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Antimicrobial Activities of Some Actinomycetes Isolated from Cultivated Soil, Egypt

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ABSTRACT

Antibiotics-resistant infections have appeared in numerous regions in the world, which draws attention to the urgent need for novel antimicrobial compounds. The current investigation aims to evaluate the activity of crude extracts from some actinomycetes isolates as potential antibacterial and antifungal agents. Fifteen actinomycetes were isolated from cultivated soil samples collected from various regions at Suez governorate. The antimicrobial properties of the crude extracts were tested against a variety of pathogenic bacteria and fungi. The selected ethyl acetate extract (EtOAc) of the isolate SUN1 showed strong antifungal activity against both *Aspergillus fumigatus* and *Fusarium solani* with inhibition zones 28 and 30 mm respectively, while showed moderate antibacterial activity against *Staphylococcus aureus* and Methicillin-resistant *Staphylococcus aureus* "MRSA" with inhibition zones 14 and 18 mm respectively. Based on the physiological, biochemical, and microscopic characterization of the isolate SUN1, it was characterized and identified on the genus level as *Streptomyces* sp. The EtOAc extract of *Streptomyces* SUN1 was characterized using the principal GC-MS chemical fingerprint. The net GC-MS data revealed that the primary concentrations are represented by phenolic and oxygenated chemicals. Eleven bioactive compounds were identified and classified as the principal components. These compounds included tributyl acetyl citrate, 1,4-benzenedicarboxylic acid, bis(2-ethylhexyl) ester, bis(2-ethylhexyl) phthalate and butyl citrate with relative amounts of 34.81, 28.39, 10.25, and 10.08% respectively. The investigation indicates that the isolated actinomycetes offer a valuable source of antimicrobial compounds that can be applied as novel antibiotics.

1. Introduction

One of the greatest issues facing the world today is antibiotics resistance, often known as antimicrobial resistance (AMR). In 2050, it is expected that AMR will destroy the lives of close to 10 million individuals. Since multidrug-resistant bacteria have become a widespread infection in recent years, treatment for them has gotten more complex and frequently results in patient death (Taneja and Sharma, 2019). According to Al-Dhabi et al. (2020), bacterial infections such as multi drug-resistant *Staphylococcus aureus* and *Enterococcus* species constitute an extensive risk. Effective management of drug resistance requires the identification or development of novel alternatives antibiotics since the most currently used are losing their efficacy against bacterial infections.

Pharmacological substances from natural sources are essential to our treatment strategies because of their biological activities and chemical diversity against diseases and their causative agents. It has been estimated that over ten thousand different antibiotics have been isolated from filamentous fungi, actinomycetes and bacteria (Pham et al., 2019).

Recently, numerous antimicrobial natural compounds were extracted from microorganisms, particularly actinomycetes. Several studies have been carried out on isolation and characterization of actinomycetes that produce antimicrobial agents. According to Lam et al. (2006) and Thomas et al. (2010), about half of the natural compounds with antimicrobial properties are extracted from actinomycetes. Actinomycetes were shown to have the greatest potential as sources of antimicrobial agents for the management of pathogenic microbes. Over the last few decades, a tremendous amount of research has been done on the synthesis of bioactive metabolites from actinomycetes, specifically genus *Streptomyces*. About 60% of the known antimicrobial agents are produced by actinomycetes, particularly *Streptomyces* species. The antibiotic streptomycin was discovered by Selman

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Waksman from the former genus (Woodruff, 2014). Other actinomycetes species can be a source of bioactive compounds, such as *Micromonospora, Nocardia, Nocardiopsis, Actinomadura, Streptosporangium, Streptoverticillium, Actinoplanes*, and *Thermoactinomyces* (Barka et al., 2016). However, further study on finding new and innovative antimicrobial products from natural sources will be beneficial in solving the problem of antimicrobial resistance (AMR) (Harikumar and Krishanan, 2022). The current study investigated the antimicrobial efficacy of various soil actinomycetes extracts against some pathogenic bacterial and fungal isolates. In addition, the chemical identification of the main bioactive components of *Streptomyces* sp. SUN1 was investigated.

2. Materials & methods

2.1. Isolation of Actinomycetes

Fifteen Soil samples were collected from several places in Suez governorate. Starch casein agar medium was used for the isolation, it consists of (g/l dist. H_2O): Soluble starch, 10.0, Casein 0.3, KNO_3 2.0, NaCl 2.0, K_2HPO_4 2.0, $MgSO_4.7H_2O$, 0.05, $CaCO_3$, 0.02, $FeSO_4.7H_2O$, 0.01 and agar-agar, 15.0 g. Using the dilution plate method (Goodfellow and Williams, 1983), actinomycete isolates were recovered on starch casein agar (SCA) supplemented by Nystatin (50 mg/ml) and Rifampicin (10 mg/ml) for avoiding the growth of fungal and bacterial contaminations (Abd-Alla et al., 2013).

2.2. Extraction of secondary metabolites

Actinomycetes isolates were cultivated for ten days at 100 rpm and 28 °C in 100 milliliters of starch casein (SC) broth medium. After the incubation period, the broth was collected and centrifuged at 4000 rpm for 30 minutes. An equivalent volume of ethyl acetate (EtOAc) was added following the supernatant collection. The two phases were shaken well for 30 min. To separate the organic and aqueous phases, the solutions were transferred in a separate funnel and left for five minutes. After removing the lower aqueous phase, the upper organic phase was collected and allowed to evaporate in a water bath at 40°C (Ghorbani et al., 2013). The residue was weighed and dissolved in methanol to solubilize the crude metabolic extract, resulting in a final concentration of 100 mg/ml for antimicrobial screening.

2.3. Antimicrobial activity assay

The antimicrobial activities of actinomycetes extracts were evaluated using the conventional well diffusion test (Flemer et al., 2012). To investigate the extent to which the metabolic EtOAc extracts of actinomycetes isolates have antibacterial capabilities, they were examined against two Gram-positive bacteria (Methicillin-resistant *Staphylococcus aureus* or "MRSA" and *Staphylococcus aureus*) and one Gram-negative bacteria (*Pseudomonas aeruginosa*). The standard control antibiotic used to compare the efficacy of the EtOAc extracts was clindamycin (2 µg). Three pathogenic fungal cultures *Fusarium solani, Aspergillus fumigatus*, and *Penicillium chrysogenum* were used to assess the antifungal capabilities. Pathogenic bacteria were swabbed onto nutrient agar (NA) plates, but fungi were swabbed onto Czapek agar (CZA) plates. Wells were created with a 6 mm diameter.

2.4. Phenotypic characterization of Streptomyces SUN1

Streptomyces SUN1 morphological and cultural characteristics were investigated by applying the strain onto specific growth media, including ISP1 (Tryptone-Yeast Extract Agar), ISP2 (Yeast Extract-Malt Extract Agar), ISP3 (Oatmeal Agar), ISP4 (Inorganic Salts-Starch Agar), ISP5 (Glycerol-Asparagine Agar), and ISP7 (Tyrosine Agar) (Pridham et al., 1948, 1957, 1961; Küster, 1959; Shinobu, 1958). The color of aerial and substrate mycelium, branching, and colony nature were noted. Using a high-power magnifying lens, the aerial mycelia were examined (Pepper and Garba 2004). The consumption of several carbohydrates as a carbon source, notably lactose, glucose (positive control), cellulose, fructose, and sucrose, was established through the carbohydrate utilization assay. Various nitrogen sources were further tested for the utilization assay such as L-asparagine, L-tyrosine, and L-tryptophane. The hydrolysis of starch, casein, urea, hydrogen sulfide generation, nitrate reduction, and the catalase test are the biochemical tests that evaluated.

2.5 Gas Chromatography-Mass Spectrometry (GC-MS) analysis

The chemical components of the *Streptomyces* SUN1 EtOAc extract were identified by GC-MS analysis. Under the identical circumstances as previously reported (Abd-ElGawad et al., 2023), the material was dissolved in hexane and injected into an Agilent Technologies GC-MS equipped with a gas chromatograph (7890B) and a mass spectrometer detector (5977A).

3. Results and discussion

3.1. Screening of antimicrobial activities

The antimicrobial properties of the actinomycete isolates were evaluated against a range of pathogenic cultures (Table 1). Strong antifungal activity against A. *fumigatus* and *F. solani* was demonstrated by the EtOAc extract of *Streptomyces* strain SUN1, with inhibition zones measuring 28±2.3 and 30±2.4 mm, respectively (Figure 1a). Similar results were obtained by Refaat et al. (2017) who investigated. The antifungal capacities of actinomycetes strains from rhizospheric soil specimens in Sinai were investigated by Refaat et al. (2017) reported strong antifungal capabilities by some isolates against *A. niger and F. oxysporium*. Furthermore, Hadizadeh et al. (2015) reported that *S. rochei* strain HF391 inhibits the proliferation of an isolate of *A. fumigatus* in clinical culture, with an inhibition zone of 15±1.6 mm.

Moreover, SUN14 and SUN23 revealed strong antibacterial activities against *S. aureus* with 24±2.2 and 21±2.1 respectively and against MRSA with 19±1.52 and 20±1.52 respectively (Figure 1b), while displaying moderate inhibitory effect against *P. aeruginosa* with 17±1.1, 18±1.23 respectively. Govindarajan et al. (2021) also documented antimicrobial properties against specific

bacteria that were multidrug-resistant. Among the tested organisms were *S. aureus*, MRSA, and *P. aeruginosa*. The isolates of actinomycetes, designated JRG-02, JRG-03, JRG-04, JRG-10, and JRG-12, demonstrated potent antimicrobial properties. Sapkota et al. (2020) carried out antibacterial investigations on two isolates of *Streptomyces* strains C2 and H16, which displayed significant efficacy against *S. aureus*.

Table 1. Antimicrobial activities of actinomycetes isolates (mm).

Crude extract code (100 µg/µl)	Zone of inhibition (mm)					
	Antibacterial activity			Antifungal activity		
	Gram +ve bacteria		Gram -ve bacteria			
	Staphylococcus aureus	Methicillin-resistant *Staphylococcus aureus* (MRSA)	*Pseudomonas aeruginosa*	*Aspergillus fumigatus*	*Fusarium solani*	*Penicillium chrysogenum*
SUN1	14±1.23	18±1.52	11±1.1	28±2.3	30±2.4	18±1.52
SUN2	14±1.23	15±1.23	15±1.23	19±1.52	22±2.1	15±1.23
SUN3	18±1.52	17±1.52	17±1.52	26±2.3	13±1.1	13±1.1
SUN8	14±1.23	13±1.1	22±2.1	13±1.1	20±1.52	15±1.23
SUN11	18±1.52	14±1.23	17±1.52	22±2.1	18±1.52	17±1.52
SUN14	24±2.1	19±1.52	17±1.1	12±1.1	21±2.1	14±1.23
SUN19	18±1.52	16±1.23	11±1.1	16±1.23	27±2.3	16±1.23
SUN20	15±1.23	13±1.1	11±1.1	10±1.1	19±1.52	14±1.23
SUN23	21±2.1	20±1.52	18±1.23	21±2.1	22±2.1	16±1.23
SUN28	17±1.52	17±1.52	13±1.1	17±1.52	23±2.1	17±1.52
SUN31	18±1.52	neg.	15±1.23	11±1.1	24±2.1	12±1.1
SUN33	23±2.1	13±1.1	12±1.1	14±1.23	27±2.3	15±1.23
SUN38	11±1.1	13±1.1	14±1.23	20±2.1	15±1.23	neg.
SUN42	13±1.1	22±2.1	18±1.52	22±2.1	19±1.52	16±1.23
SUN45	10±1.1	neg.	21±2.1	12±1.1	23±2.1	neg.
Clindamycin (DA) (2 µg)	13±1.1	neg.	9±1.1	-	-	-

3.2. Phenotypic characterization

The SUN1 crude extract exhibits interesting antibacterial and antifungal properties. Based on physiological tests, macroscopic and microscopic examination, the isolate SUN1 was suggested to be belong to the genus *Streptomyces* as described with the guidelines provided by Actinobacteria, Part A of Bergey's Manual of Systematic Bacteriology, Second Edition (Goodfellow et al., 2012). Phenotypic characterization of isolate *Streptomyces* SUN1 on various media indicated poor growth, chalkiness, and different-colored aerial and substrate mycelia (Table 2 & Figure 2 a-b).

According to Bergey et al. (1994), the hydrolysis of starch, casein, urea, hydrogen sulfide generation, nitrate reduction, and the catalase test are the biochemical tests performed to define the *Streptomyces* SUN1 strain. The biochemical characteristics were reported in Table 3. Starch can be hydrolyzed by *Streptomyces* SUN1, but casein cannot. The isolate produced negative findings in assays for H_2S formation but positive results in nitrate reduction, urease decomposition, and catalase production.

Streptomyces SUN1 can utilize a variety of carbon sources in the medium. Fructose cannot be utilized by *Streptomyces* SUN1, glucose, lactose, and sucrose can all be broken down. Actinomycetes are recognized as lignocellulose decomposers due to their ability to metabolize cellulose with efficiency (Abdulla and El-Shatoury 2007). Utilization of nitrogen sources was also investigated. Asparagine, tyrosine and tryptophane, may all be slightly degraded by *Streptomyces* SUN1.

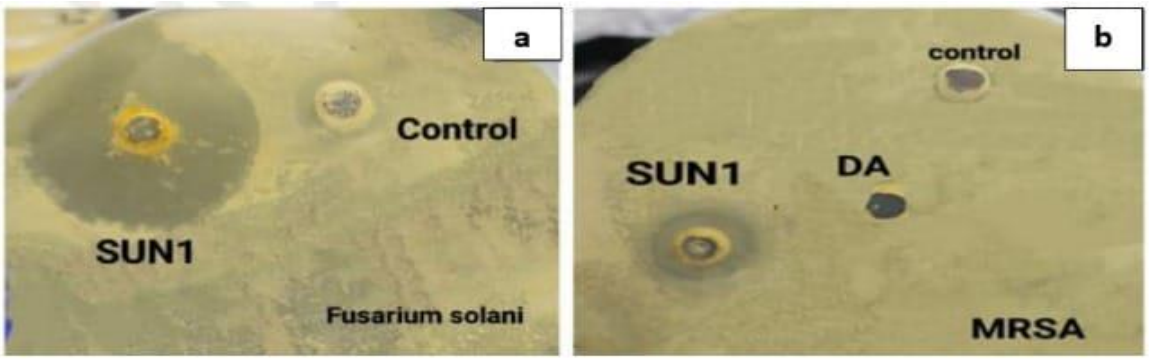

Figure 1. a. inhibition zone of EtOAc extract of *Streptomyces* sp. SUN1 against *Fusarium solani*. b. inhibition zone of EtOAc extract of *Streptomyces* sp. SUN1 against MRSA.

Table 2. Growth and morphological traits of *Streptomyces* SUN1.

Media selected	Growth	Aerial mycelium color	Substrate mycelium color	Pigments diffused
ISP 1	heavy	burlywood	beige	neg.
ISP 2	moderate	creamy	beige	neg.
ISP 3	heavy	grey	beige	neg.
ISP 4	moderate	grey	burlywood	neg.
ISP 5	moderate	white	white	neg.
ISP 7	heavy	grey	beige	neg.

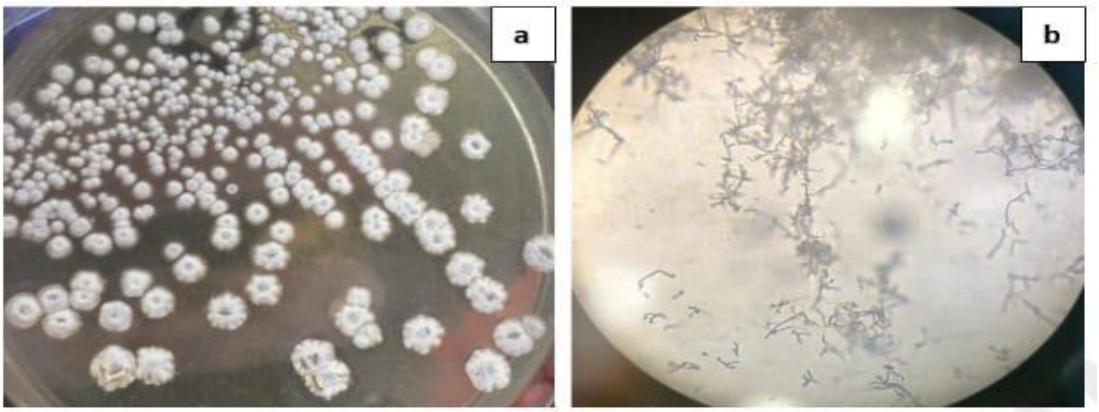

Figure 2. a. white aerial colonies of *Streptomyces* sp. SUN1. b. microscopic examination of *Streptomyces* sp. SUN1.

Table 3. Physiological and biochemical characteristics of *Streptomyces* SUN1.

Test	Reaction
Catalase production	+
Urea decomposition	+
Production of H_2S	-
Activity of nitrate reductase	+
Starch hydrolysis	+
Casein hydrolysis	-
Carbon sources utilization	
D-glucose	+
Cellulose	+
Sucrose	+
Lactose	+
Fructose	-
Use of nitrogen sources utilization	
L-tryptophane	+
L-tyrosine	+
L-asparagine	+

3.3. Gas Chromatography-Mass Spectrometry (GC-MS)

Using the GC-MS as a primary fingerprint, the hexane soluble components of the EtOAc extract of *Streptomyces* SUN1 were identified (Figure 3). Nineteen compounds, accounting for all the mass, were identified based on the GC-MS data. The retention durations and peak area percentages (relative concentration) of each detected component were entered into Table 4.

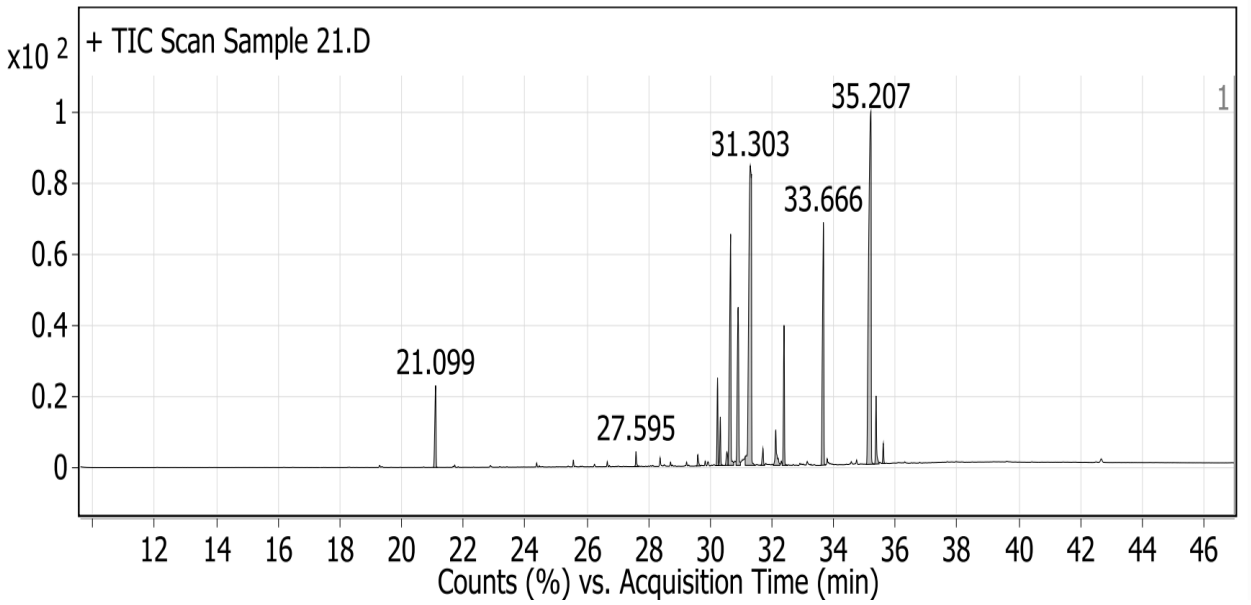

Figure 3: GC-MS of EtOAc extract of *Streptomyces* SUN1.

The two most prevalent compounds among all the detected ingredients were butyl citrate (10.08%), bis(2-ethylhexyl) phthalate (10.25%), 1,4-benzenedicarboxylic acid, bis(2-ethylhexyl) ester (28.39%) and tributyl acetyl citrate (34.81%). A minor component with the lowest concentration of all the compounds was found to be hexadecanol (0.83%). The outcomes also showed that this extract included a high concentration of phenolics and oxygenated components. The present data were in complete agreement with the literature. The documented results revealed that the of the different extracts of *Streptomyces* species are very rich with several bioactive metabolites including phenolic, nitrogenous and oxygenated metabolites (Mirsonbol et al., 2023, Sholkamy et al., 2023). The reported analysis of the antimicrobial active extract derived from *S. pactum* exhibit the presence of the oxygenated, phenolic and aromatic compounds (Mirsonbol et al., 2023). Also, the reported GC-MS of the bioactive *Streptomyces* species` extract exhibited the highly concentration of oxygenated and nitrogenous contents (Mirsonbol et al., 2023, Sholkamy et al., 2023).

Table 4: The identified components of EtOAc extract of *Streptomyces* SUN1.

Peak	RT	Name	Formula	Area	Area Sum %
1	21.099	Triacetin	$C_9H_{14}O_6$	36055550	3.16
2	27.595	1-Hexadecanol	$C_{16}H_{34}O$	9431264.2	0.83
3	30.236	Butyl citrate	$C_{18}H_{32}O_7$	27950985	2.45
4	30.327	1-Propene-1,2,3-tricarboxylic acid, tributyl ester	$C_{18}H_{30}O_6$	14998836	1.31
5	30.661	Butyl citrate	$C_{18}H_{32}O_7$	115037078	10.08
6	31.303	Tributyl acetylcitrate	$C_{20}H_{34}O_8$	397219644	34.81
7	32.126	9-Octadecenamide, (Z)-	$C_{18}H_{35}NO$	19586144	1.72
8	32.391	Hexanedioic acid, bis(2-ethylhexyl) ester	$C_{22}H_{42}O_4$	50137983	4.39
9	33.666	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	116974963	10.25
10	35.207	1,4-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	$C_{24}H_{38}O_4$	324018642	28.39
11	35.374	13-Docosenamide, (Z)-	$C_{22}H_{43}NO$	23634987	2.61

Conclusion

Actinomycetes can be an abundant source of natural antimicrobial metabolites. Herein, several *Streptomyces* species were isolated from cultivated soil and then were subjected to physiological, biochemical, and microscopic assays. The EtOAc extracts of all the *Streptomyces* species were prepared and applied for antimicrobial activities. The results revealed that all these extracts have significant antimicrobial activities. The GC-MS analysis of the most antimicrobial active extract of *Streptomyces* sp. SUN1 revealed the presence of highly oxygenated components. These findings also aid in discovering the potential use of actinomycete metabolites for the pharmaceutical production of novel antibiotics.

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