria. Five years later, in Paris and London, half of the strains of Staphylococcus were resistant to penicillin (Briand, 2009). From then on, the problem of antibiotic resistance spread to the world and became a global problem. Indeed, the WHO declared through its director-general "Margaret Chan" during the World Health Day organized on April 7, 2011: "... if we do not take urgent measures to correct this situation and protect its achievements, we are moving towards a post-antibiotic era in which many common infections can no longer be treated and will begin to kill again." Considering the above, the long-term solution to counter microbial resistances is to develop or research new molecules with antimicrobial activity, whether by synthesis of new molecules and/or hemi-synthesis from known structures; Exploit non-culturable microbial populations by the concept of metagenomics (PERIC & LONG, 2003), which consists in the extraction of DNA from a sample of the environment, the metagenome, which will be cloned into vectors Which are transformed into substitution hosts. These environmental DNA libraries are then screened for their biological activities (Zhang & Demain, 2005), or isolation of new bacterial or fungal species from less or less explored ecosystems, and analysis of their fermentation products.

Good fellow and Haynes (1984) reviewed the literature on actinomycete isolation and suggested that only 10% of actinomycetes are naturally isolated. The majority of antibiotics currently in use are derived from natural products of actinomycetes and fungi (BUTLER & BUSS, 2006; NEWMAN & CRAGG, 2007). Actinomycetes are isolated from marine sediments and soil. While the pharmaceutical industry has been analyzing soils for about 50 years, only a tiny fraction of the world's surface has been sampled. A tiny fraction of actinomycete taxa has been discovered (BALTZ, 2005; 2007). Our work is registered in the research of actinomycetes, the most promising actors for producing metabolites with antimicrobial activity.

Materials and methods

Isolation of Actinobacteria

In Kasdir, Naâma, Algeria (33° 42′ 35″, 1° 21′ 32″), poor soil samples were taken. A sterile spatula was used to remove the top 5 cm of dirt. Then, 100-150 g of dirt from 5 to 15 cm deep is gathered with a sterile spatula and placed on a sterile aluminium paper. 50 g were transported to the lab as soon as feasible (POCHON & TARDIEUX, 1962). Soil samples were room-temperature air-dried for a week. 9 ml of double-distilled sterile water contained 1 g of dirt. Diluted 10^{-1}. 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} were dispersed over starch-casein agar. Nalidixic acid and Actidione were supplemented to inhibit bacterial and fungal growth. 10-day incubation at 28 to 30°C. Based on morphological characters, Actinobacterial colony were purified on ISP2 medium. Our pilot-scale screening isolated 23 actinomycetes, KSA 1–23.

Microbial organisms

Pasteur Institute of Algiers provided the Gram- negative, Gram- positive, and fungal strains used in this study: *Escherichia coli* (ATCC 25922), *Salmonella Typhimurium* (ATCC13311), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923), *Listeria monocytogenes* (ATCC 19115), *Bacillus subtilis* (ATCC 10876), *Enterococcus faecalis* (ATCC 29212), methicillin-resistant S. aureus (MRSA) (ATCC 43300), *Candida albicans* (ATCC 2019) and *Aspergillus flavus* (ATCC 9643). Growing bacteria and fungi in Mueller Hinton and Saboraud broth at 37°C and 25 °C for 24 and 72 hours repectively.

Antibacterial activity

Actinomycetes were cultivated for 7 days on Bennett's sand GLM agar (LEE & HWANG, 2002). With a hollow punch, 3 mm cylinders of agar were placed on Mueller–Hinton media (Merck) seeded with each test bacteria. After 4 hours at 4 °C, Petri dishes were placed at 37°C for 24 hours. Measured inhibition diameters (LEMRISS et al., 2003).

Antifungal activity

Actinomycetals isolates were tested against filamentous fungi from the Pasteur Institute's Mycology Unit. Activity was tested using 9 g l^{-1} Bactocasitone, 5 g.l^{-1} yeast extract, 10 g.l^{-1} sodium citrate, 20 g l^{-1} glucose, 3.34 g l^{-1} disodium hydrogen phosphate, 0.54 g l-1 potassium di-hydrogen phosphate, and 18 g l^{-1} agar (KITOUNI et al, 2005). After 24–48 hours at 28°C, yeast and filamentous fungus inhibition zones were measured.

DNA extraction, PCR and 16S rRNA sequencing

A DNA extraction kit was used on 0.5 ml of KSA18 liquid culture (Stratec Molecular Invisorb Spin Plant kit, Berlin, Germany). DNA extraction efficiency was tested with agarose gel electrophoresis. Using two primers (27F: 5′-AGTTTGATCCTGGCTCAG-3′ and 1492R: 5′-ACGGCTACCTTGTTAGGACTT-3′), 16S rRNA was amplified in a thermocycler. The DSMZ sequencing of the PCR products was performed at DSMZ center (Brauchweig, Germany). BLAST was used to match the nucleotide sequence to GENBANK database at NCBI.

Fermentation

Primary screening showed that the isolate (called KSA18) has a zone of inhibition, indicating antibacterial activity. ISP-2 broth produced bioactive substances. 125 milliliters In a 250 ml Erlenmeyer flask, the soup was sterilized. The sterile broth included 4% two-day-old mother inoculum was placed at 28°C for 7 days and 150 rpm. Broth was filtered with Whatman N°1 after incubation. Centrifuged for 15 min to separate the filtrate.

Extraction and purification

The culture filtrate (800 ml) was extracted twice with ethyl acetate, and the pooled extracts were evaporated to dryness under reduced pressure. The antibacterial ingredient was purified using silica gel column (2.5 25) chromatography. 100-200 mm silica gel was utilized to stuff the column. Methanol and ethyl acetate (6-4 v/v) eluted. 5 g of this crude extract was diluted in 50 ml of methanol and passed through a silica gel column at 0.2 ml/min; 25 fractions (5 ml each) were collected and evaluated for antibacterial activity (AOUICHE, 2012).

Thin-layer chromatography combined with bioautography

Bio-autography and TLC were used to detect anti-S. aureus molecules. Samples were spotted on 20 cm 20 cm silica gel plates (Si60, Merck Art. 5735, Kiessel gel 60F254), washed with methanol and acetate ethyl (6:4, v/v), and air-dried overnight at 37°C. Fractionation experiments used two plates. First, bioactive chemicals were localized using retention factor (Rf) (MENDHAM, 2006). Spraying sulfuric vanillin (vanillin/H2SO4/ethanol 3:3:100, w/v/v) visualized these molecules. The second plate was placed over S. aureus-seeded Mueller–Hinton media (Merck) at 37°C for 24 hours. Antifungal compounds were discovered by matching their Rf on reference TLC plates to S. aureus-free regions.

Identification of bioactive metabolites using GC-MS analysis

The chemical composition of the TLC active eluent molecule was discovered by GC-MS (PARTHASARATHI, 2012). The program ranged from 40–280 °C, and 250 °C was chosen for injection. The GC had a capillary column and a 1 l injection volume. The sample flowed at 1 ml/s and 36.5 cm/s (BARTON, 2006). The results were compared to NIST 11.

Determination of minimum inhibitory concentrations

Minimum inhibitory concentrations (MICs) of pure bioactive substances were determined using agar dilution. Inoculated on Mueller Hinton medium for bacteria and Sabouraud medium for yeasts and filamentous fungi with 10, 20, 30, 50, 75, and 100 g/mL active chemicals. After 24–48 h at 37°C for bacteria and 48–72 h at 28°C for fungi, growth plates were inspected to find the lowest antibiotic concentration that inhibited each organism's growth. Mueller-Hinton and Sabouraud medium lacking active chemicals and target microorganisms were employed as controls.

Results

Isolation and Preliminary screening

Kasdir and Naâma, Algeria, soil samples were taken. 1g of soil was dried for actinomycetes isolation. The 23 probable actinomycetes were isolated and purified in ISP-2. The pure colonies were kept at 4°C on ISP-2 slant. The isolated cultures were KSA 1, 2, 3,... and 23. 23 cultures were tested for bacteria and fungus. Antibacterial and antifungal activity was screened by diffusion on Mueller–Hinton for bacteria and YMA and casitone for fungi. In the initial screening, 34% of the strains had weak action, 22% had moderate activity, 25% had promising activity, and 19% had no hostile activity (Table1). 8 strains with good activity were also investigated for cultural features (Table 2). The KSA18 antibacterial chemical suppressed bacteria and fungus development. KSA18 was chosen based on early screening findings to explore its extraction and antibacterial property. KSA18 methanol extract evaluated against bacteria and fungus.

Genetic Identification of KSA18 strain, Phylogenetic Analysis and Clustering

The sequencing results of the 16S rRNA gene of KSA18 straine was compared using BLAST (ZOETENDAL, 2008). Clustal W was used to align these homologous sequences with KSA18's 16S rRNA gene sequence. Figure 1 shows the phylogenetic tree based on 16S rRNA gene sequences using MEGA X (Thompson et al, 1994). illustrating the links between the KSA18 strain and the Streptomyces genus. The 16S rRNA sequence of bacterium KSA18 is 99.17% similar to *Streptomyces lasiicapitis'* sequence (accession number SUB11207111).

Extraction and purification of fermentation products

The KSA18 fermentation took 120 hours at 28 C. The culture supernatant was collected and centrifuged. Ethyl acetate (1:2, v/v) was used for the extraction and afterwards was evaporated until dry to get a yellow staining precipitate. In 50 ml of methanol, 5 g of the precipitate was separated on column chromatography using methanol and acetate ethyl (6:4,v/v). 25 5-ml portions were taken. Active portions ranged from N° 14 to N° 27. Using sil-

Table 1: Primary screening of actinomycetal isolates using agar cylinder method

	Test microorganisms									
	B. subtilis	*S. aureus*	*L. monocytogenes*	*MRSA*	*E. coli*	*P. aeruginosa*	*E. faecalis*	*S. tyfimirium*	*C.albicans*	*A. flavus*
KSA 1	–	–	–	–	–	–	–	–	–	–
KSA 2	–	+	+	–	–	–	+	–	–	–
KSA 3	–	–	–	–	–	–	–	–	–	–
KSA 4	+	+	–	–	+	–	+	–	–	–
KSA 5	–	+	–	–	+	–	+	–	–	–
KSA 6	+	+	+	+	+	–	–	+	–	+
KSA 7	–	–	+	–	+	+	+	–	–	–
KSA 8	+	+	–	–	+	–	–	–	–	+
KSA 9	–	–	–	–	–	–	–	–	–	–
KSA10	+	+	–	–	–	+	–	–	–	–
KSA11	–	+	–	–	–	–	–	+	–	–
KSA12	+	+	+	–	+	–	+	–	–	–
KSA13	–	+	+	–	+	–	–	+	–	+
KSA14	+	–	+	–	–	–	+	–	–	–
KSA15	–	–	–	–	–	–	–	–	–	–
KSA16	+	+	+	–	–	–	+	–	–	–
KSA17	–	+	+	–	–	–	–	+	–	–
KSA18	+	+	+	+	–	+	+	+	–	+
KSA19	+	+	+	+	+	+	+	+	–	+
KSA20	+	+	+	+	–	–	+	+	-	–
KSA21	+	–	–	–	–	+	–	–	+	–
KSA22	–	+	–	–	+	–	–	–	-	–
KSA23	+	+	–	–	–	–	+	+	–	–

– no inhibition; + inhibition

Table 2: Colony characteristics of most actives isolates cultivated on Isp2

Culture code	Colour	Mycelium type	Pigment production	Gram'sreaction
KSA6	White	Substrate	Orange	+
KSA7	White	Aerial	-	+
KSA8	Yellow	Aerial	Red	+
KSA13	Grey	Substrate	Yellow	+
KSA14	White	Aerial	Yellow	+
KSA16	Green	Aerial	-	+
KSA18	White	Substrate	Yellow	+
KSA19	Yellow	Substrate	Yellow	+

Table 3:.Minimum inhibitory concentrations (MIC) of the purified bioactive metabolite isolated from Streptomyces KSA18.

Test organisms	MIC (µg/ml)
Aspergillus flavus	92
Bacillus subtilis	22
Candida albicans	71
Escherichia coli	55
Enterococcus faecalis	26
Listeria monocytogenes	24
MRSA	37
Pseudomonas aeruginosa	62
Staphylococcus aureus	36
Salmonella Tyfimirium	53

ica gel column chromatography, unwanted contaminants and metabolites were removed. Obtained fractions were analyzed by TLC. Ays1 (122 mg) and Ays2 (21 mg) got substantial fractions. Fraction Ays 1 was the most antimicrobial.

Growth and antimicrobial production

The KSA18's synthesis of active metabolites was measured for 120 hours. Antibiotic production was dependent on the growth phase, with the best efficiency of the product in the end of the exponential phase and stationary periods. The highest yield was obtained after 72 hours of incubation with an inhibition zone of 22 mm (Fig. 2).

UV-visible analysis

The Uv-visible spectrum of the crude extract methanol showed distinctive absorption at 205, 210, and 240 nm. UV absorption maxima was detected at 205 nm with another peak at 240 nm, confirming a non-polyenic nature of the molecule (Fig. 3).

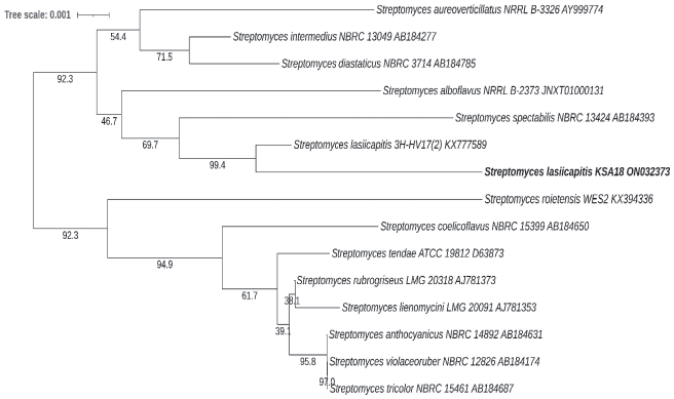

Fig. 1: A Cladogram, using neighbour-joining method of selected 16S rRNA gene sequences of the genus Streptomyses, obtained from BLAST hits, showing relationships between strains KSA18 and some closely related representative members.

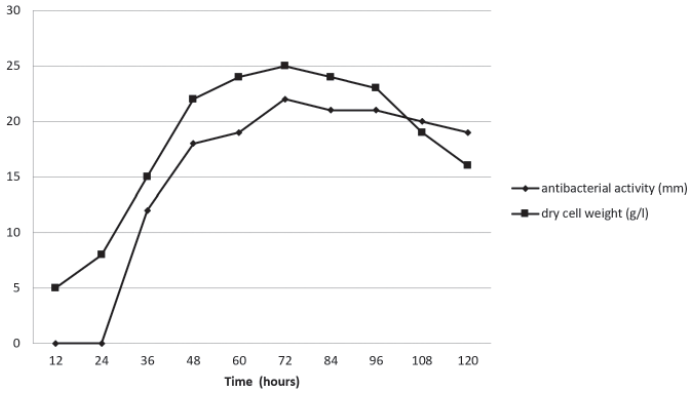

Fig. 2: Time course of growth and antimicrobial metabolites production by Streptomyces KSA18 in ISP2.

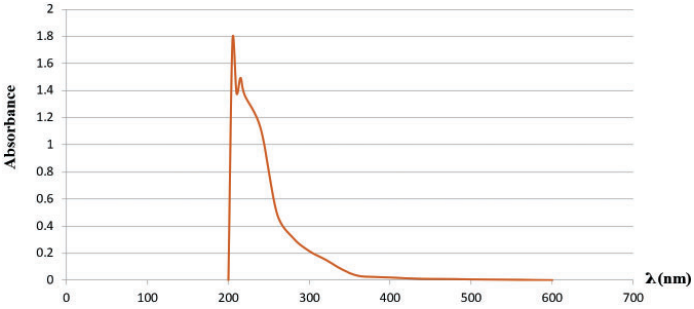

Fig. 3: UV-Visible spectrum of the crude extract of Streptomyces KSA18.

Minimal Inhibitory Concentration

The purified chemical compound showed antibacterial and antifungical activity on a wide spectrum of tested micro-organisms. The MIC for Bacillus subtilis and Listeria monocytogenes was 22 and 24 mg/ml, respectively. The MIC for Gram-negative bacteria was 53–62 mg/ml. The minimal inhibitory concentration of fungus was greatest compared to bacteria (71mg/ml for Candida albicans and 92mg/ml for A. flavus) (Table 3).

GC-MS spectrum

Analysis by GC-MS detected different chemicals (Figs. 4 and 5). 2,4-Bis (1,1-Dimethylethyl) and O-D-glucopyranoside, ß-D-fruc are important chemicals. Other compounds are in table 4.

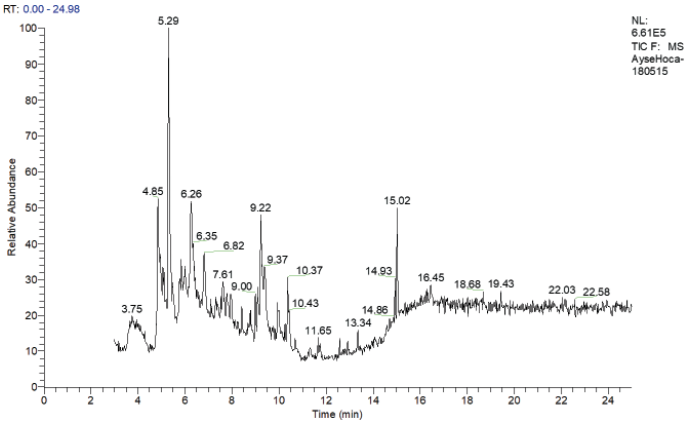

Fig. 4: GC-MS spectrum of the active compound of the crude extract of KSA18.

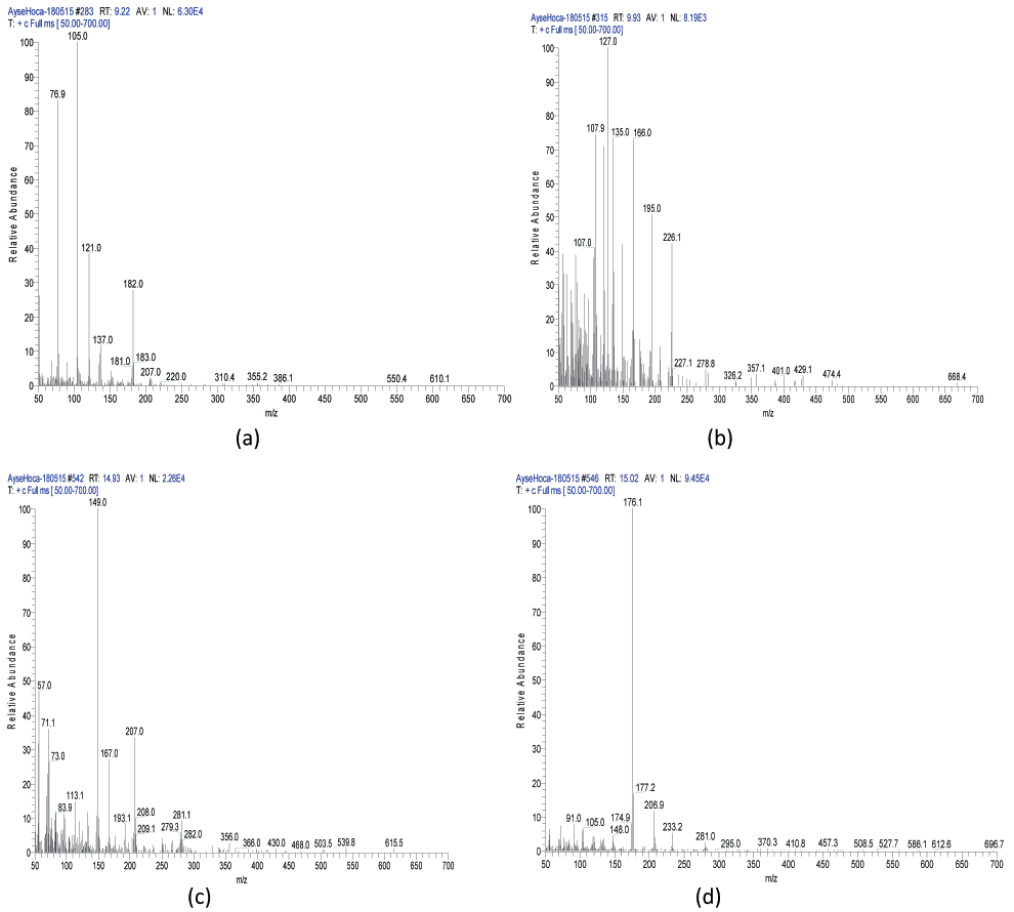

Fig. 5: MS Spectrum of Most important compounds : (a) Benzofuran, 2,3-dihydro, (b) Phenol, 2,4-Bis(1,1-Dimethylethyl) , (c) 3-Isobutylhexahydropyrrolo[1,2-A]Pyrazine-1,4-Dione, (d) 2-methyloctacosane

Table 4: GC-MS identification of bioactive compounds

Peak N°	R. Time	Name	Chemical formula	Molecular weight	Activity
1	4.85	DL-Arabinose	$C_5H_{10}O_5$	150.05	Antivirus activity
2	5.29	Xylitol	$C_5H_{12}O_5$	152.15	Antimicrobial
3	5.82	Glucitol, 6-O-nonyl	$C_{15}H_{32}O_6$	308.41	No activity reported
4	6.26	α- DGlucopyranoside,O-αD-glucopyranosyl-(1.fwdarw.3)-ß-D-fruc	$C_{18}H_{32}O_{16}$	504.40	Anticarcinogenic antimutagenic
5	6.82	N, N-Dimethylglycine	$C_4H_9NO_2$	103.12	Antioxydant
6	7.61	Glycerin	$C_3H_8O_3$		Emulsifiant
7	9.22	Benzofuran, 2,3-dihydro-	C_8H_8O	120.10	anti-inflammatory activity
8	9.29	Maltol	$C_6H_{12}O_3$	126.11	Antioxydant
9	9.37	Phenol, 2,4-Bis(1,1-Dimethylethyl)	$C_{17}H_{30}OSi$	278.50	Anti-inflammatory, antioxydant, antimicrobial
10	10.37	Levomernthol	$C_{10}H_{20}O$	156.26	Antimicrobial
11	14.93	3-Isobutylhexahydropyrrolo[1,2-A]Pyrazine-1,4-Dione	$C_{11}H_{18}N_2O_2$	210.27	Antibacterial
12	15.02	2-methyloctacosane	$C_{29}H_{60}$	408.80	Antimicrobial, antioxydant
13	16.45	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester	$C_{18}H_{28}O$	292.40	Antioxydant
14	18.68	Propanoic acid	$C_3H_6O_2$	74.07	antibacterial
15	19.43	Thieno[3,2-e] benzofuran	$C_{10}H_7NOS$	189.24	Antimicrobial
16	22.03	Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)	$C_{14}H_{16}N_2O_2$	244.29	Antioxydant
17	22.58	Bis (2-ethyl hexyl) phthalate	C24H38O4	390.6	Antibacterial

Discussion

23 actinomycetes were isolated from a poor Algerian field. Isolation of actinomycetes has been difficult compared to bacteria and fungi (KUMAR et al, 2018). It may be attributed to lengthy incubation. Strach casein agar medium with nalidixic acid 100 mgl.l-1 and actidione 20 mg.l-1 inhibited contaminating bacteria and fungi.

The isolation and morphological identification showed that 79% of identified isolates are antibacterial. This study only kept the KSA18 strain because it inhibited most examined bacteria. Morphological, physiological, and biochemical traits matched Streptomyces. KSA18 isolate generated secondary metabolites and gave antimicrobial activity against a wide range of bacteria. VALAN ARASU et al, (2008) found similar results, who recovered Streptomyces spp. (ERI-3) from Western Ghats rock soil. Actinomycete microflora from Saharian soils in southeast Algeria were identified using molecular methods. Then they checked if they killed fungus. Extract of KSA18 Streptomyces showed antibacterial and antifungal activity.

Secondary metabolite production obtained in the end of the exponential phase and continued during the stationary periods. We noticed that the production of antimicrobial compounds was significantly related to the development of the KSA18 strain.

When the crude extract's UV-VIS spectrum was checked against the maximum molecular absorption of polyenes, it was evident that the KSA18 extract's absorption peaks lacked a non polyenic structure. Since polyenes are notorious for being poisonous and difficult to dissolve, this is bad for the screening of novel antifungal compounds (KANDULA & TERLI, 2013).

The TLC results showed that the best composition of solvent used for crude extract is ethyl acetate/methanol. BOUGHACHICHE (2012), found that ethyl acetate-Methanol gives fast migration of actives compounds. Therefore, it gives good split and a suitable purification for the crude extract obtained with methanol.

The GC-MS analysis has shown a great diversity with about 17 different volatile compounds in the active fraction of the methanolic extract. Most between them could be responsible for biological activity. Devi et al. showed antifungal activity of Phenol, 2,4-Bis(1,1-Dimethylethyl) produced by Actinomycete applicable as Growth-promoting for Mangrove (BADJI et al, 2005). 3-Isobutylhexahydropyrrolo[1,2-A] Pyrazine-1,4-Dione have antimicrobial activity (DEVI et al, 2021). 2-methyloctacosane also showed antimicrobial and antioxidant activity in the work of Pelo et al, (2021). Streptomyces albidoflavus produce Dibutyl phthalate which has antimicrobial activity (ABOOBAKER et al, 2009). But this study needs to use different methods to find the structures of different molecules of technological and biological interest found in our extract.

The different MICs obtained for our extract show its effectiveness against most pathogenic microorganisms, which reveals the presence of several interesting molecules

Conclusion

Standard therapies for infections due to multiresistant strains have no longer become sufficient, even 2nd and 3rd line drugs are not active enough against them. It then becomes crucial to seek new bioactive compounds of natural origin in order to continuously stimulate the discovery and development of new drugs. This is why the main goal of this work is the isolation of Actinobacterial strains and the demonstration of their antimicrobial activity. A total of 23 actinomycetes are isolated from Saharan soil. Most of them showed microbial activity. The KSA 18 strain is retained because of their effectiveness to the majority of the pathogens tested. The molecular identification of the strain a makes it possible to classify it in the species Streptomyces lasiicapitis. A submerged fermentation of the selected bacteria allows the production and extraction of metabolites. The GC-MS technique showed a richness of the extract in various bioactive substances. This work deserves to be continued to identify the different chemical structures of the active molecules.

Author Contributions Material preparation, data collection and analysis were performed by MB, DEA and TAB. and MB. KZ and M.B. writing original draft; AO, KG. supervision, review and editing; JW. co-supervised the work; M.B., KZ. conceptualized, revised and corrected the paper. All authors listed have made a substantial, direct and intellectual contribu-tion to the work and approved the work for publication. All authors have read and agreed to the published version of the manuscript.

Funding This research received no external funding

Data Availability The datasets generated and/or analysed during the current study are not publicly available for ethical reasons, as well as privacy reasons, but are available from the author on reasonable request

Code Availability Not applicable.

Declarations

Conflicts of Interest The authors declare no conflict of interest.

Acknowledgment

The authors are grateful to the Departments of Molecular Biology and Genetics, Istanbul University, Turkey, for all their contributions to the realization of this work.

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