



Molecular identification of actinomycetes with antimicrobial, antioxidant and anticancer properties

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Abstract

The objectives of this study were to isolate and identify the actinomycetes strains from the soil and marine sediments and to evaluate the antimicrobial, antioxidant and cytotoxic activity of their bioactive secondary metabolites. Eight actinomycetes strains were isolated from soil and marine sediment samples collected from different areas in Egypt. Only three actinomycetes exhibited a wide spectrum of antimicrobial activities. They were active *in vitro* against microbial pathogen viz: *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, *Aspergillus parasiticus*, *Fusarium solani* and *Fusarium oxysporum*. These promising isolates were selected and identified using molecular identification technique and identified as *Streptomyces* spp. The crude extracts from the three *Streptomyces* exhibited potent antimicrobial activities against a set of microbial pathogens as well as antioxidant and anticancer activity in human hepatocellular carcinoma cell line (HepG2). The crude extract of *Streptomyces* isolate D showed antitumor activity with IC_{50} 0.85 μ g/ mL. Forty compounds were identified from the two most promising ethyl acetate extracts of culture broth of *Streptomyces* sp. (D-EGY) by Gas Chromatography-Mass Spectrometry analysis. It could be concluded that the streptomycetes isolated from the Egyptian environment are promising candidates as antimicrobial, antioxidant and anticancer.

Key words: *Streptomyces*; bacteria; fungi; cytotoxicity; GC-MS

Introduction

Moulds represent a great challenge confronting food and feed industry all over the world. The moulds cause great losses worldwide in food and feed supplementation due to the production of mycotoxins (Cheong et al., 2014). Contamination of foods by pathogenic bacteria (such as *Salmonella typhimurium*, *Escherichia coli*, *Campylobacter jejuni*, *Legionella pneumophila*, *Staphylococcus aureus*, etc.) results in numerous foodborne diseases (Targowski & Ptusa, 2015). Infectious diseases account for nearly 40% of the total 50 million annual estimated deaths worldwide, whereas in many developing countries microbial diseases constitute the major

cause of death (Abdulai et al., 2015).

In order to achieve improved food safety against such pathogens, several traditional means for the control of microbial spoilage and safety hazards in foods are being replaced by combinations of innovative technologies including the biological antimicrobial systems. Antimicrobial resistance among pathogenic microorganisms has led to a renewed search for new antimicrobial agents from microorganism such as actinomycetes.

Actinomycetes are Gram-positive, free-living, spore forming, saprophytic, filamentous bacteria, extensively dispersed in water, soil which has an ecological significant in soil nutrient

cycling for colonizing plants and have DNA with high GC content (69-73%). Of all the genera of the actinomycetes, *Streptomyces* is the largest number of species and varieties occurred in nature and they are playing an extensive role in the pharmaceutical as well as medical industry for their capacity to produce secondary metabolites (Janardhan et al., 2014).

Over 10,000 bioactive secondary metabolites are produced by actinomycetes, representing 45% of all bioactive microbial metabolites discovered. Many of these bioactive compounds were isolated and characterized and have been developed into drugs for treatment of wide range of diseases in human, animals and the agriculture sectors (Subathra et al., 2013). Actinomycetes are the most prolific producers of antibiotics accounting for about 80% of the known antibiotics (Subramani & Aalbersberg, 2012). Novel secondary metabolites with a wide range of biological activities may ultimately find applications as anti-infective, anticancer, antibacterial, antifungal, antiviral, anti-parasitic, immunosuppressive, enzyme inhibitory and diabetogenic (Kekuda et al., 2010). To cope up with the demand for these new pharmaceutical and agriculture compounds, researchers are looking for novel microorganisms in new environments. Therefore, the aim of the current research was to isolate and characterize new Egyptian soil and marine actinomycetes strains. The isolates were further screened for bioactive potential secondary metabolites to evaluate their ability as antimicrobial, antioxidant and cytotoxic activity.

Material and methods

Microorganisms

Gram+ve bacteria (*Staphylococcus aureus*), Gram-ve bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella typhi*), yeast (*Candida albicans*) as well as fungi (*Aspergillus niger* and *Aspergillus parasiticus*) were obtained from the culture collection of the Microbial Resources Centre (MIRCEN), Faculty of Agriculture, Ain Shams University, Cairo, Egypt. *Fusarium solani*, and *Fusarium oxysporum* were isolated from maize samples in Egypt.

Sample collection

Soil samples were collected from agricultural soil surrounding Mansoura city, Dakahlia governorate (coordinates are latitude 31° N and longitude 31° E), Egypt during April, 2015. The samples were taken at 10 cm depth using sterile spatula and each sample was placed in sterile plastic bags and transferred to the microbiology laboratory at Microbial Chemistry Department, National Research Centre, Dokki, Cairo, Egypt. The samples were sieved and air dried for 3-5 days at 25°C then was kept at 10°C in an incubator until used for the isolation of actinomycetes. Marine sediments samples were collected from Ras Sedr city, Suez Gulf, South Sinai governorate (coordinates are latitude 29° N and longitude 32° E), Egypt during May, 2015 under aseptic conditions using a piston core sediment sampler at a depth of 4-5 cm and stored at 4°C in Microbial Chemistry Laboratory, National Research Centre.

Isolation of actinomycetes

Dried soil samples (50g) were suspended separately in 200 mL of sterile distilled water and shaken on a reciprocal shaker for 2 h. The isolation of actinomycetes was carried out using starch-nitrate agar (SNA) consisting of 20 g/L starch, 1 g/L KNO₃, 0.5 g/L K₂HPO₄, 0.5 g/L MgSO₄·7H₂O, 0.5 g/L NaCl, 0.01 g/L FeSO₄, 15 g/L agar and supplemented with nystatin at a concentration of 50 µg/mL as an antifungal and nalidixic acid at a concentration of 20 µg/mL as an antibacterial (Sineva & Terekhova, 2015). However, marine sediments (1g) were suspended in 100 mL of 0.8% sterile saline solution and agitated for 30 min using orbital shaker incubator (100 rpm) at 30°C. The isolation of actinomycetes from marine sediments was carried out using starch-casein agar (SCA) consisting of 10 g/L soluble starch, 2 g/L K₂HPO₄, 2 g/L KNO₃, 0.3 g/L casein, 0.05 g/L MgSO₄·7H₂O, 0.02 g/L CaCO₃, 0.01 g/L FeSO₄·7H₂O, 15 g/L agar and 1000 mL filtered sea water supplemented with 25 µg/mL cyclohexamide and 10 µg/mL nalidixic acid to minimize contamination with fungi and bacteria, respectively. The shaken suspensions for soil or marine samples were diluted using serial dilution procedure (10⁻¹ to 10⁻⁶) and aliquots (100 µL) from the different dilutions were used to inoculate the

medium plates. The plates were incubated at 30°C for two weeks. The growing colonies were sub-cultured and transferred to slants without the antibiotic substance and kept at 4°C until use. These actinomycetes strains were maintained on SNA and SCA medium and incubated for 14 days in an incubator in the dark at 28°C. The micro-morphology and sporulation were observed by optics microscopy (Olympus, Japan).

Antagonistic activity of actinomycetes isolates

The plate diffusion method was used for the antagonistic assay of the actinomycetes isolates. In brief, the isolates were removed from their agar using a sterile cork bore (5 mm in diameter) and placed onto agar plate lawn with the pathogenic fungi or bacteria e.g. *A. parasiticus*, *F. Solani*, *F. oxysporum*, *E. coli*, *S. typhi* and *S. aureus* and incubated at 37°C for 24 h for bacteria and 28°C for 5 days for fungi. The formation of halo zone was considered as positive reaction. The diameter of the growth inhibition zones were measured averaged and the mean values were recorded.

Molecular identification of actinomycetes isolates

The three actinomycetes isolates (showed the highest antagonistic activity against the tested pathogenic fungi and bacteria) were identified according to a molecular biological protocol by DNA isolation, amplification (PCR) and sequencing of the ITS region. The forward primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3'), and 518F (5'-CCAGCAGCCGCGGTAATACG-3') and reverse 1492R (5'-TACGGYTACCTTGTACGACTT-3'), and 800R (5'-TACCAGGGTATCTAATCC-3') were used. The purification of the PCR products was carried out to remove unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore). Sequencing was performed using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA). Amplification and sequencing of the 16S rRNA gene had been done according to Myer et al. (2016). Blast

program was used to assess the DNA similarities (www.ncbi.nlm.nih.gov/blast).

Preparation of Streptomyces species extracts

The identified *Streptomyces* species were cultivated on starch nitrate agar plate medium at 28°C for 7-14 days (until complete sporulation). One-litre Erlenmeyer flasks, each containing 250 mL of ISP2 medium consisting of 4 g/L glucose, 4 g/L yeast extract, and 10 g/L malt extract, were inoculated with spore suspension from well grown slants (only one slant was used to inoculate two flasks). The flasks were incubated at 30°C using rotary shaker (150 rpm) or static in self incubator at the same temperature for 15 days. The cells were separated by centrifugation at 5000 rpm and 4°C, and both the cell-free supernatant and the cells biomasses were subject to extraction. The supernatants were extracted with ethyl acetate 3 times. However, the cell biomasses were extracted with acetone then the acetone evaporated under vacuum and the remaining water residue was re-extracted 3 times with acetyl acetate (Shaaban et al., 2013). The yielded extracts from the cell-free supernatant or the cells biomasses of the three *Streptomyces* species using different routes are presented in Table 1.

Antimicrobial activity

The antimicrobial activities of crude extracts of the isolated *Streptomyces* spp. were tested against *S. aureus*, *C. albicans*, *F. oxysporum*, *P. aeruginosa* and *A. niger*. The obtained extracts were dissolved in methanol and filter paper discs (5 mm diameter, Whatman No.1) were saturated with 10 µL from the water or the methanol extract, and dried for 1 h at room temperature under sterilized conditions (final extract concentration was 100 µg per disc). The paper discs were placed on inoculated agar plates with the tested microbes and incubated at the appropriate temperature and time for each microorganism. Bacterial strains were grown on nutrient agar; yeast strains were grown Potato dextrose agar (PDA) medium (DSMZ GmbH, Germany), and the fungal strain was grown on Czapek-Dox medium (DSMZ GmbH, Germany). After the incubation period, the diameter of the

growth inhibition zones were measured averaged and the mean values were recorded. Neomycin and cycloheximide at a concentration of 50 µg/

disc were used as antibacterial and antifungal control agents, respectively.

Table 1. Crude extracts of *Streptomyces* sp. and extraction conditions

Streptomyces sp.	Extract number	Extraction conditions
5S2-EGY	I	Extracted from supernatant incubated under rotary shaking
	II	Extracted from cells incubated under rotary shaking
	III	Extracted from supernatant incubated under static
	IV	Extracted from cells incubated under static
	V	Extracted from supernatant incubated under rotary shaking
D- EGY	VI	Extracted from cells incubated under rotary shaking
	VII	Extracted from supernatant incubated under static
	VIII	Extracted from cells incubated under static
	IX	Extracted from supernatant incubated under rotary shaking
5S1-EGY	X	Extracted from cells incubated under rotary shaking
	XI	Extracted from supernatant incubated under static
	XII	Extracted from cells incubated under static

Antioxidant activity (DPPH scavenging assay)

The crude extracts of the bioactive secondary metabolites showing high antimicrobial activity were studied for their antioxidant activity. One hundred µL of the extracts at concentrations of 5, 7.5 and 10 mg/mL were added to 5 mL of the 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) solution (0.005% in methanol). After 30 min incubation at room temperature, the absorbance was read against pure methanol at 520 nm. The radical-scavenging activities of the samples were calculated as percentages of inhibition according to the following equation:

$$DPPH\% = \frac{A \text{ blank} - A \text{ sample}}{A \text{ blank}} \times 100$$

Where: A blank is the absorbance of the control (containing all reagents except the test compound), and A sample is the absorbance of the test compound. All tests were done in triplicate.

Cytotoxic activity

Hepatocellular Carcinoma Cell Line (HepG2)

Potential cytotoxicity of actinomycetes secondary metabolites crude extracts was performed. Briefly, human hepatocellular carcinoma cell line (HepG2) (1×10^4 cells/well), obtained from the National Cancer Institute, Cairo University, Cairo, Egypt, were seeded in 96-microwell plate and allowed to grow overnight before the treatment with the crude extracts of actinomycetes to allow attachment

of the cell to the wall of the plate. Different concentrations of the crude extracts (0.39 - 50 µg/mL) were added to the cells monolayer and triplicate wells were prepared for each individual extract. Monolayer cells were incubated with the extract in humidified incubator with 5% CO₂ at 37°C and after 48 h; cells were fixed by the addition of 25 µL of cold 50% trichloroacetic acid (TCA). After incubation for one hour at 4°C, the plate was washed five times with tap water to remove TCA, medium and dead cells then the plate was dried in air. Fifty µL of 0.4% sulforhodamine B stain dissolved in 1% acetic acid was added to each well and allowed to stand for 30 min. The plate was subsequently washed four times with 1% acetic acid to remove excess stain then dried in air again. One hundred and 50 µL of 10 mM aqueous Tris-Base [tris(hydroxymethyl) aminomethane] was added to solubilize the cell-bound dye. The plate was then shaken for 10 min followed by the reading of optical density (OD) at 570 nm in a microplate spectrophotometer.

Identification of chemical composition of the crude extracts of using GC-MS

The most promising two crude extracts (V and VII) that showed high antimicrobial and cytotoxic activities were analysed by a coupled Varian gas chromatography/ mass spectrometry (Perkin Elmer Auto XL GC, Waltham, MA, USA) equipped with a flame ionization detector to identify their chemical composition. The GC

conditions were EQUITY-5 column (60 m x 0.32 mm x 0.25 μm); H_2 carrier gas; column head pressure 10 psi, the oven temperature was maintained initially at 70°C for 2 min, and then programmed from 70 to 250°C at a rate of 3°C/min. The ionization voltage was 70 eV, mass range m/z 39-400 amu. The identification of individual compounds was based on their retention times relative to those of authentic samples and matching spectral peaks available with the published data.

Statistical analysis

All data were statistically analyzed by analysis of Variance (ANOVA). The significance of the differences among treatment groups was determined by Waller-Duncan k-ratio (Waller & Duncan, 1969). All statements of significance were based on probability of $P \leq 0.05$.

Results

Isolation of actinomycetes

Four actinomycetes species were isolated from soil samples and nomeados S14,

AM2, S3 and L. Other 4 species were isolated from marine sediments samples and nomeados 5S1, 5S2, D and 25. It is of interest to mention that the genus *Streptomyces* was the dominant in all species in the marine sediments samples.

Antagonistic activity of the isolated actinomycetes

The results of antagonistic activity of the isolated actinomycetes presented in Table 2 revealed that only three isolates e.g. 5S1, 5S2 and D showed the highest antagonistic activity against most of the tested microorganisms. Two isolates e.g. AM2 and L showed only antagonistic activity against the tested fungi but not against bacteria and only one isolate (S3) did not show any antifungal or antibacterial activity (Table 2). Consequently, the three actinomycetes isolates that showed strong antimicrobial activity (5S1, 5S2 and D) were subjected to molecular identification technique.

Molecular Identification of the actinomycetes isolates 5S1, 5S2 and D

Table 2. Antimicrobial activity of actinomycetes species

Actinomycetes isolates	Inhibition zone (mm)					
	<i>A. parasiticus</i>	<i>F. solani</i>	<i>F. oysporum</i>	<i>E. coli</i>	<i>S. typhi</i>	<i>S. aureus</i>
5S1	25.0 \pm 0.0	30.0 \pm 0.0	20.5 \pm 0.05	10.5 \pm 0.05	ND	10.5 \pm 0.05
5S2	27.0 \pm 5.0	26.5 \pm 1.5	19.0 \pm 0.0	10.0 \pm 0.05	ND	10.0 \pm 0.05
D	25.0 \pm 5.0	30.0 \pm 0.0	23.0 \pm 2.0	12.0 \pm 1.0	14 \pm 0.0	12.0 \pm 1.0
S14	10.0 \pm 10	22.5 \pm 2.5	20.0 \pm 0.0	ND	ND	ND
AM2	15.0 \pm 0.0	30.0 \pm 5.0	ND	ND	ND	ND
L	ND	13.5 \pm 0.05	14.0 \pm 1.0	ND	ND	ND
S3	ND	ND	ND	ND	ND	ND
25	13.5 \pm 0.05	ND	ND	ND	ND	ND

Results are mean \pm SEM. ND: Non detected

The molecular identification of the three actinomycetes revealed that a nucleotide sequence of 967, 971 and 1054 bp of the whole 16S rRNA gene of the *Streptomyces* sp. for isolate 5S1, 5S2 and D, respectively was determined in both strands. BLAST search revealed that 99% similarity to *Streptomyces* sp. and the phylogenetic trees of these *Streptomyces* were also constructed (Figure 1a-c). The gene sequences of actinomycetes were deposited in GenBank database; as *Streptomyces* sp. 5S1-EGY (Accession No. KP336669.1), *Streptomyces* sp. 5S2-EGY (Accession No. KP336670.1), and *Streptomyces* sp. D-EGY (Accession No. KP336671.1). It is well known that the traditional

identification techniques are depending on the culture morphology and some biochemical reactions are known to be a time consuming methods that also need a talented persons with very high experience (Sugita & Nishikawa, 2003). Consequently, the use of DNA sequences in the current study was proved to be an objective, reproducible, accurate and quick tools for identification. Universal primers: 27F (5'GAGTTTGATCATGGCTCAG3'), 1492R (5'GGTTACCTTGTTACGACTT3') [for PCR] and 518F (5'CCAGCAGCCGCGGTAATACG3') and 800R (5'TACCAGGGTATCTAATCC3') were used in the study of the genotypic identification of microorganisms isolated from non-parenteral

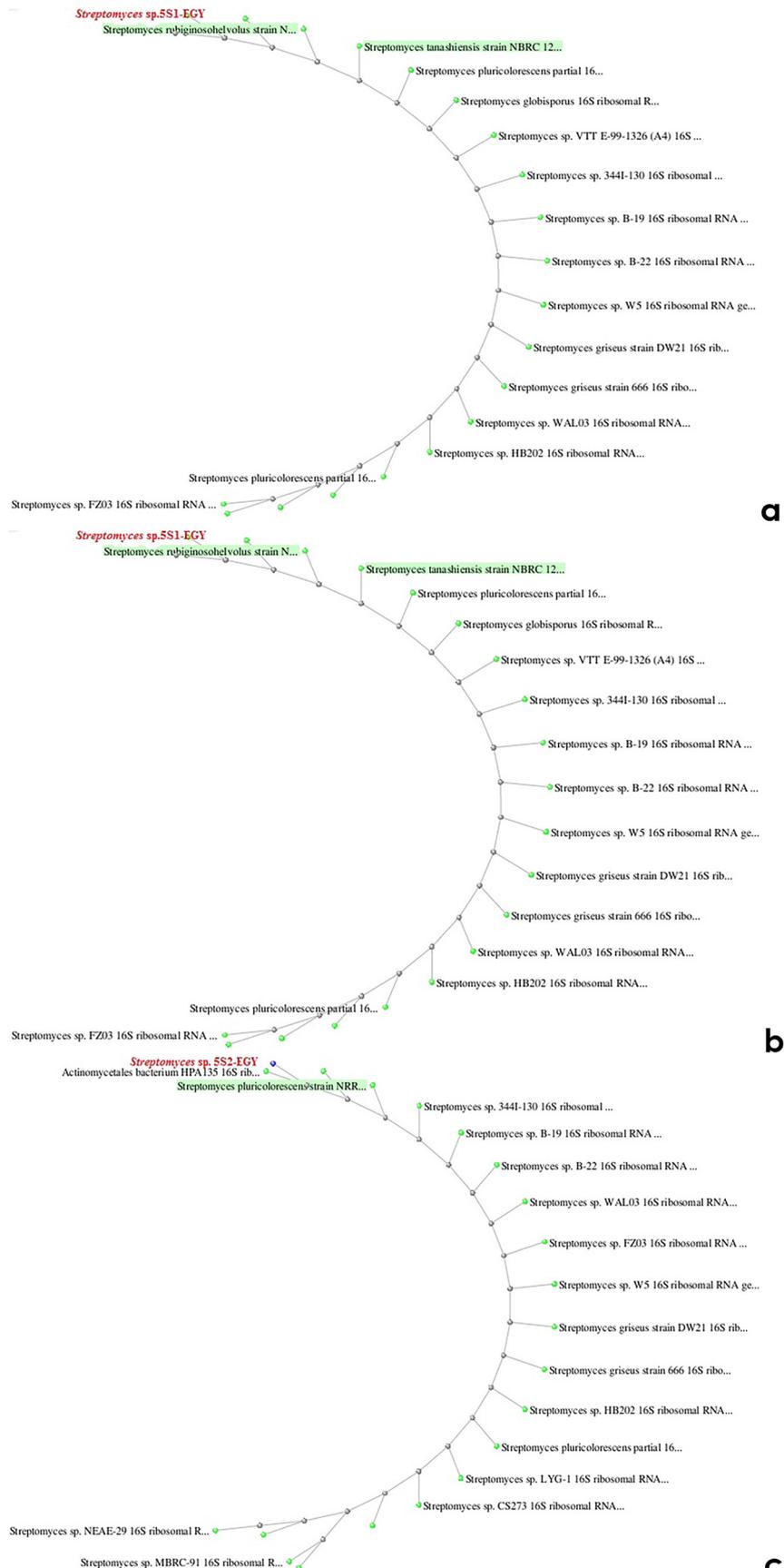


Figure 1. Phylogenetic tree showing relationship of strain 5S1 (a), 5S2 (b) and D(c), and the type strains of closely related *Streptomyces* species constructed using the neighbour-joining method and based on 16S rRNA gene sequences.

pharmaceutical formulations. Moreover, the primers 27F and 1492R were used for the identification of bacteria.

Antimicrobial activity of crude extract of Streptomyces sp

The crude extracts of the three isolates

5S1, 5S2 and D were used to investigate the antimicrobial activity against pathogenic microorganisms. The results revealed that the extracts of these isolates inhibited the growth of pathogenic microorganisms at varying degrees (Figure 2).

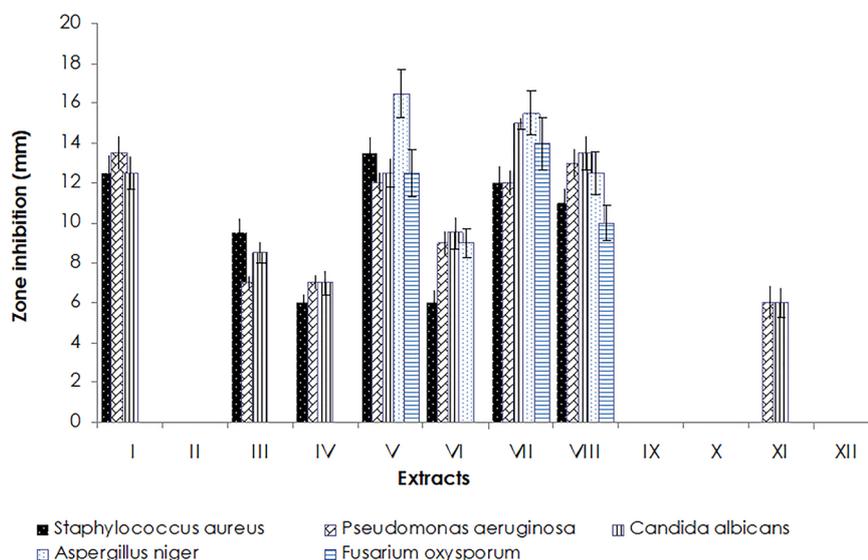


Figure 2. Antimicrobial activity of actinomycetes crude extracts. Results are mean ± SEM. Methanol and water, used as controls showed no zone inhibition

The extract isolated from the two actinomycetes strains (D, and 5S2) inhibited the growth of nearly all the tested microorganisms, whereas the third actinomycetes extract (5S1) did not show any inhibition of the tested organisms. These extracts showed antimicrobial activities against the two tested bacteria and *Candida albicans*. Neomycin as anti bacterial standard exhibited the following results: *S. aureus* (18 mm), *P. aeruginosa* (21 mm), *C. albicans* (23 mm) and *A. niger* (0 mm). On the other hand Cyclohexamide as antifungal standard exhibited the following results: *S. aureus* (0 mm), *P. aeruginosa* (0 mm), *C. albicans* (0 mm) and *A. niger* (26 mm). Negative controls (discs with water and methanol) did not exhibit any antimicrobial activity against any the tested microorganisms.

Antioxidant activity

The crude extracts showing high antimicrobial activity (i.e. extracts I, III, V, VI, VII, and VIII) were evaluated for their antioxidant activity. The results DPPH free radical scavenging activity (%) of different ethyl acetate extracts of both *Streptomyces* species are shown in Figure

3. The degree of stable DPPH* decolourization to DPPH yellow (reduced form of DPPH) indicated the scavenging efficiency of the extract. The recorded values of radical scavenging ability of different extracts I, III, V, VI, VII, and VIII at a concentration of 10 mg/mL were 82.06, 46.71, 20.52, 14.74, 18.74, and 11.01 % respectively.

Cytotoxic activity

Human hepatocellular carcinoma cell lines (HepG2) was used as a model system to examine the cytotoxic effect of the isolated *Streptomyces* crude extracts at a concentration varied from 0.39 to 50 µg. The concentration of the crude extracts leading to 50% of cytotoxicity was then determined. The results presented in Figure 4 showed that the ethyl acetate extract of the isolate D caused an inhibitory activity against HepG2 cell lines with IC₅₀ = 0.85 µg. The results presented in Figure 5 and the regression analysis presented in Figure 6 also showed the effects of these extracts on HepG2 cell line viability.

Identification of chemical composition by GC-MS

Based on the above mention results, the crude extract of static and of shaken culture supernatant of *Streptomyces* isolate D showed

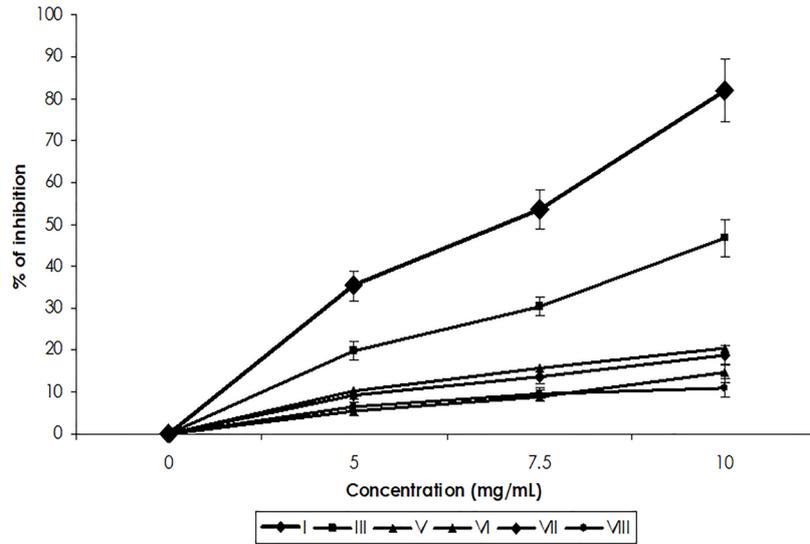


Figure 3. Antioxidant activity of actinomycetes crude extracts measured by DPPH assay. Results are mean \pm SEM

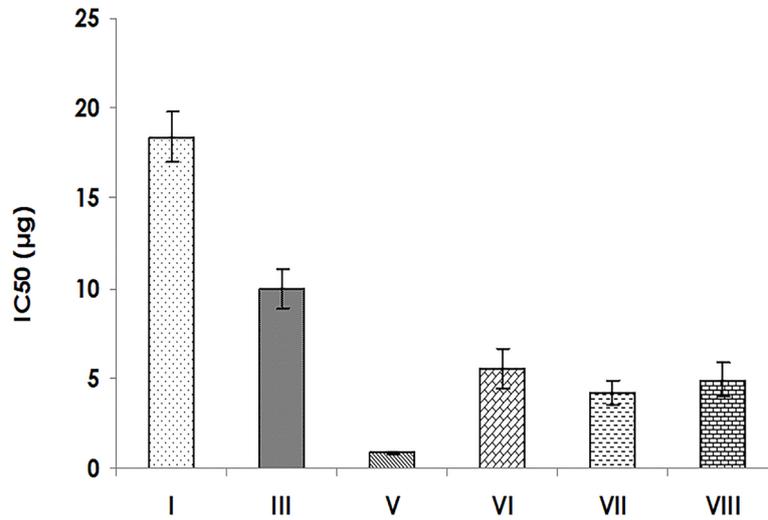


Figure 4. Evaluation of cytotoxicity (IC₅₀) against hepatocellular carcinoma. Results are mean \pm SEM

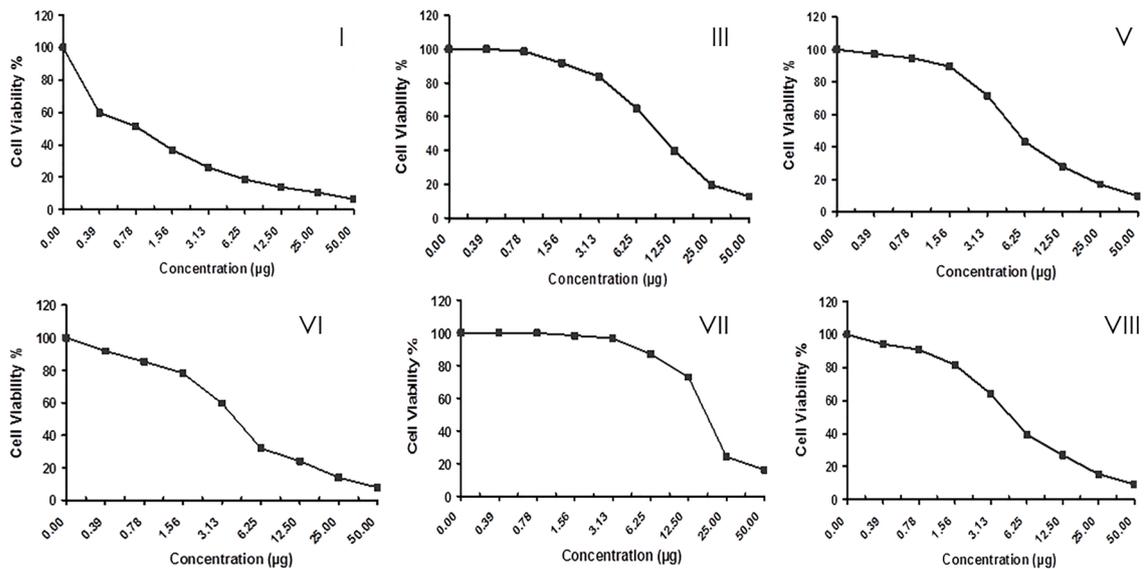


Figure 5. Effects of secondary metabolites extracted from *Streptomyces* sp. on HepG2 cell line viability

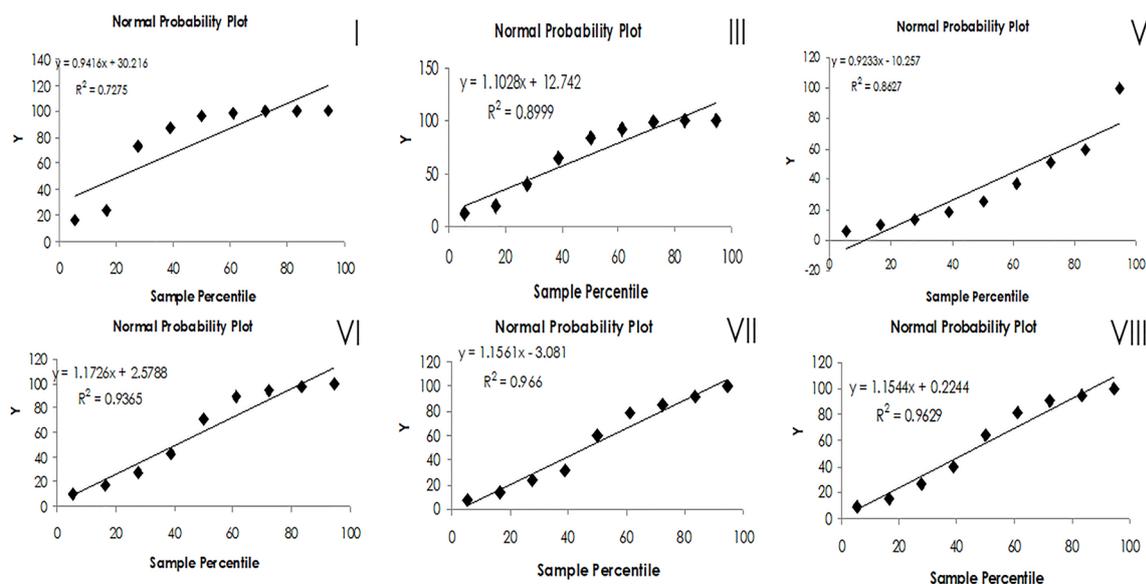


Figure 6. The regression analysis for the effect of secondary metabolites extracted from *Streptomyces* sp. on HepG2 cell line viability

the highest antimicrobial, antioxidant and cytotoxic effects. Consequently, these extracts were subjected to the identification of their active components. The chemical analysis of these crude extracts indicated the identification of 40 components for each extract, representing 98.84% and 99.98% respectively (Tables 4 and 5). The major components in the extract of rotary shaker of the culture supernatant of D isolate (extract V) were, Butane, 2, 3-bis (trimethylsiloxy) (14.89%), 3-Trifluoromethylsulfonyl-4,5-dihydro-2H-furan (11.37%), Allyl mercaptan (11.21%), 2,3-Butanediol, O-(trimethylsilyl)-, monoacetate (10.66%), Butanoic acid, trimethylsilyl ester (9.83%), Bis(2-ethylhexyl) phthalate (8.59%) and Ethanedioic acid, bis (trimethylsilyl) ester (8.22%) with the retention time 7.80, 6.41, 5.45, 11.15, 10.60, and 62.97, respectively (Table 4). On the other hand, the major components in extract of the static culture supernatant of the same isolate (extract VII) were 1, 2-Dimethyl-2-pyrrolino [3, 2-c] pyridine (19.08%), 3-(4Vinyl benzyloxy) benzyl alcohol (18.37%), 3-Octanol-3, 6-dimethyl (12.66%) and 1, 2-Benzene dicarboxylic acid, diisooctyl ester (7.38%) with the retention time 7.86, 7.80, 26.48 and 62.94, respectively (Table 5).

Discussion

Streptomyces are the largest antibiotic-producing genus has antifungal, antibacterial, antiparasitic drugs and also a wide range of other bioactive compounds such as

immunosuppressants. However, isolation of actinomycetes has always been faced with the difficulties in comparison to their competitors such as other bacteria and fungi which may be due to their long incubation period. The actinomycetes isolation ratio was increased by pre-treatment of the samples by subjecting them to dry air for three weeks.

In the current study, the isolated actinomycetes showed high antagonistic activity against several pathogenic fungi and bacteria. Patel et al. (2014) reported that 79% of the actinomycetes isolates were active against at least one of the 18 tested pathogens. Other reports indicated that actinomycetes isolates showed inhibitory activity against mycelial growth of plant pathogenic fungi like *Alternaria mali*, *Colletotrichum gloeosporoides*, *F. oxysporum* (Lim et al., 2000); against yeasts and gram positive and gram negative antibiotic resistant pathogens (Thirumalairaj et al., 2015).

The three actinomycetes isolates that showed the highest antagonistic effect against the tested microorganisms (i.e 5S1, 5S2 and D) were identified as *Streptomyces* spp. by using 16S rRNA molecular technique.

The crude extract of the bioactive compounds isolated from the three isolates of *Streptomyces* showed antimicrobial activity against different microorganisms. Similar results were reported by Khamna et al. (2009) who found that the crude extract was active as antifungal

Table 4. Chemical compositions of the ethyl acetate extract (V) of the filtrate of shaken culture broth of *Streptomyces* sp. D-EGY.

No.	RT	Area %	M.W	M.F.	Compound name
1	5.02	0.73	132	C ₆ H ₁₆ OSi	Silane, trimethylpropoxy
2	5.33	3.97	990	C ₆₀ H ₇₈ S ₆	1,4-Bis(3,3",4,4"-tetra butyl-2,2':5',2"-terthie n-2-yl)-1,3-butadiyne
3	5.45	11.21	74	C ₃ H ₆ S	Allyl mercaptan
4	6.15	0.85	162	C ₆ H ₁₈ OSi ₂	Dimethyl(trimethylsilyl) methoxy silane
5	6.41	11.37	202	C ₅ H ₅ F ₃ O ₃ S	3-Trifluoromethylsulfonyl-4,5-dihydro-2H-furan
6	6.71	8.22	234	C ₈ H ₁₈ O ₄ Si ₂	Ethanedioic acid, bis(trimethylsilyl) ester
7	7.04	0.12	148	C ₇ H ₈ N ₄	s-Triazolo[4,3-a]pyrazine, 3-ethyl
8	7.13	0.28	130	C ₇ H ₁₄ O ₂	(S)-6-Hydroxyheptanoic acid ^{1, 2}
9	7.19	0.24	131	C ₆ H ₁₀ ClN	5-Chloro-1,4-dimethyl -2,3-dihydro-1H-pyrrole
10	7.41	0.52	320	C ₂₀ H ₂₀ N ₂ O ₂	Bis[2-(1-methylethenyl)anilide]
11	7.51	0.99	172	C ₁₁ H ₁₂ N ₂	4-(1-Aminobut-3-enyl)benzotrile
12	7.57	0.68	323	C ₁₉ H ₃₃ NO ₃	Phenol, 2,6-di(tert-butyl)-4-bis (2-hydroxyethyl)amin
13	7.80	14.89	234	C ₁₀ H ₂₆ O ₂ Si ₂	Butane, 2,3-bis(trimethylsiloxy)
14	8.13	0.35	413	C ₁₉ H ₃₉ NO ₃ Si ₃	Silanamine, N-[2-[3-methoxy-4-[(trimethylsilyl)oxy]phenyl]-N,1,1,1-tetramethyl-2-[(trimethylsilyl)oxy]ethyl]-
15	8.40	3.13	2084	NA	Sscfggridrigaq Sglggn/3
16	9.84	1.20	2027	NA	Cnckapetascarr Cqah/2
17	10.60	9.83	160	C ₇ H ₁₆ O ₂ Si ₇	Butanoic acid, trimethylsilyl ester
18	11.15	10.66	204	C ₉ H ₂₀ O ₃ Si	2,3-Butanediol, O-(trimethylsilyl)-, monoacetate ¹
19	13.85	0.61	471	C ₂₁ H ₄₅ NO ₃ Si ₄	Epinephrine-Tetratms
20	13.97	0.30	415	C ₂₇ H ₄₅ NO ₂	6á-Nitrocholest-4-ene
21	14.09	0.83	266	C ₁₅ H ₂₆ O ₂ Si	1-Methoxy5-trimethylsilyloxy-3-phenylpentane
22	14.22	0.21	280	C ₁₆ H ₂₈ O ₂ Si	1-Methoxy-5-dimethyl (ethyl)silyloxy-3-phenylpentane
23	14.30	0.23	200	C ₁₁ H ₂₄ O ₂ Si	Butyl(dimethyl)silyloxy cyclopentane
24	14.63	0.60	202	C ₉ H ₁₈ O ₃ Si	Pantoyl lactone, trimethyl silyl
25	16.54	0.18	326	C ₁₅ H ₂₈ B ₂ O ₆	à-D-Mannopyranoside, methyl, cyclic 2,3:4,6-bis(butylboronate)
26	19.77	0.41	306	C ₁₃ H ₃₀ O ₄ Si ₂	2-Mono-isobutyryn, bis(trimethylsilyl)
27	22.36	0.27	314	C ₁₇ H ₃₈ OSi ₂	11-(Trimethylsilylmethyl)dimethylsilyloxy-1 -undecene
28	23.86	0.44	358	C ₂₁ H ₁₈ N ₄ O ₂	1-Amino-2-cyano-3,4-d ihydro-4-ethoxycarbo nyl-3-phenylpyrido[1, 2-a] benzimidazole ¹
29	26.29	0.82	274	C ₁₁ H ₂₃ BO ₅ Si	à-L-Galactopyranoside , methyl 6-deoxy-2-O-(trimethylsilyl)-, cyclic methylboronate
30	40.53	0.21	256	C ₁₆ H ₃₂ O ₂	Tetradecanoic acid, 12-methyl-, methyl ester
31	43.27	0.80	284	C ₁₈ H ₃₆ O ₂	i-Propyl 12-methyltetradecanoate
32	44.94	0.51	270	C ₁₇ H ₃₄ O ₂	Pentadecanoic acid, 14-methyl-, methyl ester
33	46.96	0.47	210	C ₁₁ H ₁₈ N ₂ O ₂	1,4-diaza-2,5-dioxo-3isobutyl bicyclo[4.3.0]nonane
34	47.45	0.43	330	C ₁₉ H ₃₈ O ₄	Hexadecanoic acid, 2,3-dihydroxypropyl ester
35	47.97	2.47	234	C ₁₇ H ₃₀	3-Methylene-9-pentyl piro[5.5]undecane
36	50.24	0.83	294	C ₁₉ H ₃₄ O ₂	9,12-Octadecadienoic acid, methyl ester, (E,E)
37	50.39	0.18	296	C ₁₉ H ₃₆ O ₂	16-Octadecenoic acid, methyl ester
38	52.19	0.62	308	C ₂₀ H ₃₆ O ₂	Linoleic acid ethyl ester
39	52.35	0.72	308	C ₂₀ H ₃₆ O ₂	Linoleic acid ethyl ester
40	62.97	8.59	390	C ₂₄ H ₃₈ O ₄	Bis(2-ethylhexyl) phthalate

M.F.: Molecular formula; M.W.: Molecular weight; RT: Retention time

¹Anticancer; ²Antioxidant (Data obtained from Dr. Duke's Phytochemical and Ethnobotanical databases)

Table 5. Chemical compositions of the ethyl acetate extract (VII) of the filtrate of static culture broth of *Streptomyces* sp. D-EGY.

No.	RT	Area %	M.W	M.F.	Compound name
1	5.02	1.67	158	C ₆ H ₁₀ F ₄	Hexane, 3,3,4,4-tetrafluoro
2	5.18	7.85	2904	NA	unknown
3	5.98	0.29	72	C ₄ H ₈ O	Butanal
4	6.07	0.44	383	C ₁₈ H ₃₇ NO ₂ Si ₃	1-(4'-trimethylsilyl oxy phenyl)-1-trimethylsilylox y-2-methyltrime thylsilyl-amino ethane
5	7.80	18.37	240	C ₁₆ H ₁₆ O ₂	3-(4Vinylbenzyloxy) benzyl alcohol
6	7.86	19.08	148	C ₉ H ₁₂ N ₂	1,2-Dimethyl-2-pyrrolino[3,2-c]pyridine ¹
7	9.98	0.39	192	C ₁₁ H ₁₂ O ₃	3-Isopropoxyphthalid
8	11.25	0.51	2084	NA	unknown
9	13.36	0.90	282	C ₁₉ H ₃₈ O	2-Nonadecanone
10	14.07	2.32	385	C ₂₂ H ₂₇ NO ₅	cis-1,2,7,9,10-pentamethoxy aporphine
11	15.02	0.63	990	C ₆₀ H ₇₈ S ₆	1,4-Bis(3,3",4,4"-tetra butyl-2,2':5,2"-terthien-2-yl)-1,3-butadiyne
12	20.52	0.57	122	C ₇ H ₆ O ₂	Benzoic acid
13	22.11	1.83	114	C ₆ H ₁₄ N ₂	N,N'-Dimethyl piperazine
14	22.84	0.80	340	C ₂₀ H ₂₄ N ₂ O ₃	(+)-5-Oxo-2α,7-.alph a,19,20-tetrahydro-16 epipleiocarpamine
15	23.13	0.83	257	C ₁₄ H ₂₇ NO ₃	12-Dimethylamino-10oxododecanoic acid
16	23.86	0.51	339	C ₁₅ H ₂₁ N ₃ O ₄ S	Ethyl 2-[3'-(3"-methyl-1"-hydroxycarbonylbutyl) thioureido]pyridine-3-carboxylate
17	24.24	3.42	216	C ₁₂ H ₂₄ O ₃	Propanoic acid, 2-methyl-, 2-ethyl-3-hydroxyhexyl ester
18	26.48	12.66	158	C ₁₀ H ₂₂ O	3-Octanol-3,6-dimethyl
19	39.05	0.67	242	C ₁₅ H ₃₀ O ₂	Pentadecanoic acid
20	40.54	0.60	256	C ₁₆ H ₃₂ O ₂	Tetradecanoic acid-12-methyl, methyl ester
21	43.70	0.74	270	C ₁₇ H ₃₂ O ₂	Pentadecanoic acid, 14-methyl-, methyl ester
22	43.78	0.36	242	C ₁₃ H ₂₆ O ₂ Si	(S)-5,5-Dimethyl-4-((tert-butyl)dimethylsilyloxy)-2-cyclopentenol ^{1,2}
23	44.13	2.42	242	C ₁₃ H ₂₆ O ₂ Si	(S)-5,5-Dimethyl-4-((tert-butyl)dimethylsilyloxy)-2-cyclopentenol ^{1,2}
24	44.97	0.87	270	C ₁₇ H ₃₄ O ₂	Hexadecanoic acid, methyl ester
25	46.15	0.75	873	C ₄₁ H ₄₇ NO ₂₀	Wilfortrine
26	46.24	1.54	447	C ₁₆ H ₁₉ N ₂ O ₉ S ₂	Glucobrassicin
27	46.73	1.05	378	C ₂₇ H ₅₄	Cyclohexane-1,3,5-trimethyl 2octadecyl
28	47.16	0.34	284	C ₁₈ H ₃₆ O ₂	Octadecanoic acid
29	47.28	0.63	284	C ₁₈ H ₃₆ O ₂	Hexadecanoic acid-14methyl, methyl ester
30	47.54	2.00	210	C ₁₁ H ₁₈ N ₂ O ₂	Pyrrolo[1,2-a]pyrazine -1,4-dione, hexahydro-3-(2-methyl propyl)
31	47.80	1.59	256	C ₁₆ H ₃₂ O ₂	Hexadecanoic acid
32	48.01	0.32	170	C ₆ H ₁₀ N ₄ S	2-Hydrazino-4-methyl6-methyl thiopyrimidine ^{1,2}
33	48.14	0.56	378	C ₂₇ H ₅₄	Cyclohexane, 1,3,5-trimethyl-2-octadecyl
34	49.88	1.40	270	C ₁₇ H ₃₄ O ₂	Pentadecanoic acid, 14-methyl, methyl ester
35	50.24	0.32	294	C ₁₉ H ₃₄ O ₂	9,12-Octadecadienoic acid, methyl ester
36	52.23	0.55	308	C ₂₀ H ₃₆ O ₂	Ethyl-9-cis-11-trans-octadecadienoate
37	52.42	1.01	310	C ₂₀ H ₃₈ O ₂	9-Octadecenoic acid (Z)-, ethyl ester
38	53.04	0.34	586	C ₃₆ H ₅₈ O ₆	Hexadecanoic acid, 1α,2,5,5α,6,9,10,10α-octahydro-5,5α-dihydroxy -4-(hydroxyl methyl)-1,2,3,22-Triacetoxystictane ¹
39	62.94	7.38	390	C ₂₄ H ₃₈ O ₄	1,2-Benzene dicarboxylic acid, diisooctyl ester (4S,5S)-4-(tert-Butyl dimethyl silyloxy methyl)
40	75.12	1.47	679	C ₃₅ H ₅₂ F ₃ NO ₅ Si ₂	-5-[(1R,4S,2E)-4-tert-b utyldiphenylsilyloxy-1(2,2,2-trifluoroacetylamino)pent-2-enyl]-2,2-di methyl-1,3-dioxolane ^{1,2,*}

M.F.: Molecular formula; M.W.: Molecular weight; RT: Retention time. ¹Anticancer; ²Antioxidant [Data obtained from Dr. Duke's Phytochemical and Ethnobotanical databases]. *'Smart drugs' enhance the processes associated with cognitive brain activity, allowing the user to experience positive benefits

against *R. tolonifera*, *A. flavus*, *F. oxysporum* and *Alternaria*. Moreover, several *Streptomyces* was reported to produce antifungal secondary compounds and the antagonistic activity of *Streptomyces* and the fungal pathogens is related to these compounds such as chitinase, β -1,3-glucanase, chitosanase, and protease which was documented to have the ability to lyse fungal cell walls and suppress fungal growth and both exponential and stationary culture filtrates of *Streptomyces* sp exhibited antifungal activity (Wang et al., 2011).

In DPPH scavenging assay, the antioxidants reduced the purple coloured DPPH radical to a yellow coloured compound diphenylpicrylhydrazine and the extent of reaction depended on the hydrogen donating ability of the antioxidants. Although the scavenging abilities of the crude extract reported herein was lesser, it was evident that the extracts showed hydrogen donating ability and therefore could serve as free radical scavengers and acting possibly as primary antioxidants. According to Taylor et al. (2007), there is no satisfactory drug to control cancer which is known to be the leading cause of death in the world. The limited availability to control cancer drug resistance along with the emergence of new pathogens has led the expansion of the search for novel drug candidates. According to the criteria of the National Cancer Institute (NCI, USA), the crude extract is considered promising as an antitumor at IC_{50} of $\leq 30 \mu\text{g/mL}$ however; for a pure substance IC_{50} should be $\leq 4 \mu\text{g/mL}$, which corresponds to strong cytotoxic activity (Dalal et al., 2016). The IC_{50} values of the crude extract of actinomycetes secondary metabolites in this study were found to be lower (18.4, 9.97, 0.85, 5.5, 4.21, and 4.91 $\mu\text{g/mL}$ for extracts I, III, V, VI, VII, and VIII, respectively) than that specified by the NCI for crude extracts, thus they are promising for further purification. Among the members of actinomycetes genus, *Streptomyces* sp. is a dynamic producer of functional, bio-effective metabolites with broad pharmaceutical range having antimicrobial, antihelminthic, antitumor and antiviral agents (Ravikumar et al., 2008). The current study revealed that both the rotary shaker and static crude extract of the *Streptomyces* D-EGY showed

the highest antimicrobial, antioxidant and anticancer properties. In this concern, Subashini & Kannabiran (2014) indicated that secondary metabolites from actinomycetes, especially the genus *Streptomyces*, are considered one of the most important sources for novel anticancer agents. The results of the present study suggested that the potential cytotoxic activity of extract VII against HepG2 could be due to the bioactive compound 1, 2- benzene dicarboxylic acid and hexadecanoic acid which were identified as the main components of *Streptomyces* sp. D-EGY (Krishnan et al., 2014). The mechanism by which these bioactive compounds can induce their cytotoxicity was suggested by the interference with basic cellular functions such as cell cycle, apoptosis, inflammation, angiogenesis, invasion and metastasis (Abe et al., 1993). The GC-MS results also revealed that complex mixture of numerous compounds were discovered. These results were similar to those reported by Ashok et al. (2014) who analysed secondary metabolites of *Streptomyces albus* and identified 8 compounds included 1-Octanol 2,7dimethyl which exhibited anticancer properties. Moreover, Abd-Elnaby et al. (2016) analysed *Streptomyces parvus* crude extract by GC-MS and found among others benzoic acid.

Conclusion

Streptomyces isolated from soil and marine sediments samples present varying degrees of antagonism against different pathogenic microorganisms. The crude extract of the isolates 5S1, 5S2 and D present greater antimicrobial, antioxidant and anticancer activity. Among them, the D-EGY isolate is the most effective. The chemical composition of these extracts revealed that they contain 40 chemical compounds two of them are well known as anticancer.

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