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Isolation and Characterization of Antibiotic / Antitumor Producing *Streptomyces.*

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ABSTRACT

A total of 500 *Streptomycetes* strains were isolated from twenty soil samples and screened for their antimicrobial activity. Only 56 isolates exhibited antimicrobial activity, of which two isolates showed a broad spectrum. They were identified phenotypically and genotypically as *Streptomyces fluvissimus*-FHM275 and *Streptomyces pratensis*-FHM572. MIC values of their crude extracts confirmed their antimicrobial potencies. Anticancer activities of the crude extracts were measured against human hepatocellular carcinoma cell line (HePG 2), human Caucasian breast adenocarcinoma (MCF 7), colon cell line (HCT 116) and lung carcinoma cell line (A549) as well as normal human epithelial amnion cells. The crude extract of *St fluvissimus*- FHM275 showed antitumor activity only against HCT116 and A549 with IC_{50} 15.5, 15.3 µg/ ml and SI, 3.6 and 3.7, respectively; however, *St. pratensis*-FHM572 showed a wide spectrum of cytotoxic activity towards most tested human antitumor cell lines, its extract appears to be both the most potent and most selective against A549 (IC_{50} = 7.5 µg/ml, SI = 7.9).

Keywords: Antimicrobial activity, Antitumor activity, Selectivity index, *St. fluvissimus*-FHM275, *St. pratensis*-FHM572, Lung cancer, Colon cancer

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INTRODUCTION

Microorganisms as a natural source of potential drug candidates were explored after the discovery of penicillin in 1929. Since then, large numbers of microorganisms, isolated mainly from soil, have been screened for drug discovery. Primary and secondary metabolites produced by the microorganisms are highly potent, biologically active and remain a powerful source for pharmaceutical discovery [1]. Over the last several decades and up till 2010, a significant number of natural product drugs/leads are actually produced by microbes as recently reviewed [2].

Among the well characterized pharmaceutically relevant microorganisms, actinomycetes remain the major sources of novel, therapeutically useful natural products [3, 4]. Along with actinomycetes, *Streptomyces* are the largest antibiotic-producers and untapped drug-productive species; they produce over two thirds of the clinically useful antibiotics of natural origin. *Streptomycetes* are the most economically and biotechnologically valuable prokaryotes, for their matchless ability to elaborate antibiotics with a range of biological activities that may eventually find current therapeutic applications as anti-infective, anti cancer agents, immunosuppressive agents or other pharmaceutically useful compounds [1, 5-12].

Antitumor antibiotics (cytotoxic/anticancer antibiotics) are drugs that inhibit and combat the development of tumors. The antitumor antibiotics produced by *Streptomyces* are invaluable in the medical field [13-15]. Although thousands of antibiotics have been isolated from *Streptomyces*, these represent only a small fraction of the bioactive compounds must be produced up to now. As a result, the discovery rate of new compounds from unexplored strains of *Streptomyces* would not decline for several decades. Therefore, isolation of new *Streptomyces* from natural resources and characterization of their secondary metabolites is still a valuable endeavor [2, 16, 17]. The current study was directed towards the isolation, characterization and identification of indigenous *Streptomycetes* exhibiting antimicrobial activities from soil. The metabolic products of the most potent *Streptomycetes* isolates were tested for their antimicrobial/antitumor activities.

MATERIALS AND METHODS

Sampling, isolation and maintenance of *Streptomycetes* cultures

Twenty cultivated soil samples were collected in sterile plastic bags at a depth of 15-20 cm, from five Egyptian Governorates, Assiut, Dakahlia, Sharkia, Kalubeia and Giza. Upon arrival to the laboratory, the samples were air dried for 24 h at room temperature. Isolation of *Streptomycetes* from each air dried soil sample was performed by serial dilution plate technique using starch nitrate agar medium [18], then the plates were incubated at 28 ± 2 °C for 7 days. Typical colonies were picked up based on their special morphological characteristics and purified by streaking (ca. 5-7 times) on starch nitrate agar plates. Long term preservation of isolates was achieved in soil cultures [19].

In vitro antimicrobial activity

A total of 500 purified morphologically distinct *Streptomycetes* isolates were screened for antimicrobial activity against the pathogenic test microorganisms by well diffusion method assay. The isolates were grown on starch nitrate agar for 5 days at 28 ± 2 °C, afterward, a disk of 8.0 mm diameter of each culture was cut by sterile cork borer and aseptically transferred into 250 ml Erlenmeyer flasks containing 50 ml of sterile starch nitrate broth medium. The inoculated flasks were incubated for 5 days at 28 ± 2 °C and 180 rpm on a rotary shaker (New Brunswick Scientific, Edison, N. J., USA). The cultures were filtrated through Whatman filter paper No. 1, and then the resulted filtrates were sterilized by filtration. A sterile cork borer was used to make holes (8.0 mm in diameter) in the plates seeded with the test organism, then only 0.1 ml of each filtrate was aseptically transferred into each hole and incubated at 30 °C for 1 and 3 days for bacteria and fungi, respectively. Antagonism was determined by measuring the size of inhibition zone around holes in millimeter.

The assay plates were seeded with the following microorganisms: Gram positive bacteria (*Bacillus cereus*, *Bacillus subltis* ATCC 6633, *Staphylococcus aureus* ATCC 6538), Gram negative bacteria (*Escherichia coli* ATCC 19404, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella typhimurium* ATCC 14028), yeast (*Candida albicans* ATCC 10231) and fungi (*Asperagillus flavus* NRRL A- 1957, *Asperagillus niger* ATCC 16404, , *Botrytis allii* NRRL A- 2502, *Macrophomina phaseolina* NRRL A- 62743, *Asperagillus terries*, *Rhizoctonia solani*). *B.*

cereus, A. terries and *R. solani* were kindly provided by Culture collection of Cairo MIRCIN, Faculty Agric., Ain Shams University.

Extraction of the bioactive compound(s) from potent cultures filtrates

The most potent two isolates which possessed high broad spectrum activity against both bacteria and fungi were selected for further studies. As previously mentioned, each strain was grown in starch nitrate broth medium for 5 days at 28 ± 2 °C and at 180 rpm; then broth cultures (pH 8.0) were filtrated and sterilized. Different solvents were tested for the extraction of the bioactive compound(s) from the culture filtrates. The solvents used were ethyl acetate, butyl acetate, amyl acetate, chloroform, n-butanol, petroleum ether, diethyl ether, benzene and mixture of ethyl acetate: chloroform (1:1) to determine the ideal solvent for extraction. Each crude culture filtrate was divided into 9 portions; an equal volume of each solvent system was added to each portion. Subsequently, each mixture was divided into 3 parts, the first was acidified with acetic acid or HCl to pH 2; and the second was kept as such (pH 8.0) while the third was made alkaline to pH 10 by adding NaOH. Solvent-supernatant mixture was agitated for 15 min, and then the organic phase was separated from broth by separating funnel. Solvent was centrifuged at 5000 rpm for 15 min to remove traces of fermentation broth. All extracts were assayed for their antimicrobial activity using respective solvents as control by agar well disk method [20].

Separation of bioactive compound(s) from solvent

The solvent system, ethyl acetate: chloroform (1:1) was the ideal one for extraction, therefore the organic phase evaporated at 35-40 ^{o}C under reduced pressure by rotary evaporation. The crude extracts of FHM275 (dark yellow residue) and FHM572 (Yellowish brown oily residue) were weighed and stored at −20°C for further use.

Determination of Minimum Inhibition Concentration (MIC)

The minimum inhibitory concentrations (MICs) of the ethyl acetate: chloroform (1:1) crude extracts were determined and compared with the reference antibiotics, amoxicillin trihydrate as antibacterial and metronidazole as antifungal. Three-fold dilution procedure of 10 mg/ml of each crude extract, amoxicillin and metronidazole (filter sterilized) were serially diluted in nutrient broth or Czapek's Dox broth media (2ml/tube) for bacteria and fungi, respectively. Thereafter, 0.1 ml of the test organism culture was added to the tubes containing the antimicrobial agent(s) and broth media. The tubes were incubated aerobically at 30°C for 24 h for bacteria and 48 h for fungi. Positive controls were prepared separately for both bacteria and fungi with respective organisms in the same culture media without the extract. After incubation, the tube with lowest concentration of extract shows no growth was taken as the MIC value for the respective organism. All data represented the average of three replicates.

***In vitro* cytotoxic activities bioassay**

Human normal and tumor cell lines and cell cultures

Anticancer activity of crude extracts was measured against human hepatocellular carcinoma cell line (HePG 2), human Caucasian breast adenocarcinoma (MCF 7), colon cell line (HCT 116) and lung carcinoma cell line (A549) as well as normal human epithelial amnion cells; all cell lines were obtained from ATCC. This bioassay was conducted in the Bioassay-Cell Culture Laboratory, National Research Centre, El-Tahrir St., Dokki, Cairo 12622, Egypt. Handling with cell lines was carried out under aseptic condition in a Laminar flow cabinet biosafety class II level (Baker, SG403INT, Sanford, ME, USA); HePG2, MCF7 and HCT116 cells were suspended in RPMI 1640 medium and A549 in DME medium. The media are supplemented with 1% antibiotic-antimycotic mixture (10,000U/ml potassium penicillin, 10,000µg/ml streptomycin sulfate and 25µg/ml amphotericin B), 1% L-glutamine and 10% fetal bovine serum and kept at 37 ºC under 5% CO_2.

Cytotoxic activity

The viability of cells was determined by a colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay as described by Mosmann [21]. The assay is based upon the ability of mitochondria

to catalyze the reduction of yellow MTT bromide to insoluble purple formazan, the concentration of which is measured spectrophotometrically. Cells were batch cultured for 10 days, then seeded at concentration of 10×10^3 cells/well in fresh complete growth medium in 96-well microtiter plastic plates at 37 ºC for 24 h under 5% CO_2 using a water jacketed carbon dioxide incubator (Sheldon, TC2323, Cornelius, OR, USA). Medium was aspirated, fresh medium (without serum) was added and cells were incubated either alone (negative control) or with different concentrations of sample to give a final concentration of (100, 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.78 ug/ml). After 48 h of incubation, medium was aspirated, 40 µl MTT salt (2.5µg/ml) were added to each well and incubated for further four hours at 37ºC under 5% CO_2. To stop the reaction and dissolving the formed crystals, 200 µl of 10% sodium dodecyl sulphate (SDS) in deionized water were added to each well and incubated overnight at 37ºC. A positive control which composed of 100 µg/ml of adrinamycin (doxorubicin) was used as a known cytotoxic natural agent who gives 100% lethality under the same conditions [22, 23]. The optical densities were measured using a microplate multi-well reader ELISA (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA) at a test wavelength 595 nm and a reference wavelength of 620 nm. DMSO is the vehicle used for dissolution of crude extracts and its final concentration on the cells was less than 0.2%. A statistical significance was tested between samples and negative control (cells with vehicle) using independent t-test by SPSS 11 program. A probit analysis was carried out for IC_{50}, IC_{90} determinations using SPSS 11 program. The percentage of change in viability was calculated according to the formula:

$$1- \frac{\text{Reading of extract}}{\text{Reading of negative control}} \times 100$$

Selectivity index (SI) was determined as the ratio of cytotoxicity (IC_{50}) on normal human epithelial amnion cells to each cancer cells [24].

Characterization of active isolates

The active isolates were characterized following the guidelines established by the International *Streptomyces* Project, ISP [25- 27]. Macro-morphological, micro-morphological, physiological and biochemical as well as chemotaxonomical features were studied. Morphological observation through macro-morphology based on the growth pattern on starch nitrate agar medium and recorded after incubation for 7 to 14 days at 28 ± 2 °C. The color of aerial mycelium, substrate mycelium and soluble pigment were observed by naked eye. The spore chain morphology and spore surface ornamentation were made with a light and transmission electron- microscopy (Zeiss EM-10 West Germany). Physiological and biochemical tests included carbon utilization, melanin synthesis, enzyme activities of proteolytic, lipolytic and lecithinase [28], nitrate reduction, hydrogen sulphide production, pectin, xanthine and arbutin degradation activities. Chemotaxonomical markers of isomers of diaminopimelic acid (DAP) in cell wall hydrolysates and whole-cell sugars. The strains were identified based on the keys of Szabo *et al*. [26] and Holt *et al*. [27].

Molecular identification

Preparation of template DNA

Streptomyces strains were grown in starch nitrate agar at 28 ± 2 °C for 5 days, a single colony of each strain was picked up and streaked on starch nitrate agar slants and incubated at 28 ± 2 °C for 5 days for obtaining heavy growth. Colonies were suspended in 0.5 ml of sterilized saline in a 1.5 ml and centrifuged at 10,000 rpm for 10 min. After removal of supernatant, the pellets were suspended in 0.5 ml of InstaGene Matrix (Bio-Rad, USA). Following the manufacturer's protocol, the suspension incubated at 56°C for 30 min and heated at 100°C for 10 min, then after heating, supernatant was used for PCR.

PCR amplification

PCR amplifications of 16S rRNA gene fragments were performed by adding 1 µl of template DNA in 20 µl of PCR reaction solution (Macro Gen) using the following primer sets 27 F (AgA gTT TgA TCM TGG CTC Ag) and 1492 R (TAC ggY TAC CTT gTT ACg ACT T). The amplification was performed as follows: 35 amplification cycles at 94°C for 45 sec, 55°C for 60 sec and 72°C for 60 sec. An aliquot of 5-15 µl of PCR reaction products

were electrophoresed on a 1% agarose gel containing ethidium bromide (10 mg/ml in H_2O) and the DNA bands were visualized under the UV light.

Purification of PCR products

PCR products were purified using Montage PCR Clean up kit (Millipore) according to the supplier's instructions.

Sequencing of the amplified PCR product

The purified products of approximately 1,400 bp were sequenced using the following primer sets, 518 F (CCA gCA gCC gCg gTA ATA Cg) and 800 R (TAC CAg ggT ATC TAA TCC). Sequencing were performed using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA) according to supplier's instructions. Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA).

Sequence similarities and phylogenetic analysis

The nucleotide sequence data were submitted to the BLASTN programs search nucleotide data bases (http://www.ncbi.nlm.nih.gov) within the National Centre of Biotechnology Information (NCBI), GeneBank [29]. The partial DNA sequences were assigned GenBank accession nos. KM438035 and KM046933 for *Streptomyces* FHM275 and FHM572 strains, respectively. These strains are available in Microbiological Resources Center (Cairo MIRCEN), Fac. Agric., Ain Shams University under nos. EMCC 1923 and 1924, in that order.

Results

***In vitro* antimicrobial activity**

The search for novel microbial metabolites of pharmaceutical importance especially from *Streptomyces* needs a strategy for screening of a large numbers of isolates. From twenty different soil samples collected from different area, 500 actinomycetes isolates were recovered and screened for their ability to produce antimicrobial metabolite(s). Out of the 500 isolates, only 56 exhibited antimicrobial activity against the tested microorganisms. Of which, 19 were active against only Gram negative bacteria, 25 against Gram positive bacteria, 14 against both Gram positive and gram negative organisms, 16 against *Candida* (3 against *Candida* and gram positive or Gram negative tested bacteria, 13 against *Candida* and filamentous fungi), 50 against fungi and others and 22 against filamentous fungi only (Fig. 1). Six isolates exhibited antagonistic activity against at least one of each of Gram negative, Gram positive organisms, filamentous fungi in addition to *Candida* (Fig. 2). On the whole, these 56 isolates exhibited variable activity against different tested microorganisms as illustrated in Fig. (3).

Fig. 1. Distribution of active actinomycetes isolates against different groups of tested microorganisms.

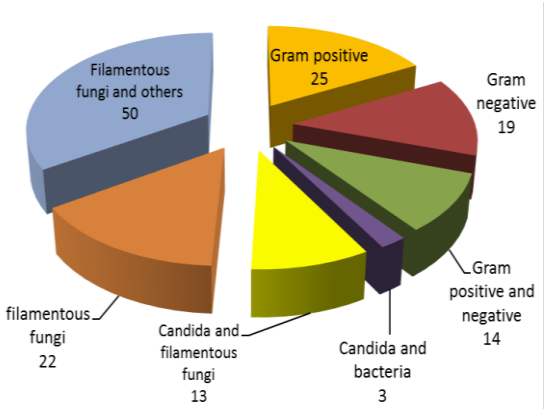

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Fig. 2. Isolates with a wide spectrum of antimicrobial activity against different tested microorganisms.

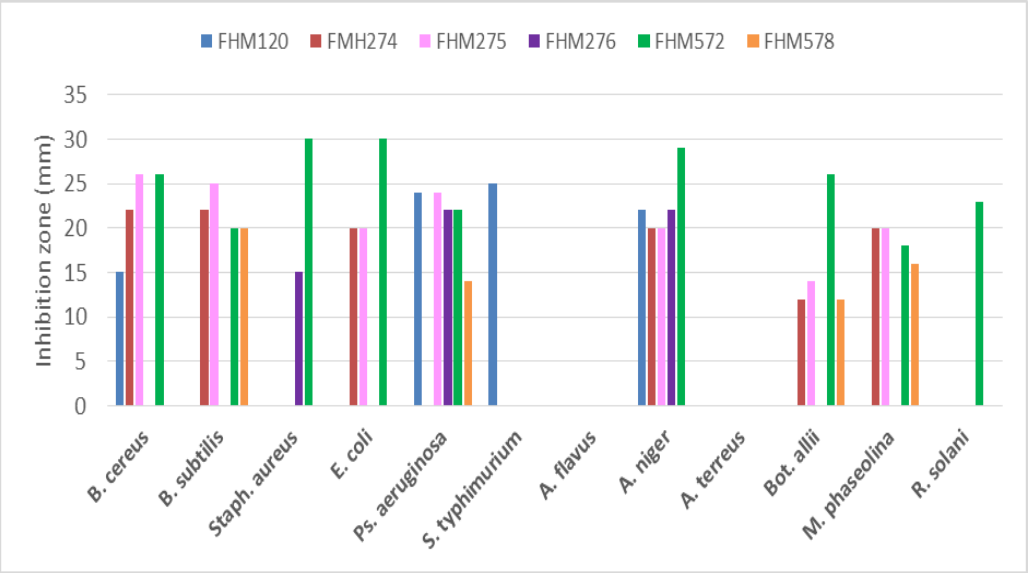
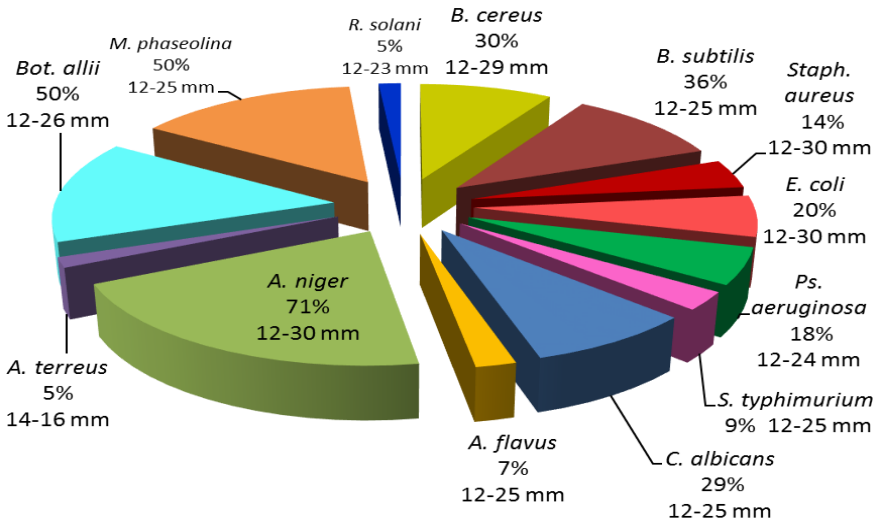


Fig. 3. Percentage of active actinomycetes isolates against the different tested microorganisms and the range of inhibition zone in mm diameter.



Extraction of the bioactive compound(s) from potent cultures filtrates

As illustrated in Table (1 and 2), different organic solvent systems at pH values of 2, 8 and 10 were used for extraction the bioactive compound(s) from the cultural filtrates of the most potent strains FHM275 and FHM572. The filtrates pH as such (8.0) was more efficient during extraction course by different solvents. Ethyl acetate, butyl acetate, amyl acetate, chloroform, n-butanol, petroleum ether, diethyl ether and benzene were unsuitable solvents for extracting the bioactive compound(s) where meager yields were obtained as indicated by the size of inhibition zones. Solvent system of ethyl acetate: chloroform (1:1) mixture was clearly quite effective**;** it produced the maximum inhibitory zones against all the tested microorganisms.

Minimum Inhibition Concentration (MIC)

The antimicrobial profile of the extracted crude bioactive compound(s) of FHM275 and FHM572 isolates in terms of MIC is shown in Table (3). MIC values of crude bioactive compound(s) against the tested

microorganisms were generally lower than those obtained by reference antibiotics which indicated their antimicrobial potencies.

Table. 1. The antimicrobial activity of bioactive compound(s) extracted by different solvents from crude filtrate of isolate FHM275.

Solvent System / Crude filtrate pH	Inhibition zone mm in diameter of											
	E. coli			*Ps. aeruginosa*			*B. cereus*			*A. niger*		
	2	8	10	2	8	10	2	8	10	2	8	10
Ethyl acetate	15	22	15	22	25	20	20	29	20	15	22	10
Butyl acetate	15	19	10	10	20	10	20	22	20	10	15	12
Amyl acetate	10	17	12	10	22	10	23	25	20	12	18	10
Chloroform	15	23	18	20	28	15	24	28	25	19	25	15
n-Butanol	10	15	10	10	15	10	18	20	15	10	10	10
Petroleum ether	10	18	10	10	15	10	20	25	20	10	15	10
Di-ethyl ether	15	20	15	20	25	15	20	25	23	10	20	12
Benzene	15	20	10	15	24	10	22	25	20	10	20	10
Ethyl acetate: chloroform 1:1	22	**29**	20	22	**32**	20	25	**34**	29	22	**30**	20
Crude filtrate (control)		**25**			**30**			**32**			**28**	

Table. 2. The antimicrobial activity of bioactive compound(s) extracted by different solvents from crude filtrate of isolate FHM572.

Solvent System / Crude filtrate pH	Inhibition zone mm in diameter of											
	E. coli			*Staph. aureus*			*B. cereus*			*A. niger*		
	2	8	10	2	8	10	2	8	10	2	8	10
Ethyl acetate	20	29	17	20	28	19	25	31	29	25	30	22
Butyl acetate	12	15	10	15	19	10	25	29	25	16	22	10
Amyl acetate	15	20	17	15	20	10	23	25	23	10	17	10
Chloroform	22	26	19	22	30	19	24	33	29	26	30	25
n-Butanol	12	15	10	12	15	10	26	23	24	10	15	10
Petroleum ether	10	15	10	10	10	10	20	15	20	10	19	10
Di-ethyl ether	15	20	18	19	27	22	23	25	25	20	26	15
Benzene	15	20	15	20	25	20	27	29	26	26	30	22
Ethyl acetate: chloroform 1:1	20	**34**	25	25	**37**	19	28	**38**	30	23	**34**	20
Crude filtrate (control)		**33**			**33**			**35**			**30**	

Table. 3. Antimicrobial activities of ethyl acetate: chloroform (1:1) extracted bioactive compound(s) produced by isolates FHM275 and FHM572.

Test organism	MIC µg/ml		
	FHM275	FHM572	Reference antibiotics*
E. coli	41	13	370
Ps. aeruginosa	41	41	123
B. cereus	13	4.5	370
B. subtilis	4.5	4.5	123
Staph. aureus	-	41	370
C. albicans	370	123	370
A.niger	123	123	370

* Amoxycillin trihydrate as antibacterial; metronidazole as antifungi.

***In vitro* cytotoxic activities against human tumor cell lines**

The crude extract of the most potent two isolates FHM275, FHM572 which possessed high broad spectrum activity against both bacteria and fungi (Fig. 2), were evaluated for cytotoxic activities against different human cell lines. The activities were evaluated by MTT cell viability assay at the following concentrations of crude extracts: 100, 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.78 ug/ml. As shown in Table (4), the crude extract of the strain FHM275 showed cytotoxicity only against the colon and lung carcinoma with IC_{50} 15.5 and 15.3 µg/ ml and the complete inhibition that obtained at 28.4 and 26.2 µg/ ml, respectively, was in accordance with IC_{90}. These values were lower than those needed to obtain an equal inhibition of normal human epithelial amnion cells.

The crude extract of the isolate FHM572 showed a wide spectrum of cytotoxic activity towards most cell lines; however the degree of cytotoxicity was differed greatly. It had less cytotoxic effect against breast cancer where it triggered inhibition at 70 µg/ ml with IC_{50} and initiate complete inhibition with IC_{90} at 114.4 µg/ ml. It is worthy to mention that these values surpassed the values required to initiate the similar inhibition with IC_{50} or IC_{90} of normal human epithelial amnion cells (Table 4). Lung carcinoma A549 was more sensitive to the tested extract than colon cancer (HCT116) and hepatocellular carcinoma (HePG2) as indicated by the measurements of cell lethality. Normal human epithelial amnion cell lines exhibited lower sensitivity than human tumor cell lines; IC_{50} ranged between 7.5 to 36.9 and IC_{90} ranged between 14.8 to 71.7 in tumor cell lines comparing to 59.5 and 105.4, respectively, in normal cell line. Crude extract from FHM572 was more effective against lung cell line A549 than that extracted from FHM275. Both crude extracts are highly selective where the selectivity index (SI) is greater than 3 (Table 5). The extract from FHM572 possesses the highest activity and highest selectivity against lung carcinoma A549 (IC_{50}= 7.5 µg/ml, SI 7.9).

Table.4. Cytotoxic activities of the crude extracts from *Streptomycetes* isolates FHM275 and FHM572.

Strains	Human tumor cell line (s)								Normal human epithelial amnion cells	
	Hepatocellular carcinoma HePG2		Colon HCT116		Caucasian breast adenocarcinoma MCF7		Lung carcinoma A549			
	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}
	(µg/ ml)									
FHM275	-	-	15.5	28.4	-	-	15.3	26.2	56.1	97.0
FHM572	36.9	71.7	16.1	29.3	70.6	114.4	7.5	14.8	59.5	105.4

IC50 : Inhibition concentration of the sample which causes the death of 50% of cells in 48 hrs.
IC90: Inhibition concentration of the sample which causes the death of 90% of cells in 48 hrs.

Table. 5. Selectivity index (SI) of the cytotoxic crude extracts from *Streptomycetes* isolates.

Extracts	Human tumor cell lines			
	HePG2	HCT116	MCF7	A549
FHM275	-	3.6	-	3.7
FHM572	1.6	3.7	-	7.9

SI = IC_{50} against normal cell line ÷ IC_{50} against each cancerous cell line

Characterization of active isolates

The cultural, morphological, physiological and biochemical properties of the potent isolates FHM275 and FHM572 are presented in Table (6) and Fig. (4 a-c). Morphological and cultural observations were based on the growth pattern on starch nitrate agar medium. They were Gram-positive filamentous bacteria and produced creamy white leathery colonies without diffusible pigmentation in starch nitrate agar medium. The mature sporulating aerial mycelia color of the strains, in that order, was found to be brownish white or yellowish white; the substrate mycelia color were belonging to the brown series when cultured in the starch nitrate agar medium (Table 6). The spore chain morphology was examined under microscopy and they showed straight or flexuous spore-bearing hyphae, respectively, and the spore surface was smooth in nature (Fig. 4 a-c). They utilized glucose, fructose, sucrose, arabinose, galactose and rhamnose; however, they varied in their ability for consuming the other tested sugars (Table 6). They were unable to produce melanoid pigments on the media used, but have the ability to produce H_2S and reduce $-NO_3$. Both strains displayed positive results for lipase, pectinase and protease and showed variability in their ability to degrade lecithin, arbutin and xanthine.

Examination of the whole cell hydrolysates on TLC plate proved that the major cell wall chemotype was identified to be L-diaminopimelic acid (L-DAP), a chemotype I cell wall as a diagnostic diamino acid of the peptidoglycan. No diagnostic sugars were found in whole-cell hydrolysate.

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Table. 6. Cultural, morphological, physiological and biochemical characteristics of active isolates.

Characteristics	Culture code		Characteristics	Strains	
	FHM275	FHM572		FHM275	FHM572
1. Cultural characteristics			Galactose	+	+
Arial mycelium color	B. white	Y. white	D- Manitol	-	+
Substrate mycelium color	L. brown	Y. brown	Inisitole	-	+
Diffusible pigmentation	-	-	**Enzyme activity**		
2. Morphological			Arbutinase	+	-
Spore chain morphology	Straight	Flexuous	Chitinase	-	-
Spore surface ornamentation	Smooth	Smooth	Lecithinase	+	-
3. physiological and biochemical properties			Lipase	+	+
Carbon utilization			Pectinase	+	+
Glucose	+	+	Protease	+	+
D- Fructose	+	+	Xathinase	-	+
Sucrose	+	+	**Melanin production in:**		
Xylose	+	-	Iron peptone	-	-
L-Arabinose	+	+	Tyrosine	-	-
Rhamnose	+	+	**H_2S Production**	+	+
Raffinose	+	-	**-NO3 reduction**	+	+

B., brownish; Y., yellowish; L., light.

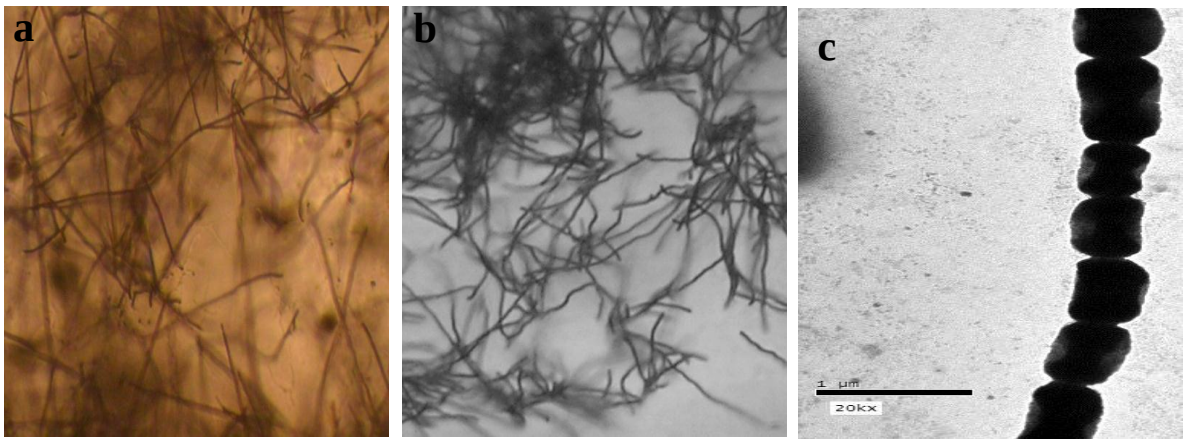

Fig.4. Photomicrographs showing sporophores types: a) straight spore-bearing hyphae (400 X), b) flexuous spore-bearing hyphae (400 X), c) and smooth spore surface ornamentations 25,000 X) of potent *Streptomyces* strains.

Identification

Based on the studied phenotypic characteristics through morphological, cultural chemotaxonomical, biochemical and physiological features, it is appeared strongly that the isolates FHM275 and FHM572 belong to genus *Streptomyces*. This assignment was also supported by amplified 16S rRNA gene sequence data. The alignment of the obtained sequences through Internet using the NCBI Blast software, for comparison with the homologous sequences contained in the data bank (GenBank) exhibited a high similarity of 100 % with *Streptomyces fulvissimus* and *Streptomyces pratensis* for the strains FHM 275 and FHM572, respectively.

The 16S rRNA sequences of these candidate *Streptomyces* strains were deposited in Gene Bank under the accession number of KM438035 and KM046933. The phylogenetic tree was constructed for partial 16S rRNA sequences of these isolates and the closest matches from BLAST searches (Fig. 5).

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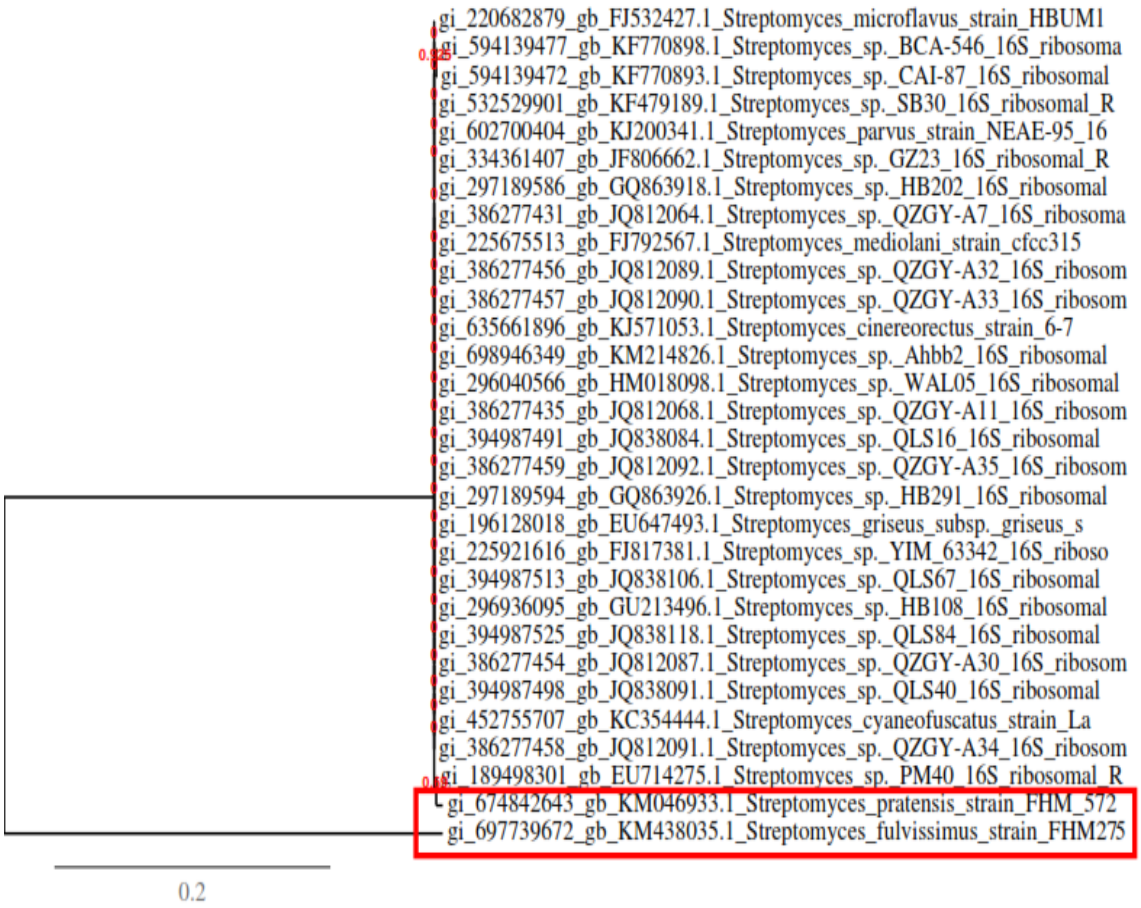

Fig.5. Phylogenetic tree based on partial 16S rDNA sequences showing the relationship of *St. fulvissmus*-FHM275 and *St. pratensis*-FHM572 with other species belong to the genus *Streptomyces*.

DISCUSSION

Streptomyces are widely represented in nature by the largest number of species and varieties. They differ greatly in their morphology, physiology and biochemical activities in producing the majority of known antibiotics [30]. That's why; they hold a famous position as targets in screening programs because of their diversity and their established capacity to produce bioactive compounds of pharmaceutical and clinical importance. In the present study, determination of antimicrobial activity of pure *Streptomycetes* cultures was performed using starch nitrate as production medium and shake culture as well as the well diffusion methods. The inhibition zone diameter is dependent upon the rate of diffusion of the antibiotic by the well diffusion assay. The strong activity expressed by a large zone of inhibition on agar plates indicated, as mentioned by Crawford *et al*. [31], that those most active isolates produce water-soluble antimicrobial metabolites. During the current screening course for new isolates for natural drug discovery, about 88.8 % of the streptomycete isolates did not show any antibiotic activity toward the tested microorganisms. This percentage is higher than those obtained in prior studies [32, 33].

It is palpable that all *Streptomycetes* probably possessed some antimicrobial properties if proper conditions were taken into consideration during culturing of these organisms for purpose of assessing their antibiotic production [34]. An early studies showed that shake flask method detected the fewest numbers of antibiotic producers as a result of a significant hyphal breakage rate occurs in shake-flask liquid cultures of many *Streptomyces*. This phenomenon accounts for these species producing antibiotics on agar but not in liquid culture [35, 36]. Besides, the composition of the medium employed was directly increased or completely inhibited the elaboration of antimicrobial substances [37, 38].

The present work focused on the biological properties of isolates in terms of the antimicrobial and antitumor activities, therefore the detail study was directed toward isolates showing a wide spectrum of antagonistic effects. Identification of the potent strains was based on phenotypic features and 16S rRNA partial gene sequences analysis. Evidently, 16S rRNA sequences provide a powerful way as they offer further verification for identification of *Streptomyces* [39]. From the present study, the most active *Streptomyces* strains FHM275 and FHM572 were confirmed as *Streptomyces fluvissimus* and *Streptomyces pratensis,* respectively.

Streptomyces fluvissimus-FHM275 showed broad spectrum activity against *B. cereus, B. subtilis, E. coli, Ps. aeruginosa, C. albicans, A. niger, Bot. allii* and *M. phaseolina*. However, *Streptomyces pratensis*-FHM572 exhibited a wider spectrum against the aforementioned tested microorganisms in addition to *Staph. aureus* and *R. solani*.

The *in vitro* toxicity assay of their secondary metabolites showed a significant antitumor activity, and the effect was a dose, strain and cell line type dependent. The crude extract of *Streptomyces fluvissimus*-FHM 275 showed cytotoxicity only against the colon cell line (HCT 116) and lung carcinoma (A549) with IC_{50} 15.5, 15.3 µg/ ml, respectively. However, crude extract of *Streptomyces pratensis*-FHM572 showed a wide spectrum of activity towards most human tumor cell lines with variable degree of cytotoxicity. Lung carcinoma (A549) was more sensitive to the tested extract followed by colon cancer (HCT116) and hepatocellular carcinoma (HePG2); IC_{50} values were 7.5, 16.1 and 36.9 µg/ml, respectively. According to the guideline criteria of the United States National Cancer Institute, the IC_{50} values of < 20 µg/ml were within the cutoff point [40, 41].

Anticancer drugs with limited side effects, inducing apoptosis and targeting selective cytotoxicity to the cancer cells are the drugs of choice. The selective activity or selectivity index (SI) is defined as the ratio of cytotoxicity on normal cells to cancer cells. Low SI indicates that the cytotoxic activity is probably due to cytotoxicity rather than activity against the cancerous cells themselves; therefore, high SI should offer the potential of safer therapy [24, 42, 43]. Our results are in line with this objective and showed good results because the crude extracts could kill cancers cells but did little damage to normal cells with selectivity index higher than 3. *St. pratensis*-FHM572 possesses the highest activity and highest selectivity against lung carcinoma A549 (IC_{50}= 7.5 µg/ml, SI =7.9).

CONCLUSION

The preliminary screening of *St. fulvissimus*-FHM275 and *St. pratensis*-FHM572 revealed their potential activities to yield potent bioactive compound(s) of anti-cancer/antimicrobial drugs; which indicates that these strains are promising for further study to isolate, purify and identify active compound(s) with potential anticancer/antimicrobial properties.

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