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Bioactive compounds from a haloalkalitolerant *Streptomyces* sp. EMSM31 isolated from Um-Risha Lake in Egypt

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ABSTRACT

Wadi Al-Natrun, Egypt is considered an extreme environment, where, salinity and alkalinity are high. It was aimed in this work to explore such an environment for novel actinobacteria producing bioactive compounds. A total of 31 actinobacteria isolated from sediment of Um-Risha Lake at Wadi Al-Natrun were screened for antimicrobial activity against some Gram-positive and Gram-negative bacteria. The most potent isolate was identified based on the phenotypic and phylogenetic analyses using the 16S rRNA gene sequence and compared to those in the publicly available databases. It showed 99% similarity to *Streptomyces violaceoruber* strain EA169 and was identified as *Streptomyces* sp. EMSM31. The antimicrobial activity of this strain was assessed and was highly dependent upon the nutrients and conditions used for cultivation. Strain EMSM31 showed a broad spectrum antagonistic activity against both Gram-positive (*Bacillus subtilis*, *Staphylococcus aureus*) and Gram-negative (*Escherichia coli*, *Klebsiella pneumoniae*, *Enterococcus faecalis*) bacteria recording inhibition zones of 22, 18, 19, 19, and 18 mm, respectively. The maximal activity of secondary metabolite (23mm) was achieved in an optimized production medium containing fructose and yeast extract at pH7 on the fifth day of fermentation at 30°C. Moreover, gas chromatography-mass spectrometry (GC–MS) profiles of produced metabolites revealed apparent differences when compared to the public database of existing natural products. Analysis showed the presence of 3- hydroxy dodecanoic acid (19.49%) as a major compound. This study establishes that streptomycetes from extreme environments may be a rich reservoir for the production of biologically active compounds with pharmaceutical potentials.

INTRODUCTION

Actinobacteria are Gram-positive of high G+C DNA content having the potential to produce many clinically important bioactive molecules. They are the most important source of bioactive natural compounds with a long track record of producing novel molecules **(Amin *et al*., 2020; Maiti *et al*., 2020; Sharma and Thakur, 2020)**. They are ubiquitous in all types of environments, while the soil contains around 90% of total actinobacteria **(Suga *et al*., 2018)**. These organisms have received great attention in recent years as producers of antibiotics, enzymes, and other proteins which have immense

industrial importance (**Inahashi *et al.*, 2011**; **Malisorn *et al.*, 2020**). They are responsible for the production of more than 20,000 natural products widely used in pharmaceutical and agrochemical industries **(Manteca *et al.*, 2008, Charousová *et al.*, 2017).** Among Actinobacteria, *Streptomyces* only produces about 80% of all actinobacteria-derived antibiotics **(Law *et al.*, 2019; Almalki, 2020)**. Members of the genus *Streptomyces* represent significant source for supplying bioactive natural products and produce around 50–55% of known antibiotics with clinical and pharmaceutical applications (**Bao *et al.*, 2018; Sivalingam *et al.*, 2019)**. Aminoglycosides, glycopeptides, anthracyclins, macrolides, β-lactams, nucleosides, peptides, poly ethers, polyenes, and tetracyclines are the most common antibiotics produced by *Streptomyces* **(Namitha *et al.*, 2021)**. More novel molecules with potential therapeutic applications are still on the row to be discovered from actinobacteria **(Cortés-Albayay *et al.*, 2019)**. Undoubtedly, screening, synthesis, and production of bioactive compounds and natural products represent one of the most critical research trends in biopharmaceutical studies around the world **(Axenov *et al.*, 2020)**. These products have long been recognized as mainstay for drugs with a wide variety of biological activities **(Thompson *et al.*, 2017)**. They are considered to be an ideal scaffold for the formulation and production of antibiotics, anticancer agents, antioxidants, enzyme inhibitors, immunomodulators, plant growth hormones, and insect control agents **(Siddharth *et al.*, 2020)**. Therefore, effective drugs are urgently needed in the future, to improve the appropriate use of antimicrobial and to devise methods for discovering new antibiotics through previously untapped targets **(Fikry *et al.*, 2018)**. For this, *Streptomyces* species are one of the most important targets for mining new antibiotics **(Jacob *et al.*, 2017)**.

The existence of natural compound producing microbes is obtained by exploiting the unexplored or non-exploiting habitats **(Al-Dhabi *et al.*, 2019)**. Extreme environments such as hypersaline lakes, inland saline zones, volcanic zones, hyperarids and glaciers are largely unexplored for novel actinobacteria (**Hamedi *et al.*, 2013; Jose and Jebakumar, 2013)**. These unusual environments are promising in the prospect of discovering novel compounds. In this respect, Wadi Al-Natrun, Egypt, is an interesting region for many researchers, we thus initiated a program for the discovery of novel species in this place. In a recent study (**Arayes *et al.*, 2021)**, the bacterial biodiversity of Al-Hammra Lake in Wadi Al-Natrun was reported. Hence, in the present study, we are involved in isolation and characterization of actinobacteria producing antimicrobial secondary metabolites from sediment Um-Risha Lake, Wadi Al-Natrun, Egypt.

MATERIALS AND METHODS

Bacteria

Streptomyces sp. EMSM31 used in this study was isolated from Um-Risha Lake, Wadi Al-Natrun and identified using 16SrDNA. The human pathogenic strains: *Staphylococcus aureus* ATCC-25923, *Enterococcus faecalis* ATCC-29212, *Bacillus subtilis* ATCC-6633

(Gram-positive); *Escherichia coli* ATCC-8739, *Klebsiella pneumoniae* ATCC-13883 (Gram-negative) were obtained from National Institute of Oceanography and Fisheries, Alexandria. The strains were maintained on nutrient agar plates at 4 °C and subcultured every month.

Samples collection and processing

Sediment samples (10g) were collected, from Um-Risha, in sterile bags at 10–15 cm depth, transported to the laboratory and stored in a refrigerator at 4°C. Samples were air dried and mixed with calcium carbonate at the ratio of 1:1, and incubated in closed sterile petri dishes for 7 days at room temperature. High relative humidity was maintained in Petri-dishes by water saturated filter paper (**Wadetwar *et al*., 2013**).

Isolation and maintenance of actinobacteria

Isolation of actinobacteria was performed by the spread plate technique using starch casein agar medium (SCA) **(Küster *et al*., 1964)**. Media was adjusted to pH7 and supplemented with Nystatin (50 µg/ml) and Amoxycillin (10 µg/ml) to inhibit fungal and Gram-negative bacterial growth, respectively **(Ahmad *et al*., 2017)**. One gram of treated sample was added to 9 ml sterilized distilled water and followed by serial dilution up to $10^{-۳}$. One milliliter aliquots of 10^{-3} suspension were spread on SCA plates, incubated at 30 ± 2°C for 7-21 days, and observed regularly for colony development. Colonies with differentiable morphologies were picked up using sterile tooth picks and purified on International *Streptomyces* Project Medium- 2 (ISP-2) **(Shirling and Gottlieb, 1966)**.

Screening for antimicrobial activity

Preliminary screening

The isolates were first screened for antagonistic activity by cross streak method **(Ganesan *et al*., 2017; Talpur *et al*., 2020)**. Briefly the isolates were inoculated in straight line on ISP-2 agar plates and incubated at 30°C for 7 days; the test pathogen was inoculated by a single streak at 90° angles to the line of the producer isolate, and finally the plates were incubated at 37°C for 24 h. The antimicrobial activity was observed by noting the absence or presence of pathogenic bacterial growth and measuring the distance of inhibition in mm.

Secondary screening

The secondary screening was carried using agar well diffusion method **(Pathalam *et al*., 2017)**. A spore suspension (OD_{600} = 0.2) of each strain was prepared, by transferring spores from 14 days old colonies cultured on ISP-2 agar plates to a tube of sterilized distilled water containing 0.05% (v/v) tween 20 **(Thakur *et al*., 2009; Saurav and Kannabiran, 2010)**. The tube was vortexed for 2–3 min to prepare a homogenous suspension ($2x10^6$ /ml). Two ml of the spore suspension of each strain were inoculated into 100 ml Erlenmeyer flask containing 20 ml sterile ISP-2 broth (pH7) and incubated at 30°C for 14 days on a rotary shaking at 120 rpm. The fermented broths were centrifuged

at 4000 rpm for 10 min and 25µl of each supernatant were loaded into 6mm diameter wells made on nutrient agar plates seeded with tested pathogen (OD_{600} = 1±0.1) using a sterile cork borer. The plates were kept in a refrigerator for 2 h to permit homogenous diffusion of the antimicrobial agent before growth of the pathogen and then incubated at 37°C for 24 h. The antimicrobial activity was assessed by measuring the inhibition zone diameter (IZD) in mm. The bioassay was performed in triplicate and the mean value was calculated.

Identification and characterization of isolate EMSM31

The most potent isolate EMSM31 was identified based on phenotypic and genotypic analysis.

Phenotypic Characterization

Morphological signs were carried out according to methods given by the International *Streptomyces* Project (ISP) (**Shirling and Gottlieb, 1966)**. ISP2-ISP7 media were used to determine growth and color of aerial, and substrate mycelia, formation of soluble pigments and melanin production. Micromorphology was examined by cover slip insertion method **(Williams and Cross, 1971; Mohamed *et al.*, 2017)** to observe the spore chain and the shape of spore on ISP-2 media using scanning electron microscopy (SEM). Various physiological criteria and biochemical tests were performed for the identification, including tolerance to pH (6-9) and NaCl levels (0-10%) using ISP-2 medium. Enzymatic profile was determined including catalase, oxidase, amylase, cellulase, protease, mannanase, inulinase, keratinase, xylanase, lipase, and chitinase **(Williams *et al.*, 1983)**. Antibiotic sensitivity was determined as reported by **Maiti *et al.*, (2020)**.

Molecular identification

Genomic DNA was extracted according to the manufacturer's protocol of **Deininger (1990)**. 16S ribosomal DNA was amplified by PCR using universal bacterial primers PA as forward primer (F-5'AGAGTTTGATCITGGCTCAG-3') and PH as reverse primer (R– 5'ACGGITACCTTGTTACGACTT3'). The product amplified by PCR was qualitatively evaluated by agarose gel electrophoresis 1% (w/v) TAE buffer, and then, sequenced. The parameters of the following cycle were used: a) denaturation at 94 °C for 1 min., b) annealing at 55 °C for 1 min., and c) extension at 72 °C for 2 min for 35 cycles. Sequencing was performed by using the BigDye Terminator technique and ABI PRISM 377 DNA Automated Sanger's Sequencer. The sequence was compared for similarity level with the reference strains of actinomycetes from genomic database banks, using the NCBI Blast available at the http://www.ncbi.nlm.nih.gov/ blast web site and analyzed using the CLUSTAL W v.1.4 software available at http://www.ebi.ac.uk/clustalw. 22. The phylogenetic tree was displayed using MEGA7 software **(Kumar *et al.*, 2016)**.

Optimization of *Streptomyces* sp. EMSM31 activity against *B. subtilis*

The one factor at a time approach was adopted to maximize the activity of *Streptomyces* sp. EMSM31. All experiments were performed in 100 ml Erlenmeyer flasks containing 20 ml of sterile broth medium, inoculated with 2ml spore suspension (OD_{600}= 0.2,≈ $2x10^6$ spores/ml) and incubated at 30°C on a rotary shaker (120 rpm). Using the well diffusion method, the activity against *B. subtilis* was expressed as inhibition zone diameter (IZD).

Effect of different fermentation media

Seven types of growth media namely, SC, ISP-2, YGP, MYGP, BENNET, ISP-4 and Gause' synthetic (GSB) were tested **(Ganesan *et al*., 2017)**.

Effect of incubation period

ISP-2 broth was inoculated with strain EMSM31, growth was monitored as dry weight of the mycelium every 48h for 14 days. The biomass was separated after centrifugation at 4000 rpm for 10 min and then was transferred to dry, weighted filter paper, and set in an oven at 60˚C till a constant weight. The amount of growth was expressed as mg/20 ml culture medium according to **Dezfully *et al*. (2015)**. The antimicrobial activity was examined against *B. subtilis*.

Effect of pH

Portions of ISP-2 broth as growth media were adjusted to different pHs (6-9) to improve antimicrobial activity production. The initial pH was adjusted using 0.1 N NaOH/0.1 N HCl.

Effect of carbon source

Glucose, the carbon source in ISP-2 medium (pH7) was substituted with starch, galactose, fructose, sucrose, lactose, maltose and mannitol at 4 g/l concentration. The sugar was sterilized separately by autoclaving and added to the production media in order to study its effect on the production of antimicrobial compound. Media without sugar was also included.

Effect of nitrogen source

Yeast extract, malt extract, peptone, beef extract, tryptone, casein, ammonium chloride, sodium nitrate, ammonium nitrate and ammonium sulfate were added to the medium at a concentration equivalent to the concentration of the basal nitrogen source (yeast extract and malt extract, 0.812 g N/l) to study the effect of different nitrogen sources on antimicrobial activity with fructose as the carbon source.

Thermal stability of the antimicrobial substance

Thermal stability was estimated by exposing aliquots of cell free supernatant at various temperatures (50°C to 100°C) for 15 min. The antibacterial activity was assayed by well diffusion method **(DH and Dhundale, 2013; Fikry *et al*., 2018)**.

Extraction of the bioactive compound(s)

Streptomyces sp. EMSM31 was cultured in the optimized media and incubated at 30°C for 5 days shaken at 120 rpm. Culture media was harvested, centrifuged for 15 min at 8,000 rpm and the collected supernatant was extracted with different solvents such as ethyl acetate, ethanol, methanol, acetone, chloroform, and hexane (1:1, v/v) and shaken vigorously for 1 h for complete extraction **(Mohamed *et al.*, 2017)**. The crude extract was recovered using a rotary evaporator at 60°C and dissolved in the appropriate solvent. The antagonistic activity of the crude extract was determined using the well diffusion method.

Determination of minimum inhibitory concentration and minimum bactericidal concentration of the crude extract

MIC of the extracted product was determined against all the five test pathogens using macro broth dilution method **(Andrews, 2001)**. Test tubes containing 5 ml of nutrient broth were prepared, 100µl of methanol extract (20 mg/ml) were added to the first test tube. Then, serially diluted to reach concentrations of 2, 1, 0.5, 0.25, 0.12, and 0.06 mg/ml followed by the addition of 100µl of overnight test cultures (OD_{600} = (1±0.1) to each tube. The negative control contained inoculated broth without extract. After 24 h incubation at 37°C, bacterial inhibition was determined by recording the optical density at 600 nm. The lowest concentration of extract that inhibited bacterial growth in comparison with a growth control was considered MIC. The MBC was determined by inoculating samples that did not show any growth of pathogens on nutrient agar and observing growth after incubation at 37°C for 24 h. The MBC is the minimum concentration yielding no growth. Gentamycin as a standard antibiotic, was used for comparative effect. All experiments were performed in triplicate.

Hemolytic activity assay

The hemolysis assay was determined according to **Heidarian *et al.* (2019)**. The red blood cells (10%) were collected by centrifugation at 3,500 rpm for 15 min and washed with PBS buffer three times. The crude extract of *Streptomyces* sp. EMSM31 was prepared at final concentrations of 40, 20, 10, 5, and 2.5 mg /ml and then added to the red cell suspension in phosphate buffer saline (PBS) buffer. The mixtures were incubated for 1 h at 37°C and then centrifuged at 1,500 rpm for 5 min. The OD_{545} of the supernatant was measured. Ferrous sulfate solution (65 mM) served as a positive control whereas; buffer alone was the negative control. The average value was calculated from triplicate assay. The % hemolysis was calculated by using the formula **(Chalasani *et al.*, 2015)**:

$$\text{Hemolysis } \% = \frac{\text{Test} - \text{negative control}}{\text{Positive control} - \text{negative control}} \times 100$$

Identifying the bioactive substance via GC–MS analysis

Gas chromatography-mass spectrometry (GC-MS) was used for the detection of the individual components present in the methanolic extract of *Streptomyces* sp. EMSM31 as previously described **(Sharma and Thakur, 2020; Al-Dhabi *et al.*, 2020)**. The percentage of each compound was calculated as the ratio of the peak area to the whole chromatographic area. GC-MS peaks were assigned through comparison with several pronounced information and similarity percentages have been detected from the Wiley 275 libraries.

RESULTS

Isolation of actinobacteria and screening for antimicrobial activity

A total of 31 morphologically unique colonies developed on starch casein agar plates were screened for antimicrobial activity against some human pathogens using cross streak method on ISP-2 agar plates. Only 10 isolates (32.25%) showed good antimicrobial activity against Gram positive (*B. subtilis* ATCC-6633, *S. aureus* ATCC-25923), Gram negative (*K. pneumoniae* ATCC-13883, *E. coli* ATCC-8739, and *E. faecalis* ATCC-29212) **(Fig. 1**). Four strains were chosen for secondary screening. Based on maximum inhibition zone, isolate EMSM31 (**Fig. 2)** was selected for further experiments, using *B. subtilis* ATCC6633 as an indicator bacterial strain.

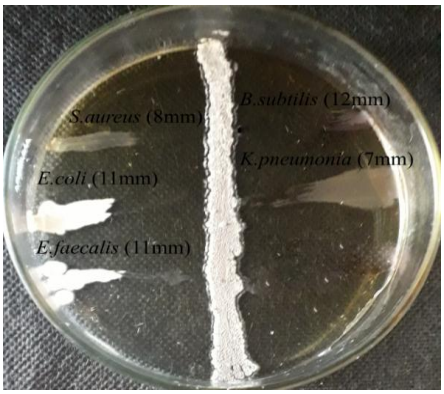

Fig. 1. Primary screening of isolate EMSM31 for antimicrobial activity using perpendicular streak method.

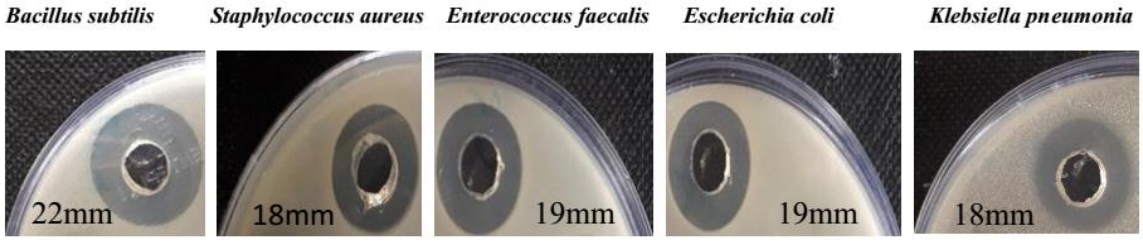

Fig. 2. Antibacterial activity of EMSM31 supernatant against some pathogens expressed as IZD. The strain was grown on ISP-2 media for 14 days at 30°C at 120 rpm.

Identification of isolate EMSM31

The bacteria formed colonies of whitish gray filamentous aerial mycelium on ISP-2 agar plates; sporulation was observed after 2 to 3 days of incubation. No diffusible pigment was detected on the same media **(Table 1)**. Scanning electron micrograph showed spore chain like a hook (Retinaculum-Apertum) carried on aerial mycelia with warty spore surfaces **(Fig. 3)**. These morphological characteristics led to strongly suspect that EMSM31 is a member of genus *Streptomyces.* Physiological and biochemical characteristics are summarized in **Table "2"**.

Table 1. Morphological characteristics of EMSM31 on different media after 14 days of incubation at 30°C.

Medium	Growth	Aerial mycelium	Substrate mycelium	Spores	Soluble pigment
ISP1	Good	Moderate, Whitish grey	Light brown	Whitish grey	None
ISP2	Good	Abundant, Whitish grey	Dark brown	Whitish grey	None
1SP3	Good	Abundant, Grey	Black	Grey	None
ISP4	Good	Abundant, Grey	Black	Grey	None
ISP5	Poor	None	White	None	None
ISP7	Poor	None	White	None	None

Abbreviations: ISP-1-7: International Streptomyces Project medium

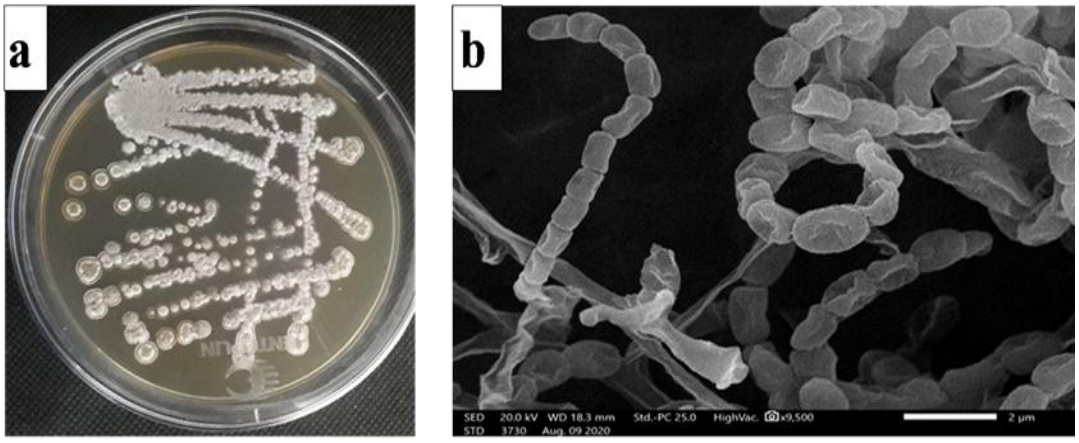

Fig. 3. Colony morphology of 14 days old culture of EMSM31 on ISP-2 (a) scanning electron micrograph X 9,500 (b).

Table 2. Some phenotypic characteristics of EMSM31

Character/ test	Result
Morphological characteristics	
Sporophore morphology	Retinaculum-Apertum
Spore	Doliform
Biochemical characteristic	
Catalase test	+
Oxidase test	-
Melanin production	-
Enzyme production	
Protease	+
Mannanase	+
Xylanase	+
Cellulase	+
Amylase	+
Inulinase	+
Lipase	+
Keratinase	+
Chitinase	+
Growth at:	
pH range, optimum	(6 – 9) opt. (7)
NaCl (%; w/v), optimum	(0 – 9 %) opt. (0%)
Antibiotic sensitivity	
Penicillin	R
Amoxicillin /Clavulanic acid	R
Rifampicin	R
Gentamycin	S
Streptomycin	S
Doxycycline	S

*R = resistant and S = sensitive

Phylogenetic analysis

Analysis of partial 16S rRNA gene sequence (1037 bp) of EMSM31 showed 99% similarity to *Streptomyces violaceoruber* strain EA169. The evolutionary tree (**Fig. 4**) represents the phylogenetic affiliation of EMSM31 with other *Streptomyces* strains. The 16S rDNA sequence was deposited in GenBank under accession number MW722937. The strain was thus designated as *Streptomyces* sp. EMSM31.

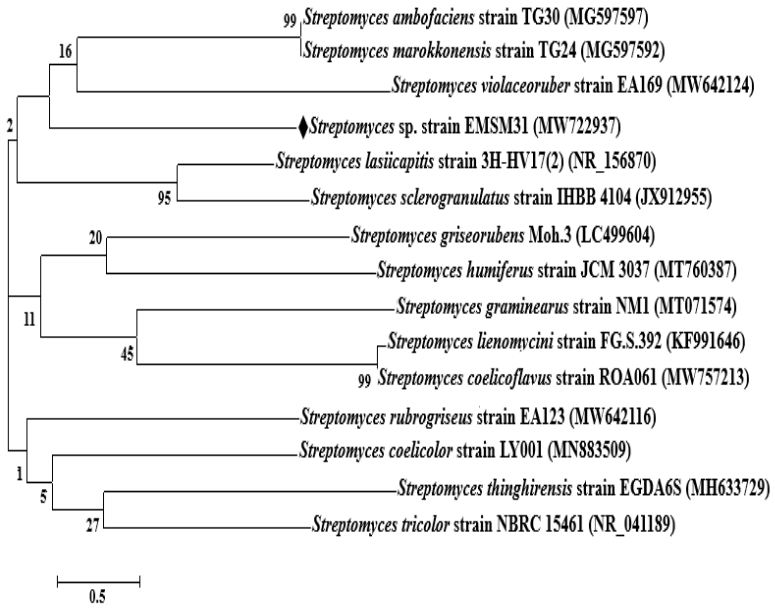

Fig. 4. A phylogenetic tree showing the relationship between EMSM31 and related taxa based on 16S rDNA sequences. Numbers at nodes are bootstrap percentages based on 1000 replications. The bar indicates 0.5 nucleotide substitutions per site.

Optimization of *Streptomyces* sp. EMSM31 activity against *B. subtilis*

Among the different fermentation media examined, ISP-2 broth supported the production of bioactive compound of maximum antimicrobial activity (IZD of 22mm) against *B. subtilis* **(Fig. 5)**. The production of bioactive compound was growth associated with highest titer at the end of the second phase of growth **(Fig. 6)**.

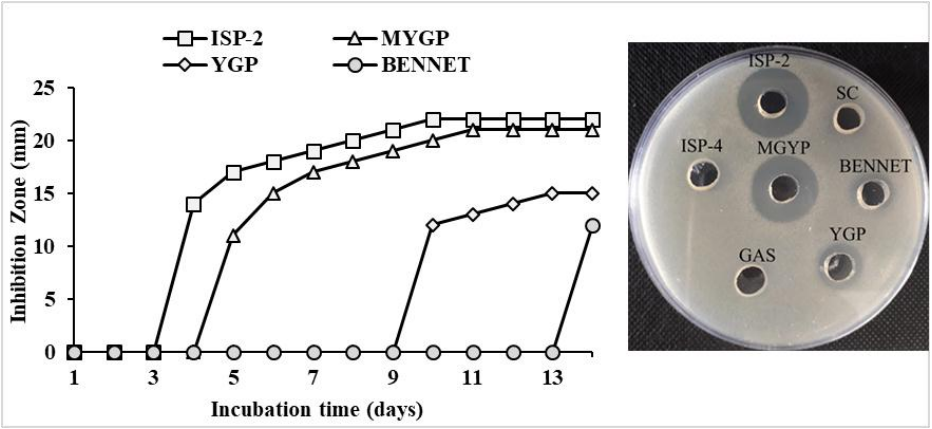

Fig. 5. Inhibition zone (mm) of *St.* sp. EMSM31culture filtrates obtained from different growth media against *B. subtilis* ATCC-663. Inset shows bioactivity of 14 days old *St.* sp. EMSM31 culture supernatant against *B. subtilis.*

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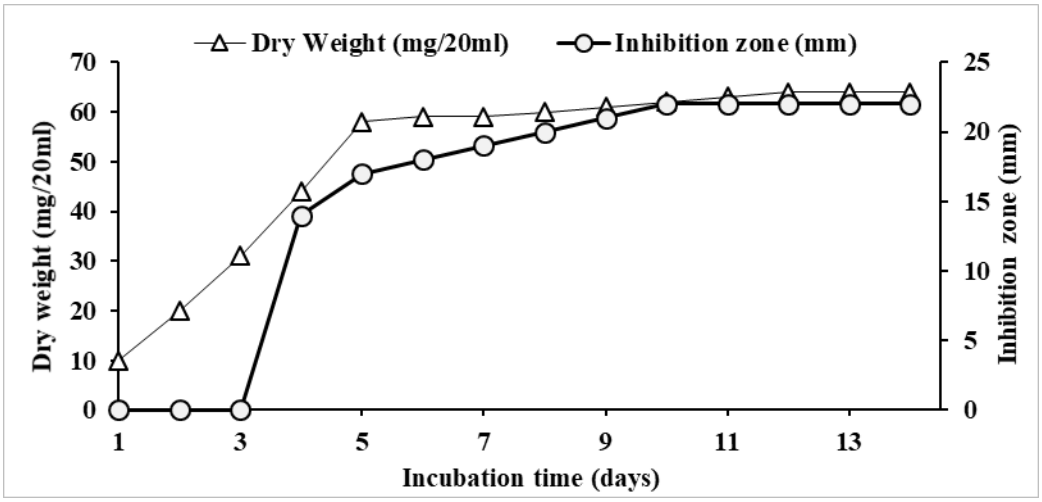

Fig. 6. Effect of incubation time on growth and production of *Streptomyces* sp. EMSM31 bioactive compound against *B. subtilis* ATCC-6633.

Adjusting the initial pH of fermentation media to 7, 8, and 9 resulted to the production of antibacterial compounds with highest activity (IZD 22mm) after 10 days of incubation **(Fig. 7**), confirming the alkalitolerant nature of *Streptomyces* sp. EMSM31.

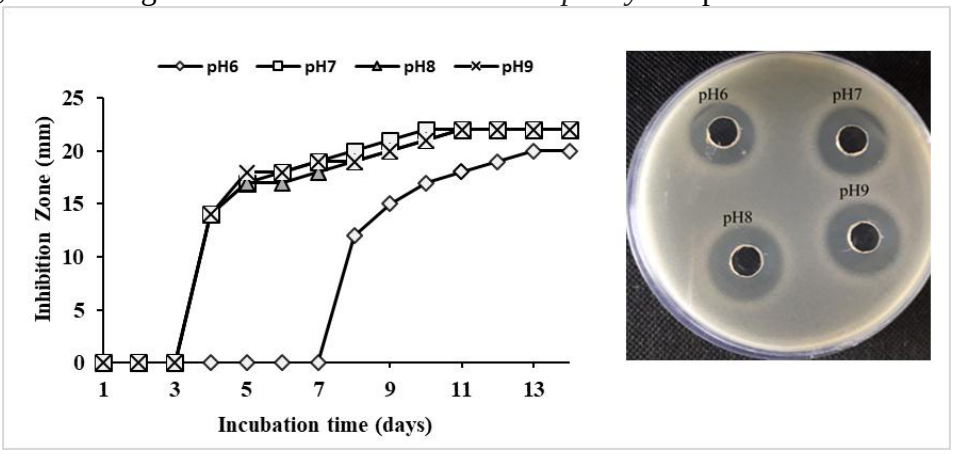

Fig. 7. Antagonism of *St.* sp. EMSM31 culture supernatant against *B. subtilis* ATCC-6633 as affected by the initial pH value of ISP-2 broth after incubation at 30°C on rotary shaker at 120 rpm. Inset, visualized the antibacterial bioactive compound inhibition zone from *Streptomyces* sp. EMSM31 against *B. subtilis.*

Among the tested carbon sources, fructose gave the highest zone of inhibition (22mm) against the indicator strain at the 5th day of incubation **(Fig. 8**). All the carbon sources used for the study supported antimicrobial activity of *Streptomyces* sp. EMSM31.

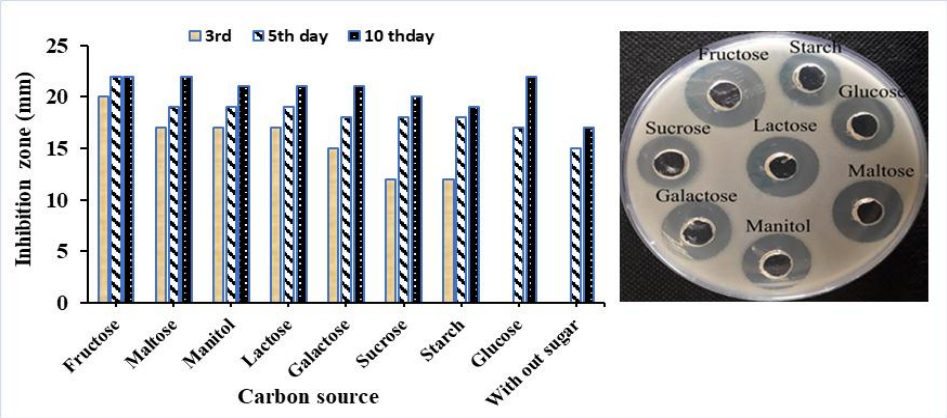

Fig. 8. Effect of carbon source on *St.* sp. EMSM31 bioactive compound against *B. subtilis* ATCC- 6633 after different incubation periods at 30°C on rotary shaking at 120 rpm.

Among the evaluated nitrogen sources, no activity was observed upon using peptone, casein, and inorganic sources containing media. Maximum activity (23mm) was recorded with yeast extract containing medium followed by malt extract and yeast + malt extract containing media **(Fig. 9**).

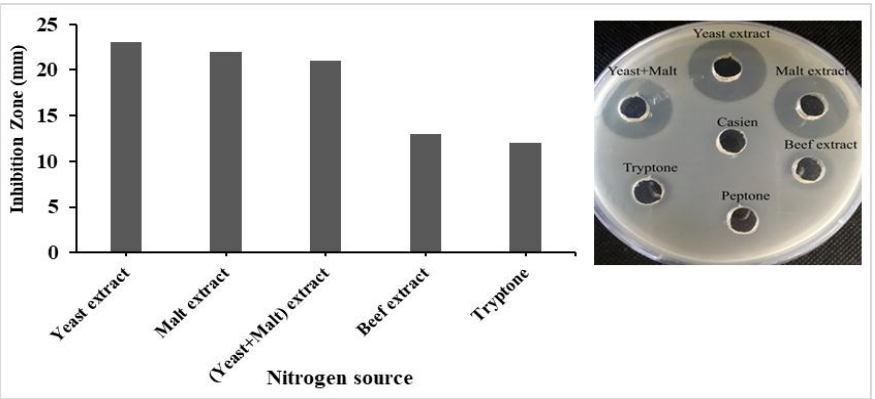

Fig. 9. Effect of nitrogen source on *St.* sp. EMSM31 bioactive compound against *B. subtilis* ATCC-6633 after 5 days of incubation at 30°C on rotary shaking 120 rpm.

Extraction of *Streptomyces* sp. EMSM31 bioactive compounds

The thermal stability of the supernatant was first tested before extraction by heating at various temperatures (50°C to 100°C). The filtrate was thermally stable at all heat treatments even after autoclaving for 15 min. Successful extraction of the bioactive compound was achieved using the water-miscible solvents such as acetone, ethanol, and methanol with variable degrees but not with ethyl acetate, hexane, or chloroform. The maximum activity was recorded upon using culture filtrate: methanol at a ratio of 1:1 (v:v).

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The efficacy of *Streptomyces* sp. EMSM31 methanolic extract was generally evaluated in terms of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The results showed potent antimicrobial action with an MIC range between 0.25mg/ml and 0.5mg/ml and MBC of 0.5mg/ml to 1.0 mg/ml against indicator strains. However, the MIC of gentamicin ranged between 0.06 mg/ml to 0.12 mg/ml and MBC of 0.12 mg/ml to 0.25 mg/ml against same indicator strains **(Table 3)**. Interestingly the standard gentamicin showed MIC and MBC values lower than the crude extract. The results demonstrated that *Streptomyces* sp. EMSM31 crude extract contained particular molecules that inhibit the growth of the Gram positive and Gram-negative microbial pathogens. Therefore, the methanolic extract was submitted to identification by chromatographic techniques.

Table 3. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of strain EMSM31 methanolic extract

Indicator strain	MIC (mg/ml)		MBC (mg/ml)	
	Methanolic extract	Gentamycin	Methanolic extract	Gentamycin
S. aureus (ATCC-25923)	0.50	0.06	1.0	0.12
B. subtilis (ATCC-6633)	0.25	0.06	0.5	0.12
E. faecalis (ATCC-29212)	0.25	0.06	0.5	0.12
E. coli (ATCC-8739)	0.50	0.12	1.0	0.25
K. pneumoniae (ATCC-13883)	0.25	0.06	0.5	0.12

Hemolytic activity

As the antimicrobial substances are used for biomedical applications to treat infectious diseases, it is essential to evaluate the degree of hemolysis on freshly drawn human red blood cells. The bioactive compound showed no hemolytic activity even at 40mg/ml concentration against human erythrocytes, indicating the non-toxic of the extracted compound.

Identification of *St.* sp. EMSM31 major metabolites using gas chromatography mass spectrometry (GC–MS) analysis

The metabolic profiling of EMSM31 extract was analyzed by Gas chromatography mass spectrometry (GC–MS) (**Fig. 10)**. The detailed identified chemical compounds of the extract are presented in **Table "4"**. The main chemical constituents observed were 3-hydroxy dodecanoic acid (19.49%), 1,4-dimethyl-3-n-octadecylcyclohexane (16.34%), 16-nitrobicyclo [10.4.0] hexadecan-1-ol-13-one (10.99%), decanoic acid, 10-(2-hexylcyclopropyl) (5.65%), Z,Z,Z-4,6,9-nonadecatriene (5.00%), 2-(2-butynyl) cyclohexanone (4.68%), and octadecanoic acid, 9,10-epoxy-, cis- (4.48%).

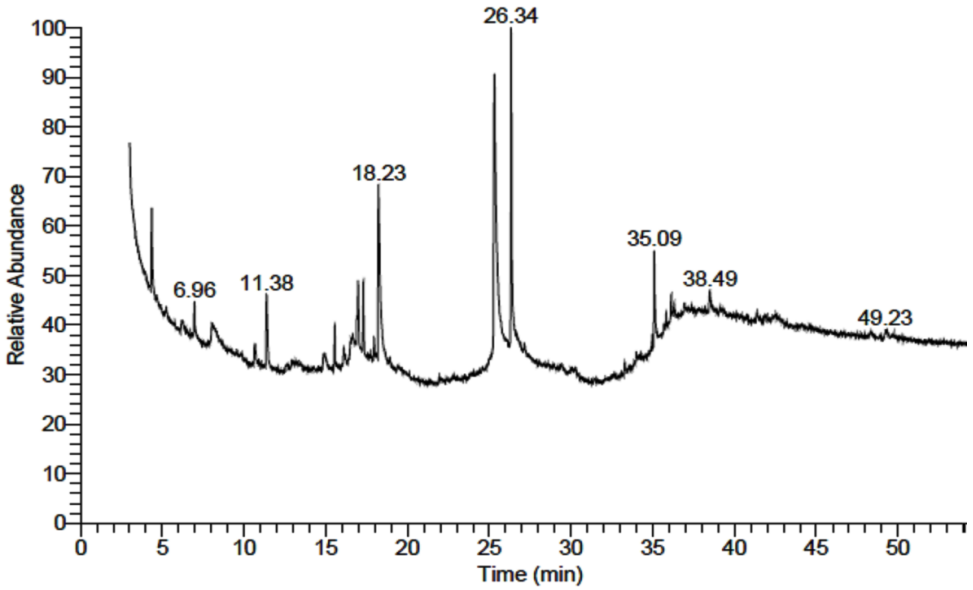

Fig. 10. GC–MS spectrum of *St.*sp. EMSM31 methanolic extract.

Table 4. Chemical compounds detected in methanol extract of *Streptomyces* sp. EMSM31 culture supernatant using GC-MS analysis.

Compound name	Retention time	Area%	Chemical Formula	Molecular weight
Octadecanoic acid, 9,10-epoxy-, cis-	4.35	4.48	$C_{18}H_{34}O_3$	298
Docosahexaenoic acid, 1,2,3-propanetriyl ester	5.24	0.62	$C_{69}H_{98}O_6$	1022
Milbemycin B, 6,28-anhydro-15-chloro-25-isopropyl-13-dehydro-5-O-demethyl-4-methyl	6.20	1.49	$C_{33}H_{47}ClO_7$	590
Docosahexaenoic acid, 1,2,3-propanetriyl ester	6.97	2.11	$C_{69}H_{98}O_6$	1022
Gibberellic acid	8.02	0.53	$C_{19}H_{22}O_6$	346
Gibberellic acid	8.08	2.35	$C_{19}H_{22}O_6$	346
Milbemycin B, 6,28-anhydro-15-chloro-25-isopropyl-13-dehydro-5-O-demethyl-4-methyl	10.66	1.49	$C_{33}H_{47}ClO_7$	590
Z,Z,Z-4,6,9-Nonadecatriene	11.38	5.00	$C_{19}H_{34}$	262
2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trienyl]cyclohex-1-en-1-c arboxaldehyde	14.87	2.29	$C_{23}H_{32}O$	324
Arachidonic acid methyl ester	15.55	2.43	$C_{21}H_{34}O_2$	318
Gibberellic acid	16.10	1.19	$C_{19}H_{22}O_6$	346
[5,9-Dimethyl-1-(3-phenyl-oxiran-2-yl)-d eca-4,8-dienylidene] -(2-phenyl-aziridin-1-yl)-amine	16.20	0.42	$C_{28}H_{34}N_2O$	414
Pseudosolasodine diacetate	16.53	0.95	$C_{31}H_{49}NO_4$	499
Paromomycin i	16.58	0.51	$C_{23}H_{45}N_5O_{14}$	615
Paromomycin i	16.65	1.18	$C_{23}H_{45}N_5O_{14}$	615
2-(2-Butynyl) cyclohexanone	16.98	4.68	$C_{10}H_{14}O$	150
7-(2,4-Dinitrophenoxy)-2,2-dimethyl-2,3-dihydro-1-benzofuran	17.30	3.62	$C_{16}H_{14}N_2O_6$	330
Pseudosolasodine diacetate	17.72	0.64	$C_{31}H_{49}NO_4$	499
10,13-Octadecadiynoic acid, methyl ester	17.96	1.23	$C_{19}H_{30}O_2$	290
16-Nitrobicyclo [10.4.0] hexadecan-1-ol-13-one	18.22	10.99	$C_{16}H_{27}NO_4$	297

2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trienyl]cyclohex-1-en-1-carboxaldehyde	18.91	0.58	$C_{23}H_{32}O$	324
3- Hydroxy dodecanoic acid	25.31	19.49	$C_{12}H_{24}O_3$	216
1,4-Dimethyl-3-n-octadecylcyclohexane	26.34	16.34	$C_{26}H_{52}$	364
Ethyl iso-allocholate	27.15	0.85	$C_{26}H_{44}O_5$	456
2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl) 1hexa-1,3,5-trienyl]cyclohex-1-en-1-carboxaldehyde	33.30	0.78	$C_{23}H_{32}O$	324
2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trienyl]cyclohex-1-en-1-carboxaldehyde	34.00	0.75	$C_{23}H_{32}O$	324
N-[(2-Nitrophenyl) sulfanyl] leucine	34.96	0.74	$C_{12}H_{16}N_2O_4S$	284
Decanoic acid, 10-(2-hexylcyclopropyl)	35.09	5.65	$C_{19}H_{36}O_2$	296
9,12,15-Octadecatrienoic acid, 2-phenyl-1,3-dioxan-5-yl ester	35.82	0.88	$C_{28}H_{40}O_4$	440
9,12,15-Octadecatrienoic acid, 2-phenyl-1,3-dioxan-5-yl ester	36.15	1.44	$C_{28}H_{40}O_4$	440
Gibberellic acid	36.31	0.80	$C_{19}H_{22}O_6$	346
Triamcinolone Acetonide	36.96	0.61	$C_{24}H_{31}FO_6$	434
Androst-5,7-dien-3-ol-17-one	38.49	1.79	$C_{19}H_{26}O_2$	386
Lycopene, 1,1',2,2'-tetrahydro-1,1'-dimethoxy-, all-trans-	41.36	0.57	$C_{42}H_{64}O_2$	600

DISCUSSION

Searching for untapped producers of novel antimicrobial compounds, unexplored extreme environments attracted attention for the isolation and screening of bioactive novel actinobacteria **(Patel *et al*., 2014)**. This study aimed for discovering actinobacterial strains producing novel bioactive compounds from Wadi Al-Natrun, an extreme environment in Egypt characterized by high salinity and alkalinity. Thirty isolates were recovered on starch casein medium, the most suitable media reported for the isolation of actinobacteria (**Ramesh and Mathivanan, 2009; Rajaram *et al*., 2020)**.

Applying the cross-streak method as a preliminary screening, 10 isolates (32.25%) showed potential antagonism against Gram positive (*B. subtilis* ATCC-6633, *S. aureus* ATCC-25923) and Gram negative (*K. pneumoniae* ATCC-13883, *E. coli* ATCC-8739, *E. faecalis* ATCC-29212) bacteria. *B. subtilis* was the most sensitive to antimicrobial compounds tested. Based on the secondary screening by agar well diffusion method, isolate EMSM31 showed the highest antimicrobial activity against *B. subtilis* (22mm) after 10 days of incubation.

Morphological, biochemical, and physiological characteristics are commonly used for preliminary identification **(Taddei *et al*., 2006; Masand *et al*., 2018)**. Phenotypic data was confirmed by 16S rDNA sequencing and showed 99% sequence similarity to *Streptomyces violaceoruber* strain EA169 and the strain was designated as *Streptomyces* sp. EMSM31. Procurement of actinobacteria, especially *Streptomyces* from previously unexplored habitats and the investigation of their biosynthetic potential have gained much attention in the renewed antibiotics search programs **(Masand *et al*., 2018)**. *Streptomyces* is an undoubtedly potent genus to hunt for novel pharmaceutically essential compounds

derived from underexplored extreme environment habitats for next-generation drugs to counteract the worldwide increase of drug resistance and to meet the demand for novel drugs with no or fewer side effects. **Cortés-Albayay *et al.* (2019)** discovered novel *Streptomyces* from Atacama Desert of inhibit growth of Gram-positive and Gram-negative bacteria and fungi and showed also the cytotoxicity against hepatocellular carcinoma and or mouse fibroblast cell lines.

The optimized production of secondary metabolites generally involves the selection of suitable fermentation medium. In this study, out of seven fermentation media tested, cell filtrate from ISP-2 broth showed maximum antimicrobial activity. ISP-2 medium is regarded as a good production medium in many publications (**Badji *et al.*, 2006 and 2007; Maataoui *et al.*, 2014; Ganesan *et a*l., 2017)**. This could be related to the chemical composition of the medium. Further, the production of bioactive compound was found to be growth associated and compounds produced were maximized at the end of the second phase of growth. This result is in good agreement with many previous studies on other *Streptomyces* species **(Bouras *et al.*, 2013; Sharon *et al*., 2014; El-Waseif *et al.*, 2018)**. The pH of the culture medium influences numerous cellular processes like regulation and biosynthesis of secondary metabolites **(Al-Askar *et al.*, 2013).** Surprisingly, *St.* sp. EMSM31 had the ability to grow and produce the antimicrobial compound at a pH range 7-9. This may be related to the high alkalinity of Wadi Al-Natrun soil. Similarly, **Dezfully *et al.* (2015)** reported that *Streptomyces flavogriseus*, strain ACTK2 could grow and produce the antimicrobial compound up to pH 10.0.

All the carbon sources used for this study supported antimicrobial production, however, the results demonstrated that fructose supported the highest yield in shorter time. The obtained results are in agreement with those reported by **Li *et al*. (2015)**, who proved that fructose was the most favorable carbon source for AP-3 production by *Actinosynnema mirum* compared to other carbon sources. Among the evaluated nitrogen sources, yeast extract slightly increased the antimicrobial production and activity of EMSM31 strain. These results agree with those of some authors asserting that the highest antimicrobial activity from *Streptomyces* sp. was obtained with yeast extract (**Ripa *et al.*, 2009; Abdelwahed *et al.*, 2012**). It may be due to its content of nitrogenous compounds, sulfur, carbon, vitamin B complex, trace nutrients, and other essential growth factors for microorganisms **(Zarei *et al*., 2016; Li *et al*., 2020)**. Organic nitrogen sources supported the production of bioactive metabolite as compared to inorganic nitrogen sources, a result supported by the wok of other researchers **(Thakur *et al*., 2009; Bundale *et al*., 2015)**.

Moreover, the bioactive compound produced by *Streptomyces* sp. EMSM31 strain was heat stable even at autoclaving for 15 min. These results are in agreement with **Traub *et al.* (1995)** who reported that the antimicrobial compounds such as beta-lactam, oxacillin and ozloscine produced by actinobacterial species are thermally stable and tolerated autoclaving for 15 min. **Kouadri *et al.* (2014)** as well reported that strain S34

related to *Streptomyces rochei* produced thermal stable antimicrobial activity. In the current study, methanol proved to be the best solvent for extraction of antimicrobial substances of *Streptomyces* sp. EMSM31 strain. Similarly, **Omran *et al*. (2016)** found that methanol was the most effective solvent for extraction of antimicrobial substances from rare actinobacteria *Pseudonocardia alni*.

The standard gentamicin showed MIC and MBC values lower than the methanol extract of *St.* EMSM31. Our finding is similar with the result of **Pudi *et al*. (2016), Adeyemo *et al*. (2020) and Al-Dhabi *et al*. (2020)** who found that the crude extracts produced from actinobacteria showed significantly higher MIC and MBC values against indicator strains comparable to standard antibiotic, streptomycin or gentamycin. This may be due to the degree of purity of the antimicrobial substances and the different indicator strains used. **Vijayakumar *et al*. (2012**) reported that the MIC for a given agent is not constant because it is influenced by the type of organism used, inoculum size, extract concentration, and aeration.

Furthermore, the mehanol extract showed no hemolytic activity against human red blood cells even at a concentration as high as 40 mg/ml. **El-Shahidy *et al*. (2015)** found that most of *Actinomadura* sp. had no hemolytic effect. However, **Heidarian *et al.* (2019)** reported that the extract of *Glycomyces sediminimaris* UTMC 2460 had hemolytic toxicity on human red blood cells at concentration of 300 and 400 µg/ml . The absence of hemolytic toxicity would make the antimicrobial compound from *Streptomyces* sp. EMSM31 a potential candidate for human use as a food preservative and oral disinfectant.

The chemical analysis of the extract showed that fatty acids were the dominant compounds. **Tanvir *et al*. (2018)** reported fatty acids and their derivatives as therapeutic agents that cover a wide range of indications such as cancer, bacterial infections, parasitic infection, inflammations to name a few. **Mao *et al*. (2020)** reported that 3-hydroxydecanoic acid and decanoic acid, isolated from *Lactobacillus plantarum* had good antibacterial activity. Fatty acids had been reported to inhibit bacterial growth by changing the permeability of bacterial membrane or inhibition of fatty acid synthesis (**Zheng *et al*., 2005; Teh *et al*., 2017**). Octadecanoic acid, 2-hydroxy-1,3-propanediyl ester and hexadecanoic acid, 2,3-dihydroxypropyl ester detected in the crude extracts of *Streptomyces* sp. SCA3-4 by GC–MS analysis, possessed antimicrobial activity (**Qi *et al*., 2019**). **Narendhran *et al.* (2014)** reported that the presence of palmitic acid and hexadecanoic acid in the fractions must also be responsible for the antimicrobial, antioxidant and anticancer activity of *S. roxburghiana*. **El-Naggar *et al*. (2017)** detected 2-decenoic acid, octadecanoic acid and octadecanoic acid methyl ester from *Streptomyces anulatus* NEAE-94 by GC–MS analysis. They act as antibiotic against multidrug-resistant *Staphylococcus aureus*. **Kumar *et al*. (2010)** reported that the 9,12,15-octadecatrienoic acid has antimicrobial, antioxidant anticancer, hypercholesterolemic, and antiulcerogenic.

The antimicrobial potentials of the extract may be attributed to the individual or synergistic effect of the above- mentioned chemical groups.

CONCLUSION

This study was a trial to explore extreme habitats for interesting streptomycetes as the possible candidates for the discovery of novel antimicrobial compounds. Extreme environments could be a rich source of biologically potential actinobacteria, which have the capabilities of producing secondary metabolites with antimicrobial functions against different pathogens. Our results highlighted that the most potent isolate *Streptomyces* sp. EMSM31 produced bioactive compounds antagonistic to a number of pathogens. The results suggested the possibility of using this strain as a candidate for use as a food preservative and oral disinfectant due to non-toxicity to the human red blood cells. The chemical analysis of the extracts revealed that fatty acids were the dominant compounds, which cover a wide range of indications such as cancer, bacterial infections, parasitic infection, inflammations, diabetes, and obesity. There is a need to increase the researches on actinobacteria to prepare for economic production of bioactive compounds for industrial and pharmaceutical applications in the current and future scenario.

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