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IDENTIFICATION AND CHARACTERIZATION OF DIFFERENT POTENTIALLY ANTIBACTERIAL COMPOUNDS FROM A MARINE *STREPTOMYCES* SP. SP1

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ABSTRACT

Due to the overuse of antibiotics and the rise in the frequency of multidrug-resistant bacteria (MDR), the development of novel antibiotics is one of the most pressing needs today. In the present study, the marine actinomycete *Streptomyces* sp. Sp1 was isolated for the first time from Lake Burullus, Nile Delta, Egypt. *Streptomyces* sp. Sp1 was identified via morphological and biochemical techniques. Furthermore, it was identified on the basis of 16S rRNA gene sequence that was analyzed using the BLAST-N tool (Basic Local Alignment Search Tool-Nucleotides) from the NCBI website (National Center for Biotechnology Information). *Streptomyces* sp. Sp1 exhibited high antibacterial activity against three serious multidrug resistant pathogens, *Listeria monocytogenes*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The potential antibacterial compounds produced by *Streptomyces* sp. Sp1 were analyzed using gas chromatography-mass spectroscopy (GC-MS). It confirmed the presence of five bioactive compounds; broxyquinoline, 9-aminoacridine, 9,10-anthracenedione, harmine and ricinoleic acid that have different antimicrobial mechanisms. The great antibacterial activity of *Streptomyces* sp.Sp1 could be explained by combination of the different antimicrobial mechanisms of these compounds.

Keywords: *Streptomyces* sp.SP1, characterization, antibacterial, identification
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INTRODUCTION

Actinomycetes are Gram-positive bacteria that are characterized by high G+C (>55%). Actinomycetes are regarded as the connecting ring between bacteria and fungi due to their prokaryotic structure and growth behavior (Blunt *et al.,* 2007). Actinomycetes can be found in a variety of habitats, including surface soils, deep soils, fresh water, marine water, sewage, air, and the surface of plants, but they are more prevalent in soil than in other habitats. In comparison to other genera in soil, *Streptomyces* is the most dominant (Sanglier *et al*., 1993). Many insecticides, antifungals, anthelmintics, anticancers and antibiotics produced by actinomycetes have been isolated and identified. For this reason, actinomycetes are considered as one of the potential pathways for obtaining new bioactive compounds that are able to overcome the multidrug resistance in pathogenic bacteria (Jagannathan *et al*., 2021). Bérdy, (2005) reported that more than 7600 bioactive compounds have been produced and identified by *Streptomyces* species alone. Although most research is interested in soil-isolated species, they have mostly been exhausted as a source of easily new detectable bioactive metabolites. In contrast, marine actinomycetes started to be the effective and realistic source for new unidentified bioactive metabolites, where many marine species remain unexplored (Baltz, 2008). As more than 70% of bacterial pathogens exhibit resistance to at least one existing antibiotic (Sharma *et al*., 2018), the demand for novel antibiotics becomes essential against the multidrug resistant pathogens. These bacteria are usually associated with hospital infections, so these bacteria have become one of the main causes of infection in the community. With the spread and development of multidrug-resistant bacteria, which has resulted in an increase in the use of ineffective antibiotics, as well as morbidity and mortality rates and health-care costs, it has become necessary to isolate new strains of actinomycetes and extract new effective antibiotics with multiple destroying mechanisms against multidrug-resistant pathogens (Duin and Paterson, 2016). Therefore, the present study aims to investigate the antibacterial activity of the marine actinomycete strain *Streptomyces* sp. Sp1 and its novel bioactive compounds.

MATERIALS AND METHODS

Sampling and isolation : Marine sediment samples were collected of deep with 5–15 cm from three different locations of Burullus Lake, north of Egypt, during the Summer 2018. Samples were air-dried at 35°C for 34 h, crushed and sieved via 2 mm pores. Soil particle sizes of 0.1 to 2 µm were used for isolation. One gram of sieved soil particles were suspended in 9 ml of sterile distilled water (Saadoun *et al*., 1999 and Williams *et al*., 1983). Serial dilutions were carried out up to 10-4 dilutions. A

50 µl from each dilution was spread on starch nitrate agar and incubated at 30°C for 7 days (Williams and Davis, 1965). Obtained colonies were purified and investigated.

Morphological and biochemical characterizations : The texture, aerial mycelium, substrate mycelium, growth rate and colour of colonies on starch-nitrate medium were investigated to detect the morphological features. Also, the colours of the aerial and reverse cultures were observed under standard illumination conditions (Shiriling and Gottlieb, 1966). Biochemical features of *Streptomyces* sp.Sp1 was examined using API 20A kit (Biomerieux). Api stripes were inoculated following by manufacturer's manual. Stripes were incubated at 30 °C for 24-48h. After incubation period, reagents were added to vials. After 5-10 min, stripes were evaluated according to the manufacturer's instructions.

Molecular identification: *Streptomyces* sp. Sp1 cultured on starch-nitrate broth medium for seven days was assembled by centrifugation at 3000 rpm for 20 min. Obtained pellet was washed twice by sterile deionized water before the extraction of genomic DNA using EZ-10 Spin Column Bacterial Genomic DNA Miniprep Kits. The 16S rRNA regions were amplified using the universal forward primer 27F 5'-AGAGTTTGATC (AC) TGGCTCAG--3') and the reverse primer 1492R (5'-ACGG (CT) TACCTTGTTACGACTT-3'). The PCR components and amplification conditions were performed according to the protocol of Al-Dhabi *et al*., (2016). A mixture of 4 µl of dNTPs (1.0 mM), 2 µl of 10X buffer , 0.2 µl of each primer (0.5 µg), 0.2 µl of Taq polymerase (5 U/µl), 1 µl of 50 ng *Streptomyces* sp. Sp1 DNA was added to sterile Milli-Q water reaching a final volume of 19.8 µl. All chemicals were obtained from Roche, Penzberg, Germany. Amplification occurred through the following program. 94°C for 3 min, 30 cycles of 94°C for 30s, 50°C for 30s, and 72 for 60s, and 72°C for 7 min. The fragments purification was performed via Qiagen PCR-purification kit. The forward 27F primer and the Big Dye Terminator Cycle Sequencing kit v1.1 were used for sequencing the 16S rRNA genes from *Streptomyces* sp.Sp1 by 3500xL Genetic Analyzer, Applied Biosystems, Foster city, California. The resulted nucleotide sequences were aligned throughout the GeneBank data, the BLAST-N program (Basic Local Alignment Search Tool-Nucleotides) from the NCBI website (National Center for Biotechnology Information).

Antimicrobial evaluation of *Streptomyces* sp. Sp1 filtrate: Three MDR pathogens; *Listeria monocytogenes* (ATCC 19115), *Staphylococcus aureus* (ATCC 25923) and *Pseudomonas aeruginosa* (ATCC 10145) were kindly obtained from strains bank at Microbial center at the Faculty of Aquatic and Fisheries Sciences, Kafrelsheikh University. One ml of *Streptomyces* sp. Sp1 spore suspension was inoculated in 50 ml of autoclaved starch-nitrate broth medium cultured in 250 ml Erlenmeyer flask. Culture was shaked at 30°C, 150 rpm for 7 days. After incubation, supernatant was separated from *Streptomyces* sp.Sp1 biomass by centrifugation at 3000 rpm for 20 min. For pathogens, one ml of old broth cultures 18hrs of the tested bacterial pathogens was swabbed separately on freshly prepared nutrient agar medium. In the separately inoculated plates, wells with a diameter of 5 mm were drilled with a sterile cork drill. Each well was injected by 100 µl of the *Streptomyces* sp.Sp1 supernatant. The plates were incubated at 37°C for 24 hours. After incubation period, the diameter of the zone of inhibition (mm) was measured (Holmalahti *et al*., 1994).

Extraction of antibacterial compounds: *Streptomyces* sp. Sp1 was cultivated then used as inoculum for one liter total volume of the culture broth. Culture was then incubated on rotary shaker incubator at 150 rpm at 30°C for 7 days. Filtrate was separated from *Streptomyces* sp. Sp1 biomass by filtration through a filter paper, Whatman no.1, followed by centrifugation at 8000 rpm at 4 °C for 15 min. Then supernatant was aseptically transferred into 250 ml flasks and mixed with equal volume 1:1 (v/v) of n-butanol. The mixture was vigorously shaken for 20 min and kept stationary for another 15 min. The aqueous and organic layers of the crude extract were separated and concentrated to solvent free content by evaporation in an oven at 40°C. The residue was vacuum dried, weighed and dissolved in methanol (1mg/ml). Dissolved compounds were evaluated for their antimicrobial activity using the well diffusion method. Methanol was used in each test as a control against the tested pathogenic bacteria (Sathiyanarayanan *et al*., 2014).

GC-MS Analysis: Detection of active compounds of *Streptomyces* sp. Sp1 that showed bactericidal activity was performed using gas chromatography-mass spectroscopy (GC-MS) at National Institute of Oceanography and Fisheries, Alexandria, Egypt. GC/MS analysis was accomplished using GC instrument (Agilent 7890A) equipped with an HP-5MS column (30 m × 250 µm × 0.25 µm film thickness) and coupled with MS detector (Agilent 5975C). The initial oven temperature was programmed to be 90°C for 1 min then risen to 300 °C for 30 min at a rate of 8°C /min. The carrier gas, helium, was used in a flow rate of 1.5 ml/ min. The injection volume of sample was 1 µl in the splitless mode where the injector temperature was 290°C. Mass spectrum was operated at 70ev and mass range from60-600 amu (Hassan and Shobier, 2018).

Statistical analysis: Data were recorded in three replicates. Statistical analysis was carried out by Statistical Package for the Social Sciences (SPSS) program. (Lorowitz *et al*., 2005).

RESULTS

Identification of Streptomyces sp. Sp1

Morphological and biochemical characteristics: *Streptomyces* sp. Sp1 grown on Starch-nitrate agar medium showed whitish grey colour appeared for both aerial mycelium and substrate mycelium, with no production of melanin pigment (Fig. 1) and positive reaction with gram stain. The biochemical analysis showed positive reactions against glucose, salicin, xylose, maltose, starch hydrolysis, mannose, galactose, N-acetyl-ß-glucosamindase activity, glycerol utilization and esculin hydrolysis, however it showed negative reactions against mannitol, lactose, saccharose, arabinose, cellobiose, melezitose, raffinose, sorbitol, rhamnose, trehalose and fructose (Table 1).

Figure 1. The colony of *Streptomyces* sp. Sp1 grown on starch nitrate medium. Bacterial colony appears as whitish grey colour without melanin pigment.

Molecular Identification

16S rRNA sequence analysis of *Streptomyces* sp. Sp1: The 16S rRNA gene amplified via PCR resulted in an approximately 1500 bp fragment (Fig. 2). The amplified DNA was purified and sequenced using the Applied Biosystems a 3500xL Genetic Analyzer, Foster city, California. The resulted sequence was analyzed using online database (NCBI) and compared to other bacterial isolates. The sequencing result revealed that the isolate belongs to the phylum Actinobacteria, the family Streptomycetaceae and the genus *Streptomyces*. It revealed a 99.04% similarity with *Streptomyces* sp.Sp1 according to NCBI GenBank (Table 2). The resulted sequence was aligned to 18 of other closely related *Streptomyces* spp. using MUSCLE algorithm method (MUltiple Sequence Comparison by Log-Expectation). Their sequences were retrieved from the NCBI GenBank database and assembled in MEGA-X software of Kumar *et al*., (2016) for phylogenetic analysis using the Neighbor-Joining method and the evolutionary distances were computed using the Kimura 2-parameter method. The obtained phylogenetic tree (Fig. 3) confirmed the similarity of the isolate to *Streptomyces* sp. Sp1. The GenBank accession number for the partial 16S rRNA gene sequence of *Streptomyces* sp. sp1 is KU182926.

Table 1. Biochemical characteristics of *Streptomyces* sp. Sp1, depending on color change in wells according to API 20A kits manufacturer's instructions.

Test	Reaction	Test	Reaction
Glucose	–	Sorbitol	+
Mannitol	–	Rhamnose	–
Lactose	–	Trehalose	–
Saccharose	–	Fructose	–
Salicin	+	Galactose	+
Xylose	+	N-acetyl-β-glucosamindase	+
Maltose	+	Glycerol utilization	+
Starch hydrolysis	+	Urease	+
Arabinose	+	Esculin hydrolysis	–
Cellobiose	–	Indole formation	–
Mannose	–	Catalase	+
Melezitose	–	Nitrate reduction	–
Raffinose			–

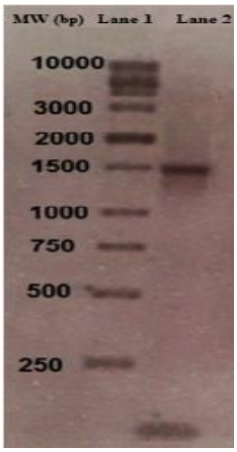

Figure 2. PCR amplified 16S rRNA gene. Lane 1: Molecular weight of SiZer-1000 DNA marker, Lane 2: amplified DNA fragment of ~1500bp from a single colony of *Streptomyces* sp. Sp1

Table 2. Gene sequence alignments of 16S rRNA gene of the isolated strain, *Streptomyces* sp. Sp1 to the data available at NCBI (BLASTN).

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Streptomyces sp. SP1(2016) 16S ribosomal RNA gene, partial sequence	1489	1489	88%	0.0	99.04%	KU182926.1
Streptomyces griseorubens strain MA-21 16S ribosomal RNA gene, partial sequence	1487	1487	88%	0.0	99.04%	KY783422.1
Streptomyces sp. strain M2 16S ribosomal RNA gene, partial sequence	1485	1485	88%	0.0	99.03%	MN371300.1
Streptomyces sp. strain G18 16S ribosomal RNA gene, partial sequence	1485	1485	88%	0.0	99.03%	MN371289.1
Streptomyces sp. SR-R48 16S ribosomal RNA gene, partial sequence	1485	1485	88%	0.0	99.03%	KF733397.1

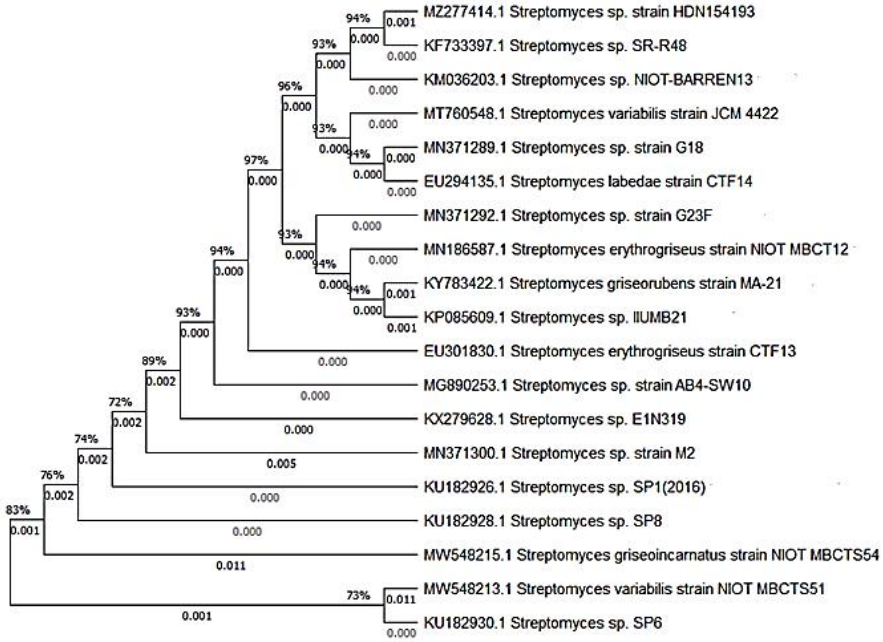

Figure 3. Phylogenetic tree of *Streptomyces* sp. Sp1 illustrates how close *Streptomyces* sp. Sp1 toward other *Streptomyces* neighbors. It has been reconstructed using MEGA-X software.

Antimicrobial activity: Significant antimicrobial activities against *Listeria monocytogenes*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* using *Streptomyces* sp. Sp1 crude extract were observed via different diameters of inhibition zones of about 12.3mm, 11.23mm and 11.06mm, respectively (Table 3) (Fig. 4).

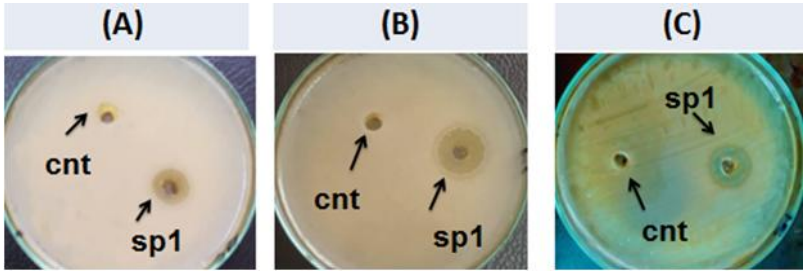

Figure 4. Evaluation of the antimicrobial effect of *Streptomyces* sp. Sp1 crude extract against; (A) *Staphylococcus aureus* , (B) *Listeria monocytogenes*, (C) *Pseudomonas aeruginosa*.

Table 3. Antimicrobial activity of *Streptomyces* sp. Sp1 crude extract against different multi-drug resistant *Listeria monocytogenes, Staphylococcus aureus* and *Pseudomonas aeruginosa.*

Pathogen	Inhibition zone (mm)
Listeria monocytogenes	**12.3±0.36**
Staphylococcus aureus	**11.23± 0.25**
Pseudomonas aeruginosa	**11.06± 0.20**

GC-MS Analysis: Because the n-butanol extract of *Streptomyces* sp. Sp1 filtrate had the best antibacterial activity against all of the MDR pathogenic bacterial species tested, it was investigated using gas chromatography–mass spectrometry (GC-MS).The obtained GC-MS spectrum of *Streptomyces* sp. Sp1 was compared with that of standard database of NIST mass spectral library (National Institute of Standards and Technology). Obtained data confirmed the existence of five bioactive compounds having antibacterial activities, one shows insecticidal activity and another one has antioxidative potential (Fig. 5, Table 4).

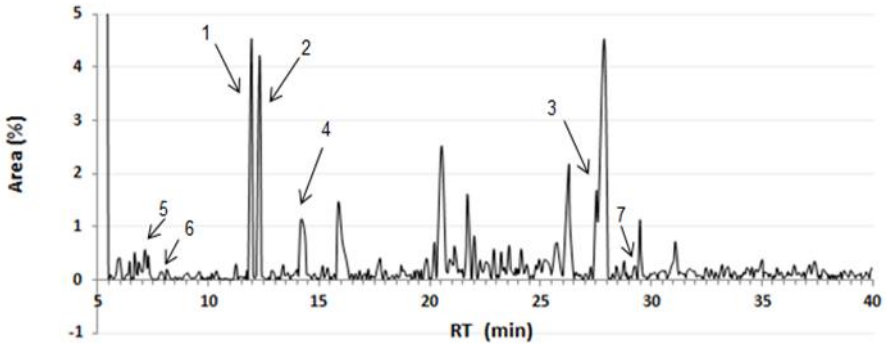

Figure 5. GC-MS chromatography of extracellular extract of *Streptomyces* sp. Sp1

Table 4. Bioactive compounds identified in the n-butanol extract of *Streptomyces* sp. Sp1 by gas chromatography.

N	Name of the compound	Molecular formula	Molecular weight g/mol	RT (Min)	Peak area (%)	Activity
1	**Broxyquinoline**	$C_9H_5Br_2NO$	302.95	11.88	4.54	Antibacterial
2	**9-Aminoacridine**	$C_{13}H_{10}N_2$	194.23	12.31	4.22	Antibacterial
3	**Phenol, 2,2'-methylene**	$C_{15}H_{16}O_2$	228.29	27.45	1.68	Antioxidant
4	**9,10-Anthracenedione**	$C_{14}H_8O_2$	208.21	14.17	1.12	Insecticidal
5	**Physostigmine**	$C_{15}H_{21}N_3O_2$	275.34	6.66	0.52	Antibacterial
6	**Harmine**	$C_{13}H_{12}N_2O$	212.25	7.13	0.57	Antibacterial
7	**Ricinoleic acid**	$C_{18}H_{34}O_3$	298.5	29.61	0.47	Antibacterial

The observed amount of bioactive compounds that have antibacterial activities of *Streptomyces* sp. Sp1 were Broxyquinoline (4.54%), 9-Aminoacridine (4.22%), 9,10-Anthracenedione (1.12%), Harmine (0.57%) and Ricinoleic acid (0.47%). In addition to antibacterial compounds, Phenol, 2,2'-methylene (1.68%) showing antioxidative Potential and insecticidal Physostigmine (0.52%) were detected.

DISCUSSION

This study has shown the isolation and identification of the bioactive strain *Streptomyces* sp. Sp1 from marine sediments of Burullus Lake using various biochemical and molecular techniques. The 16S rRNA gene amplified via PCR resulted in an approximately 1500 bp fragment that was purified and sequenced. The sequencing result revealed a 99.04% similarity with *Streptomyces* sp. Sp1 according to NCBI GenBank. According to Kawuri and Darmayasa (2019), *Streptomyces* sp. Sp1 identified using PCR with primers 63F and 1387r produced a DNA fragment 1300 bp that was purified and sequenced. The sequencing result revealed a similarity of about 99% with *Streptomyces* sp. Sp1. The crude extract of *Streptomyces* sp. Sp1 showed promising results against the tested pathogens; *Listeria monocytogenes* (ATCC 19115), *Staphylococcus aureus* (ATCC 25923) and *Pseudomonas aeruginosa* (ATCC 10145). Because these pathogens are dangerous organisms that infect both humans and animals,

controlling them with new and multiple antibacterials is critical to reduce the risk of mutation and/or adaptability. The observed results showed the existence of various antimicrobials that have several modes of action against MDR bacteria.

Broxyquinoline, an organic derivative of quinolones, showed high effect against a wide range of Gram-negative bacteria even at the ng / ml range (minimal inhibitory concentrations, MICs), while in case of Gram positive bacteria, the reported reasonably effect was in the mg / ml range (Anderson *et al*., 2012). Methyl 8-(3-methoxy-3-methylbutyl)-2-methylquinoline-4-carboxylate, a quinoline derivative that was isolated from *Streptomyces* sp. neau50 and showed cytotoxicity against human lung adenocarcinoma cell line A549 with an IC (50) value of 29.3 µg mL-1 (Wang *et al*., 2011). Anderson and Osheroff, (2001) clarified that broxyquinoline inhibited the replication of DNA and the topoisomerase.

Acridines are a large family that has an antibacterial activity against both Gram-positive and negative bacteria (Sharhan *et al*., 2020). Since acridine is one of the heterocyclic aromatic oils, this enables it to interact with a large number of macro- biological compounds by intercalation or pistacking. 9-Aminoacridine exhibited antibacterial activity against *S. aureus* and *P. aeruginosa*, with MIC ≤ 4 µg/mL and 125µg/mL respectively. This compound has a bactericidal power on these pathogens (Moukrad *et al*., 2015).

9,10-anthracenedione is also an anthraquinone derivative that was reported to have an effective antibacterial activity (Friedman *et al*., 2020). Their mechanism of action has been principally correlated to their ability to stimulate DNA cleavage mediated by the enzyme topoisomerase II (Malonne and Atassi,1997).

2-hydroxy- 9,10- anthraquinone exhibited antibacterial activity against *P. aeruginosa, B. subtilis, S. aureus* and *E. coli* (ESBL-3984), with MIC 12.5 µg/mL, >100 µg/mL, >100 µg/mL and 25 µg/mL respectively. It was isolated from *Streptomyces olivochromogenes* (Balachandran *et al*., 2016). Its antibacterial activity depends on binding to adhesins to the cell wall and inactivation of enzymes (Cowan, 1999). Harmine and its metal-organo complexes were also isolated from *Peganum harmala*. The antimicrobial mechanism of harmine metal complexes might be due to cell wall inhibition or bactericidal and/or bacteriostatic. However, some researcher's studies regarded the changes of the bacterial cell membranes upon metal ion treatment which might be the cause or consequence of cell death (Zaidi *et al*., 2012), that could be the main reason of antibacterial activity against *S. aureus* and *P. aeruginosa*, with MIC 100-200µg/100µl respectively (Salman *et al*., 2016).

Ricinoleic acid [R (Z)-12-hydroxy-9-octadecanoic acid] is considered as the major component of castor oil. The antibacterial activity of ricinoleic acid was reported against *S. aureus* and *P. aeruginosa*, with MIC 2.68 µM and 2.60 µM respectively (Narasimhan *et al*., 2005). The surfactant behavior of ricinoleic acid moiety due to the presence long lateral hydrophobic methylene units in its structure that could enable permeability of the polyester into the cell membrane and inhibited the growth of bacteria (Khan *et al*., 2012).

Phenol,2,2-methylenebis[6-(1,1-dimethylethyl)-4-methyl- the phenolic compound, was reported as antioxidant compound produced by *Streptomyces* sp. MUM292. As a group of aromatic ring compounds with one or more hydroxyl groups, these phenolic compounds perform antioxidant activity by scavenging free radicals, donating atoms or electrons, or chewing iron minerals. That's why it can be suggested that these phenolic compounds contribute to the full antioxidant capacity of *Streptomyces* sp. MUM292 extract by scavenging radicals and chelating metal cations (Hern Tan *et al*., 2018).

Physostigmine was isolated from the seeds of *Physostigma venenosum* and the soil actinomycete *Streptomyces* sp. AH-4 and has been also obtained from the culture filtrate of *Streptomyces psedogriseolus* subsp. Iriomotensis (Zang *et al*., 2021).

Conclusion: Up to date, Lake Burullus has not been screened for actinomycetes strains with antibacterial activity. The present study showed that the marine sediment of Lake Burullus is considered as a potential source of actinomycetes which may produce novel and potent antibacterial agents. GC-MS analysis of isolated *Streptomyces* sp. Sp1 filtrate showed several bioactive compounds, five of them have strong bactericidal properties. Because of the existence of these compounds, our findings potentially open the way for the use of *Streptomyces* sp. Sp1 as a biocontrol agent in fish farms.

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Conflict of Interest: Authors state that there is no conflict of interest.

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