A NEW TRIAL FOR BIOFORMATION OF ANTIMICROBIAL AGENT CONTROLLING MULTI DRUG RESISTANT MICROORGANISM

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Abstract:

In the present investigation, a trial was done to find a new antimicrobial agent producing microbe from soil microbiota of local habitats to control the problem of multiple drug resistance. The term of Antimicrobial resistance (AMR) is used to describe microorganisms that can resist the effects of drugs and chemicals designed to kill them. Seventy six actinomycetes isolates were isolated from fifteen soil samples different localities in Egypt were primary screening for antimicrobial activity by agar plug diffusion method against test microorganisms. Sixteen isolates were selected for secondary screening in small scale submerged fermentation system and assayed against pathogenic tested microorganisms using agar well diffusion method. Among of these isolates tested, the isolate (S1SHA1) showed the highest antimicrobial activity against pathogenic test organisms. This isolate was identified as *Streptomyces griseoplanus* by morphological, physiological, biochemical characters and 16s rRNA gene sequence. Physical and nutritional factors affecting activity of antimicrobial agent were studied. The results showed that, optimum activity of antimicrobial agent achieved with pH 7, incubation temperature 28 C, for 7 days, at 150 rpm agitation, carbon and nitrogen source starch 1.5% and potassium nitrate 0.4%, as well as phosphorus 2 g/l and NaCl at concentration of 1 %. Antimicrobial agent from batch culture was subjected to extraction and purification processes using ethyle acetate and preparative TLC, respectively. Determination of minimum inhibitory concentrations (MIC) and Mode of action of antimicrobial agent produced by *S. griseoplanus* (S1SHA1) on the test microbial strains using Transmission Electron Microscopy (TEM). Cytotoxic studies showed that no cytotoxic effects were observed for the compound when tested even at high concentrations

Keywords:

Pathogenic microorganism, Antimicrobial agent, Multidrug resistant, Cytotoxicity

INTRODUCTION:-

Scientists all over the world are endeavoring continuously to search for new antibiotic compounds in order to tackle the serious consequences and dynamic nature of antibiotic resistance. The term of Antimicrobial resistance (AMR) is used to describe microorganisms that can resist the effects of drugs and chemicals designed to kill them (**Thornber *et al.*, 2020 ; Vela Gurovic and Olivera, 2017**).

Actinomycetes comprise an extensive and diverse group of Gram-positive aerobic mycelial bacteria. They are widely distributed group in nature, particularly the soil inhabit (**AitBarka *et al.*, 2016**). They have produced many important bioactive substances with high pharmaceutical values and approximately two-thirds of antibiotics (**Saadoun *et al.*, 2015**). Almost 80% of the world's antibiotics are known to produce from actinomycetes, mostly from the genera *Streptomyces* and *Micromonospora* (**Saadoun *et al.*, 2015**).

Actinobacteria are known as biofactories of enzymes, with applications in the textile, bio-refineries, food, pulp and paper, agriculture, detergent and pharmaceutical industries (**Richa and Vivek, 2018**).

The majority of the actinomycetes inhabit in soil that are essential drug sources remain uncultivable, as a result, inaccessible for novel antibiotic discovery. Goodfellow and Haynes, 1984 reviewed the literature on isolation of actinomycetes and suggested that only 10% of the actinomycetes are isolated from nature. Most of the antibiotics in use today are derivatives of secondary metabolites produced by actinomycetes and fungi **(Butler and Buss, 2006; Newman and Cragg, 2007)**. Actinomycetes can be isolated from soil and marine sediments. Although the pharmaceutical industry has screened soils for about 50 years, only a small fraction of the surface of the globe has sampled, and only a small fraction of actinomycetes taxa has been discovered **(Al-Ghazali and Omran, 2017).**

Filamentous soil bacteria belonging to the genus *Streptomyces* widely recognized as industrially relevant microorganisms because of their ability to produce many kinds of novel secondary metabolites including antibiotics and enzymes (**Williams *et al.*, 1983**). Indeed, different *Streptomyces* species produces about 75% of commercially and medically useful antibiotics **(Miyadoh, 1993).**

Under different conditions, antibiotics production by *Streptomyces*, it is possible to improve or lose altogether, so promoting the growth and production of secondary metabolites from *Streptomyces* can be carried out by manipulating the nutritional, chemical, and physical parameters of the culturing conditions. The cultural factor optimization plays a remarkable role in the productivity and economics of the essential process **(Al-Ghazali and Omran, 2017).** Hence, we need to screen more and more actinomycetes from different habitats for antimicrobial sources in the hope of getting some actinomycetes strains producing new antibiotics that have not been discovered yet and active against multiple drug-resistant pathogens. Therefore, our study was aimed to the isolation of *Streptomyces* sp. having the ability to produce antibacterial metabolites and optimize some

environmental parameters for excellent antimicrobial metabolite production.

Materials and Methods:-

Samples:

A total of 15 soil samples were collected from the rhizosphere of plants and agricultural soils from field sites of locations in Al-Sharqia Governorate - Anshas El Raml 11 samples (Lat,30°22'33.2"N; Long. 31°26'40.3"E) and locations in Qaliobia Governorate - Abu Zaabal 4 samples (Lat, 30°17'06.6"N; Long. 31°23'00.4" E) The samples were placed in sterile plastic containers separately, tightly sealed and transported to the laboratory. Samples were air dried for one week. After a week, heat treatment was performed by holding the sediment samples in a water bath at 50ºC for 60 min for prevention of other bacterial flora (**Takizawa *et al*., 1993**).

Media used for isolation:-

For isolation of Actinomycetes, the following two media were used, Starch Casein Agar medium (SCA) pH was adjusted to 7.0 before sterilization (**Arifuzzaman *et al*., 2010**) and Actinomycetes Isolation Agar (AIA) were used and pH was adjusted to 7 before- sterilization (**Awad *et al*., 2009**).

Isolation and Maintenance of Actinomycetes Isolates:-

Actinomycetes were isolated by serial dilution plate technique (**Arifuzzaman *et al*., 2010**). About 1g of each soil sample was suspended in 10 ml of sterile normal saline (0.85%) and shaken on orbital shaker for about 30 min at 121rpm; the suspension was left for 30 min in the Laminar Air Flow hood. Consequently, 1ml of sample was taken and diluted 4 fold in 9 ml of sterile normal saline (0.85 %). The suspensions were agitated with vortex and 0.1 ml of sample was taken from 10-1, 10-2, 10- 3, and 10 - 4 dilutions that was then spread on each of the Starch Casein Agar medium and Actinomycetes Isolation Agar medium. The plates were incubated, at 30 °C for six to ten days (**Dhanasekaran *et al*., 2009**). All isolates were identified as actinomycetes based on colony morphology and color of mycelium (**Williams and Cross, 1971**). This work was done at the AL-Azhar center for Fermentation Biotechnology & Applied Microbiology, AL-Azhar University, Cairo, Egypt.

Primary screening of the isolated microorganisms for Antimicrobial activity:-

A total of 76 isolates were primarily screened for antimicrobial activity against four test microorganisms *Bacillus subtilis* NRRL B 543, *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922 and *Candida albicans* MTCC183 using agar plug diffusion method according to **Egorov, (1985**). The test bacteria and fungi were obtained from The Regional center for Mycology and Biotechnology, AL-Azhar University, Egypt.

Secondary screening of the isolated microorganisms for Antimicrobial activity by agar well diffusion method:-

Sixteen isolates were selected for secondary screening in small scale submerged fermentation system. Two hundred milliliter of Starch Casein Broth was dispensed in to 500 ml Erlenmeyer Flask , to which a loop full of six days grown isolates were inoculated and incubated on a platform shaker at 100 rpm at room temperature for 6 days according to (**Remya and Vijayakumar, 2008; Dhanasekaran *et al.*, 2009**). were used agar well diffusion method was used to determination size of inhibition zone against five test microorganisms *Bacillus subtilis* NRRL B 543, *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Candida albicans* MTCC183 and *Aspergillus niger* NRRL 595.

Identification of selected isolates:-

The cultural, morphological and physiological characteristics of the selected isolates were studied using all the media and methods of International *Streptomyces* Project (ISP) as described by **Shirling and Gottlieb (1966).** Identification was carried out using the keys suggested by **Buchanan and Gibbson (1974), Williams *et al.* (1989) and Hensyl (1994).**

Studies concerning the phylogenetic characteristic:-

The locally isolated actinomycete was grown for 7 days on a starch agar slant at 30°C. Two milliliter of a spore suspension was inoculated into starch nitrate broth and incubated for 3 days on an incubator shaker at 200 rpm and 30°C to form a pellet of vegetative cells. The preparation of the total genomic DNA was conducted in accordance with the methods described by **Sambrook *et al.* (1989)**. The PCR amplification of 16S rDNA gene of the local actinomycete strain was conducted using two primers: F27 with a sequence 5-AGA GTT TGA TCM TGG CTCAG-3 and R1492 with a sequence 5-TACGGGYTACCTTGTTACGACTT-3. Purification of the PCR products and sequencing of the PCR products for the isolate under study were performed at the Sigma Company of Scientific Service (Cairo-Egypt).

Sequence data were analysed in the 'GenBank database' by using the BLAST program available on the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov). The unknown sequence was compared with all the sequences in the database to assess the DNA similarities. Multiple sequence alignment and molecular phylogeny were evaluated using BioEdit software (**Hall 1999**).

Optimization of culture conditions affecting antimicrobial compound production:-

The optimization studies for enhancing the antimicrobial activity were done in a series of experiments by using the approach of changing one variable at a time and keeping the other factors fixed at a specific set of conditions. At the end of each experiment, the antimicrobial activity was determined by measuring the inhibition zones after 24 h. This work was done at the AL-Azhar Center for Fermentation

Biotechnology & Applied Microbiology, AL-Azhar University, Cairo, Egypt.

Optimization of physical parameters:-

This included optimization studies for the best medium, incubation period, temperature, pH and agitation speed. For selecting the best production medium, the test was done by carrying out the fermentation separately in different media i.e. starch casein nitrate (SCA) broth, tryptone yeast extract broth (ISP 1), yeast extract malt extract broth (ISP 2), modified soluble starch (MSS), and starch nitrate broth medium (SNB) (**Suthindhiran *et al.,* 2009**). The value of the pH of the media was changed from 4 to 9 to arrive on the optimum pH for maximum production. The temperatures used for optimization were 25, 28, 31, 34 and 40ºC. Flasks were incubated in shaker incubator at 100 rpm for 6 to 10days (**Saadoun *et al.,* 2008**).

Optimization of biochemical parameters:-

The biochemical parameters taken for optimization were the different sources of carbon, nitrogen, different NaCl and phosphate concentrations. Different carbon source were studded such as glucose, sucrose, maltose, glycerol and starch were supplemented separately to a final concentration of 1% (w/v). While the tested nitrogen sources malt extract, yeast extract, potassium nitrate, ammonium sulphate, and peptone were supplemented to a final concentration of 0.2%. For the effect of different NaCl concentration, the broth medium was supplemented with different concentration of NaCl as 0.0, 0.5, 1.0, 1.5, 2.0 and 2.5 % (w/v) individually. While the best concentration of phosphate determined by using different concentration as 0, 2.0, 4.0, 6.0 and 8.0 (g/l) individually. The best potassium nitrate concentration was determined by using different potassium nitrate concentration as 0.2, 0.4, 0.6, 0.8 and 1% and for the best starch concentration determined by testing at 1.0, 1.5, 2.0, 2.5 and 3 % **(Usha et *al.,* 2010)**.

Extraction and purification of antimicrobial agents:-

The antimicrobial agent produced in the optimized medium by the selected isolate was extracted from the filtrate by the solvent extraction method of **Maneerat and Dikit (2007)**. Five liters of culture broth was used to extract and purify the antimicrobial agent. The culture was first centrifuged for25 min at 5000rpm at 4°C.The crude material was extracted by mixing with an equal volume of ethyl acetate and mixed in the separator funnel, shaken vigorously and allowed to stand for 30 min. The aqueous layer formed at the bottom of the separator funnel was removed and the white layer of antimicrobial agent just above the aqueous layer was collected in a glass Petri dish and left in the oven at 40–45°C until dry to evaporate the pre-coated silica gel TLC plates. The concentrated extract was run on silica TLC plates using ethyl acetate: toluene: formic acid in varying ratios: 5: 4: 1 (**Sherma and Fried 1996**).

The TLC plates were examined under a UV lamp at a wavelength of 254–365 nm. All spots from the about 20 TLC plates were scraped and examined for antimicrobial activity. The active compound was dissolved in a minimum amount of methanol and examined for purity on TLC. Finally, the purified compound was

collected and used for characterization purposes. The compound separated by TLC was collected by scraping and it was preserved in a clean screw-capped glass vial as dried powder (**Fernandes *et al.,* 2007**).

Minimum inhibitory concentration MIC:-

The minimum inhibitory concentrations (MIC) of antimicrobial compound of actinomycete were determined by well diffusion method. The antimicrobial compound was dissolved in methanol different concentrations of extracts was diluted using a two-fold serial dilution to give final testing concentrations Different weights of the purified antimicrobial compound range from 1.95 µg up to 1000 µg/mL were applied. The plates were incubated 24–48 h at 37 °C for bacteria and 48–72 h at 28 °C for fungi, the plates were examined (**Bordoloi*et al.,* 2001**).

Mode of action of antimicrobial agent produced by *S. griseoplanus* (S1SHA1) on the test microbial strains by Transmission Electron Microscopy (TEM):-

The effect of purified antimicrobial compound produced by *Streptomyces griseoplanus* on bacterial and yeast cells were evaluated by electron microscopy. Both *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922 and *Candida albicans* MTCC183 were grown in Nutrient Agar for bacteria and Sabouraud Dextrose Agar for yeast at 37°C. The cultures were collected during the exponential phase by centrifugation at 9000 rpm for 7 minutes and were then re-suspended in peptone water. Samples were treated with purified compound at 62.5 µg/ml and were incubated at 37°C for 12 hour. Samples were then collected by centrifugation at 9000 rpm for 7 minutes and were fixed with 2% of glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4 for 2 hours at 4°C. The cells were collected and washed twice with PBS buffer, and were then postfixed in 1% osmium tetroxide for 1 hour at 4°C. The cells were dehydrated through as ascending ethanol series solutions and followed by propylene oxide treatment. The cells were embedded, cut and stained with 2% uranyl acetate and lead citrate. The sections were observed using Transmission Electron Microscopy TEM (JEOL 1010, Japan) at the Regional center for Mycology and Biotechnology, AL-Azhar University, Egypt.

Cytotoxicity evaluation using viability assay:-

The cytotoxicity assay of **Mosmann (1983)** was performed as follows: the normal MRC-5 cell line cells were seeded in 96-well plate at a cell concentration of 5×10^4 cells per well in 100 µL of growth medium. Fresh medium containing different concentrations of the test sample was added, after 24 h of seeding. The Microtiter plates were incubated at 37 ºC in a humidified incubator with 5% CO_2 for a period of 24 h. Three wells were used for each concentration of the purified compound. Control cells were incubated without test sample and with or without DMSO. Then, the viable cells yield was determined by a MTT colorimetric assay. Treated samples were compared with the cell control in the absence of the tested compound. All experiments were carried out in triplicate. The cellular cytotoxic effect of the tested compound was calculated. The percentage of viability was calculated as

$$[(OD_t/OD_c)] \times 100\%$$

Where: OD_t is the mean optical density of wells treated with the tested sample and OD_c is the mean optical density of the untreated cells. The 50% cell cytotoxic concentration (CC_{50}) is the concentration required to cause toxic effects in 50% of intact cells that was estimated from graphic plots of the dose response curve for each concentration using Graphpad Prism software (San Diego, CA. USA).

Results and Discussion:-

The increase in the frequency of multi- resistant pathogenic bacteria is created an urgent demand in the pharmaceutical industry for more rational approaches and strategies to the screening of new antibiotics with a broad spectrum of activity, which resist the inactivation processes exploited by microbial enzymes (**Saadoun and Gharaibeh, 2003; Motta *et al.*, 2004**). The species belonging to the genus *Streptomyces* constitute 50% of the total population of soil actinomycetes and 75-80% of the commercially and medicinally useful antibiotics have been derived from this genus (**Mellouli *et al.*, 2003**). However, screening and isolation of promising actinomycetes with potential antibiotics is still a thrust area of research and it is suggested that the exploration of materials from different areas and habitats have a vital role to play in the search for new microbes and novel metabolites and is urgent to counter the threats posed by the fast emerging phenomenon of antibiotic resistance (**Saadoun and Gharaibeh, 2003**; **El-Naggar *et al.*, 2006**).

Thus, in the present work, different microorganisms were isolated and then screened with regard to their potential to generate antibiotics. The different soil localities at Al-Sharqia and Qaliobia governorate from which, isolation of different organisms was carried out. Also, several studies revealed that the microflora present in the rhizosphere can produce antagonistic molecules that will inhibit or kill the pathogens present (**Rondon *et al.*, 1999; Jaben *et al.*, 2004**).

Primary screening of the isolated microorganisms for Antimicrobial activity:-

Seventy six isolates of microorganisms were obtained from soil samples and screened for antimicrobial activity against four test organism *Bacillus subtilis* NRRL B 543, *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922 and *Candida albicans* MTCC183. Inhibition zones indicate that microorganisms produce antimicrobial compound after 24 hrs of incubation.

Sixteen out of seventy six actinomycetes isolates showed noticeable antimicrobial activities against Gram positive and Gram negative bacteria and yeast by agar plug diffusion method. Actinomycetes from Anshas El Raml soil sediments showed strong antimicrobial activity against the panel of pathogen. But few of the strains have not antimicrobial activity.

Secondary screening of the isolated microorganisms for Antimicrobial activity by agar well diffusion method:-

Based on the results of primary screening, it was found that 16 strains suppressed the test microorganisms in a wide range. The results of ethyl acetate crude extracts of 16 actinomycetes for antimicrobial activities were screened against 5 test microorganisms as presented in (Table 1).

Moreover, only five strains viz S1SHA1, SHSA9, SHSA29, SHSA49 and SHSA66 were confirmed for their higher antimicrobial activity (Table1).The highest antimicrobial activity was seen in the strain S1SHA1.

Among the tested actinomycetes, the ethyl acetate extract of the actinomycetes strain S1SHA1 showed 38, 37, 35, 32 and 35 mm zone of inhibition broad spectrum of antimicrobial activity against *Bacillus subtilis* NRRL B 543, *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Candida albicans* MTCC183and *Aspergillus niger* NRRL 595, respectively.

Table (1): Antimicrobial activity of ethyl acetate extract obtained from the selected sixteen actinomycetes and tested using agar well diffusion method.

No,	Tested isolates	Zone of inhibition (mm)				
		B. subtilis	***S. aureus***	***E. coli***	***C. albicans***	***A. niger***
1	S1SHA1	38	37	35	32	35
2	S2SHA4	21	25	10	28	14
3	S2SHA6	32	28	29	32	31
4	S3SHA2	15	18	14	12	14
5	S3SHA5	19	14	30	19	18
6	S3SHA6	13	11	17	11	00
7	S6QA3	11	14	15	15	19
8	S6QA4	25	17	31	29	28
9	S7QA1	10	20	12	15	27
10	S7QA6	17	13	11	13	00
11	S7QA7	13	15	15	21	29
12	S9SHA3	29	33	30	32	32
13	S10SHA2	16	13	19	23	19
14	S12SHA1	16	14	10	11	00
15	S13SHA1	27	30	32	28	30
16	S14SHA4	12	16	11	17	25

Taxonomic characterizations of the actinomycete isolate S1SHA1:-

Micro-morphological study of actinomycete isolate, S1SHA1 using a scanning electron microscope (JSM-5500) revealed that the spore chain was composed of elongate shaped spores with a smooth surface (Fig. 1). The data represented in Table 2 illustrate the cultural characteristics of the actinomycete isolate S1SHA1grown on different ISP media. The aerial hyphae of the isolate were grey with brown substrate mycelia but no diffused pigment was recorded. Carbon and nitrogen utilization, degradation test, sensitivity assay, tolerance to NaCl, growth pH and growth temperature were studied. The entire hydrolysate cell of this strain contained LL-diaminopimelic acid but no characteristic sugars could be detected (Table 3). Cell wall composition analysis is one of the main methods that can be employed to identify the chemotaxonomic characteristics of *Streptomyces*.

16S rRNA gene sequencing and phylogenetic analysis:-

To confirm the identification of the actinomycete isolate, the 16S rRNA sequence of the selected S1SHA1 isolate was compared with the sequence of Streptomyces sp. through multiple sequence alignment. Experimental analysis of the PCR amplification was studied through agarose gel electrophoresis. Figure 2shows the multiple sequence alignment, which showed that this isolate was close to Streptomyces griseoplanus NRRL- ISP5009 by query 93%.

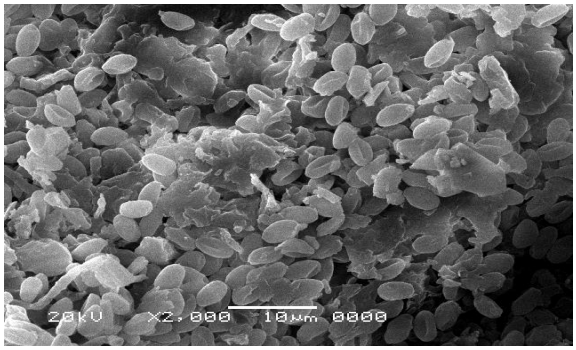

Figure (1): Scanning electron micrograph of *Streptomyces* griseoplanus, (S1SHA1) grown on starch nitrate casein agar medium for 4 days (X5, 000; bar 5 μm).

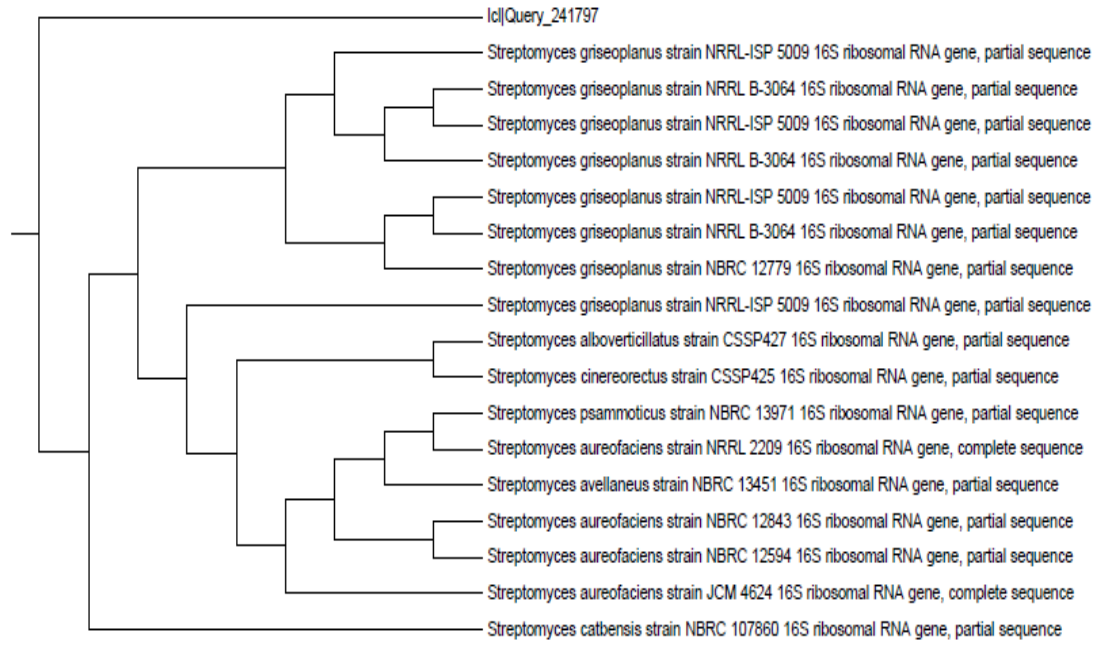

Figure (2): 16S rRNA gene tree showing the phylogenetic relationship neighbor- joining method between strain S1SHA1 and other known sequence of *Streptomyces sp.*

Table (2): Cultural and physiological characteristics of selected isolate S1SHA1.

Medium	**Growth**	**Substrate mycelium**
Yeast-malt extract agar (ISP 2)	Good	Brown 59
Oat meal agar (ISP 3)	Good	Dark yellow
Inorganic salts-starch agar (ISP 4)	Good	Dark brown 72
Glycerol-asparagine agar (ISP 5)	Moderate	Brown 59
Peptone yeast extract iron agar (ISP 6)	Good	Light brown
Tryptone yeast extract broth (ISP 1)	Moderate	Yellow brown

Table (3): The morphological, physiological and biochemical characteristics of the selected isolate S1SHA1.

Chemotaxonomic analysis			
Cell wall hydrolysis for:		Utilization of nitrogen source	
Diaminopimelic acid (DAP)	LL-DAP	L-Valine	—
Sugar pattern	ND	L-Methionine	+
Morphological characteristics		L-Phenylalanine	+
Spore chains	Elongate	L-Histidine	+
Spore surface	Smooth	L-Asparagine	+
Spore mass colour	Gray	L-Serine	+
Substrate mycelialcolour	Brown	L-Leucine	+
Diffusible pigment produced	—	L-Proline	+
Biochemical characteristics		L-Aspartic acid	+
Lipolysis	+	Utilization of carbon source	
Gelatin hydrolysis	—	Glucose	+
Nitrate reduction	+	Raffinose	—
Starch hydrolysis	+	Xylose	+
Catalase	+	Lactose	+
Sensitivity assay		Arabinose	+
Growth pH (5–10)	+	Cellobiose	—
Growth temperature (15–40°C)	+	D-Mannose	+
Growth with NaCl (4–9%, w/v)	+	D-Galactose	—
L-Cysteine	+	Starch	+

Optimization of physical and biochemical parameters for antimicrobial production by *Streptomyces griseoplanus*S1SHA1:-

Optimization of various physical parameters including different types of media, incubation period, temperature, pH and agitation speed were determined.The

difference in activity of antimicrobial agent in different types of media. The results illustrated in Fig. 3 indicate that SCA was the best production medium, giving a maximum inhibition zone on the test organisms followed by SNB medium (Fig. 3). Also, the difference in activity of the antimicrobial activity with different incubation periods culture conditions showed that it reaching its maximum activity after 7 day (Fig. 6) there are no significant variation shown in the antimicrobial activity at range 7 – 9 days. Streptomyces griseoplanus NRRL-ISP 5009, S1SHA1 produced antimicrobial agents with highest activity at pH 7 while decreased gradually at danger pH value (Fig. 4). The temperature was one of the critical parameters that has been controlled in the bioprocess. The difference inactivity of antimicrobial compound at different incubation temperatures was the highest value when the isolate grew at 28 ℃ as shown in (Fig. 5). On the other hand, the antimicrobial agent activity production was not severely affected by increasing the temperature up to 40°C. Agitation plays a very important role in the production of antimicrobial compound, which facilitates the oxygen transfer from the gas phase to the aqueous phasein the present study the highest antimicrobial compound production was obtained at an agitation speed 150 rev min-1(Fig. 7). The results obtained for demonstrated that the optimal carbon source for antimicrobial compound production was starchwith best concentration 1.5 % (Fig. 8 and 13). While the best nitrogen source for highest production of antimicrobial compound is potassium nitrate with best concentration 0.4 % (Fig. 9 and 12), were the optimal NaCl concentration for production of antimicrobial compound was 2g/l (Fig. 10).

Extraction and purification of the crude antimicrobial agent:-

The antimicrobial compound produced by S. griseoplanus NRRLISP5009, S1SHA1 was extracted with equal volume of ethyl acetate which resulted in the extraction of a brown colour precipitate. The TLC analysis performed for the extract showed the presence of eleven bands after charring with ethyl acetate: toluene: formic acid with ratio 5:4: 1 as the best mobile phase. The eleven bands were observed under UV light, the spot with Rf value of 0.36Showedthe highest inhibition zone. The most active compound was purified through a preparative TLC. The purity of the compound was checked by performing TLC analysis which showed asingle spot at an Rf value of 0.36.

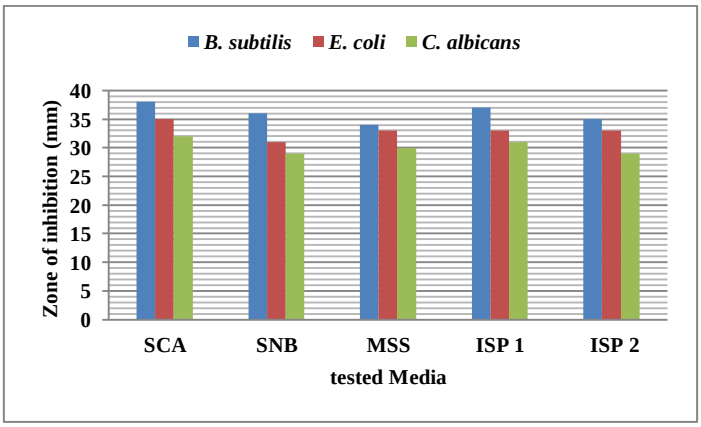

Figure (3): Effect of various media.

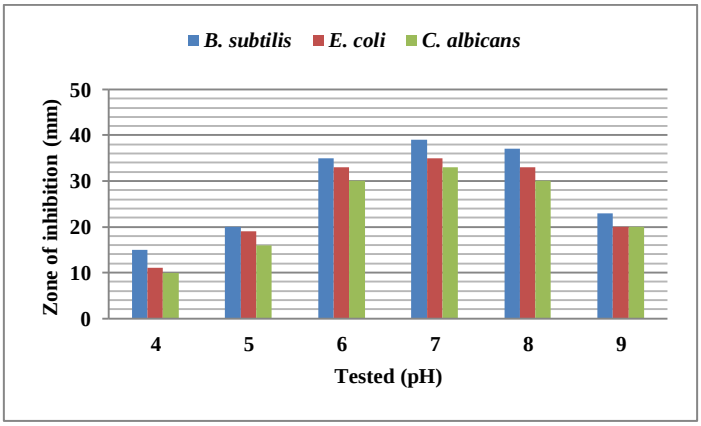

Figure (4): Effect of pH.

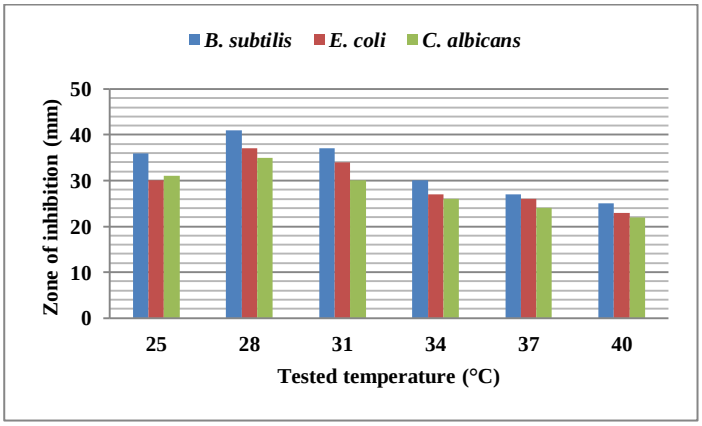

Figure (5): Effect of temperature.

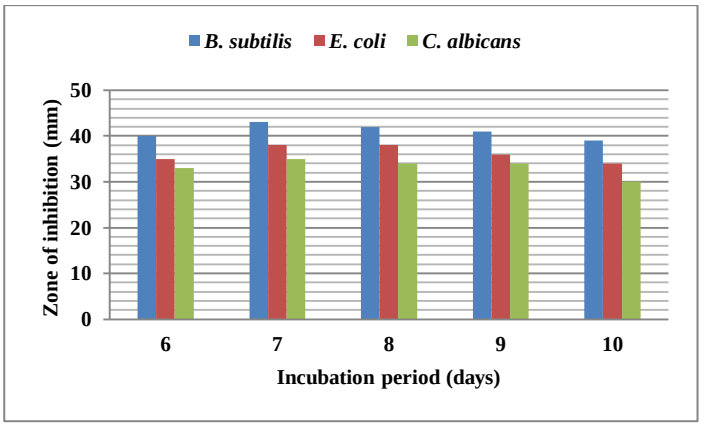

Figure (6): Effect of incubation period.

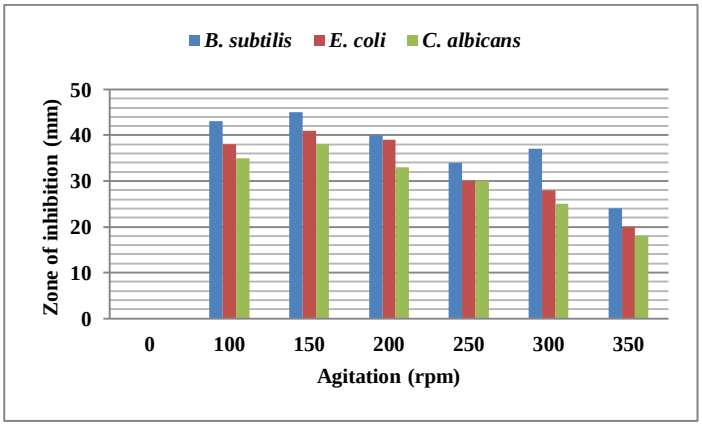

Figure (7): Effect of agitation.

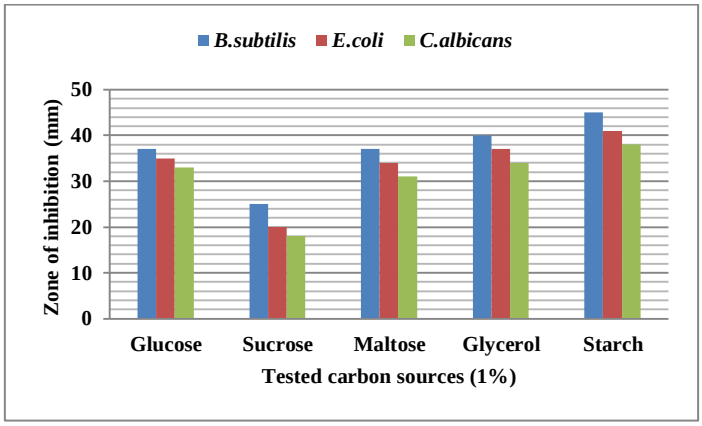

Figure (8): Effect of different carbon sources.

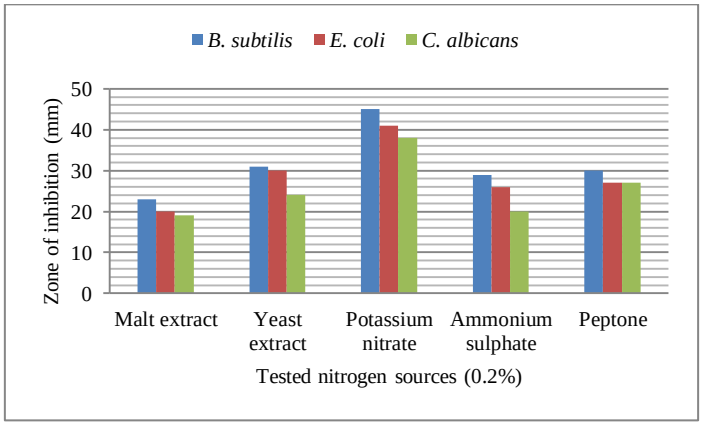

Figure (9):Effect of different nitrogen sources.

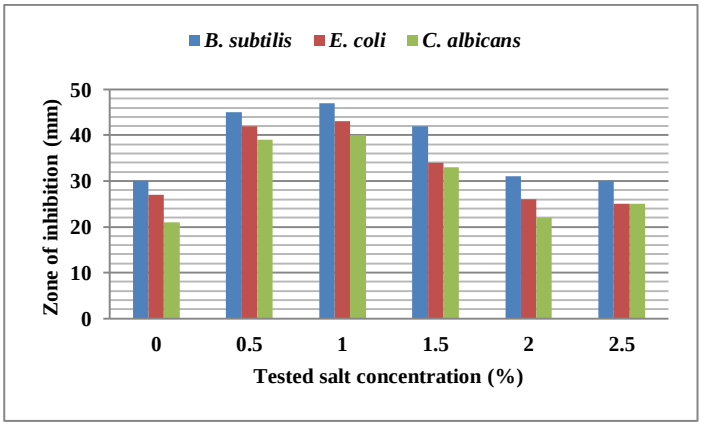

Figure (10): Effect of different salt concentration.

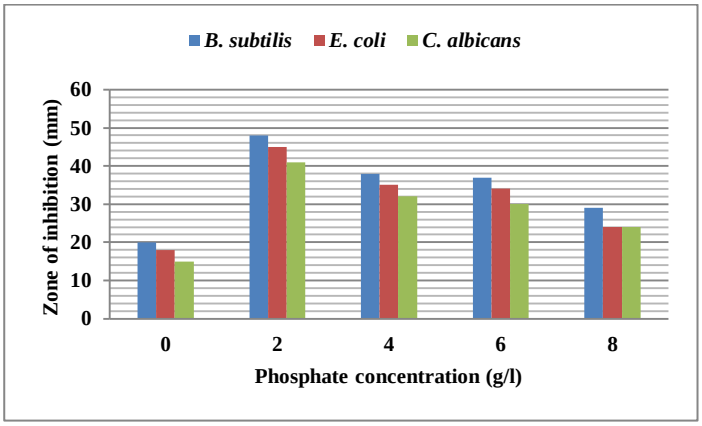

Figure (11): Effect of different phosphate concentration (K2 HPO4).

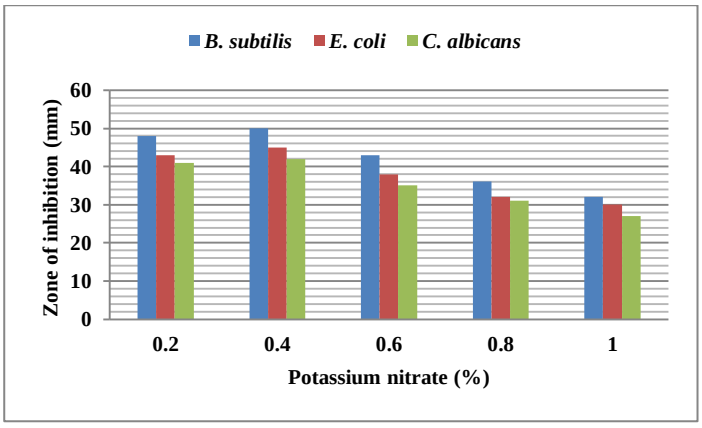

Figure (12): Effect of different potassium nitrate concentration.

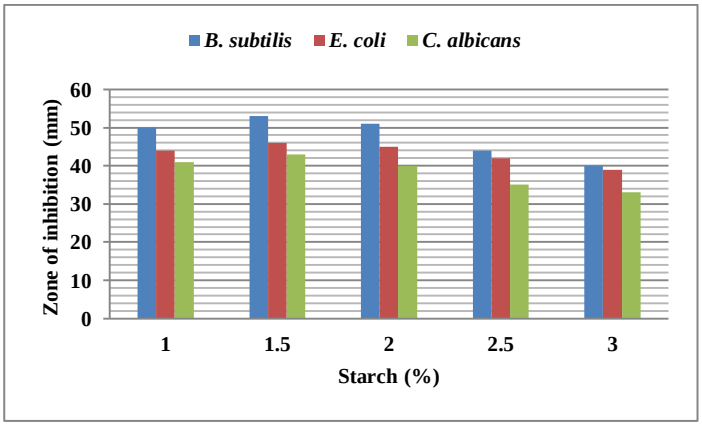

Figure (13): Effect of different Starch concentration

Determination of the MIC of the purified compound

MIC values of the antimicrobial compound from S1SHA1 against different target organisms were determined by agar well diffusion. *Bacillus subtilis* NRRL B 543 and *Klebsiella pneumonia* were the most sensitive to antimicrobial compound produced by S1SHA1 followed by other bacteria (Table 4).

Table (4): MIC values of the antimicrobial compound from *Streptomyces griseoplanus against* different pathogenic test organisms.

Test organism	MIC (µg/ml)
Bacillus subtilis NRRL B 543	62.5
Staphylococcus aureus ATCC 29213	62.5
Escherichia coli ATCC 25922	62.5
Pseudomonas aeruginosa ATCC	62.5
Candidaalbicans MTCC183	62.5
Aspergillusniger NRRL 595	125

The MIC is not a constant for a given agent, because it is affected by the nature of the test organism used, the inoculum size, and the composition of the culture medium, the incubation time, and aeration (**Pandey *et al.*, 2004**).

It is important to obtain a full characterization of an antimicrobial compound metabolite before it can be put into use since its behavior under different conditions will determine its application and ultimate usefulness (**Betina, 1964; Porter, 1971; Simone *et al.*, 1998**).

Effect on Ultrastructure of Bacterial and yeast Cells:-

The effect of purified antimicrobial compound on bacterial cell structure was

tested using transmation electron microscopy on Gram-negative tested bacteria *Escherichia coli* ATCC 25922, Gram-positive tested bacteria *Staphylococcus aureus* and yeast *Candida albicans* MTCC183

The untreated *Staphylococcus aureus* ATCC 29213 appeared cocci that displayed normally dividing cells with sharp delineation between cell wall, cytoplasmic membrane and the cytoplasm (Fig. 14). After incubation of the bacterial cells with purified antimicrobial compound, dramatic cellular alterations became visible on electron microscopic image (Fig. 14). The treated cells appeared oblong; edges become abnormal, elongated. Cell wall disrupted and exhibited thickened in some parts and breakdown in other due to leakage of cytoplasm.

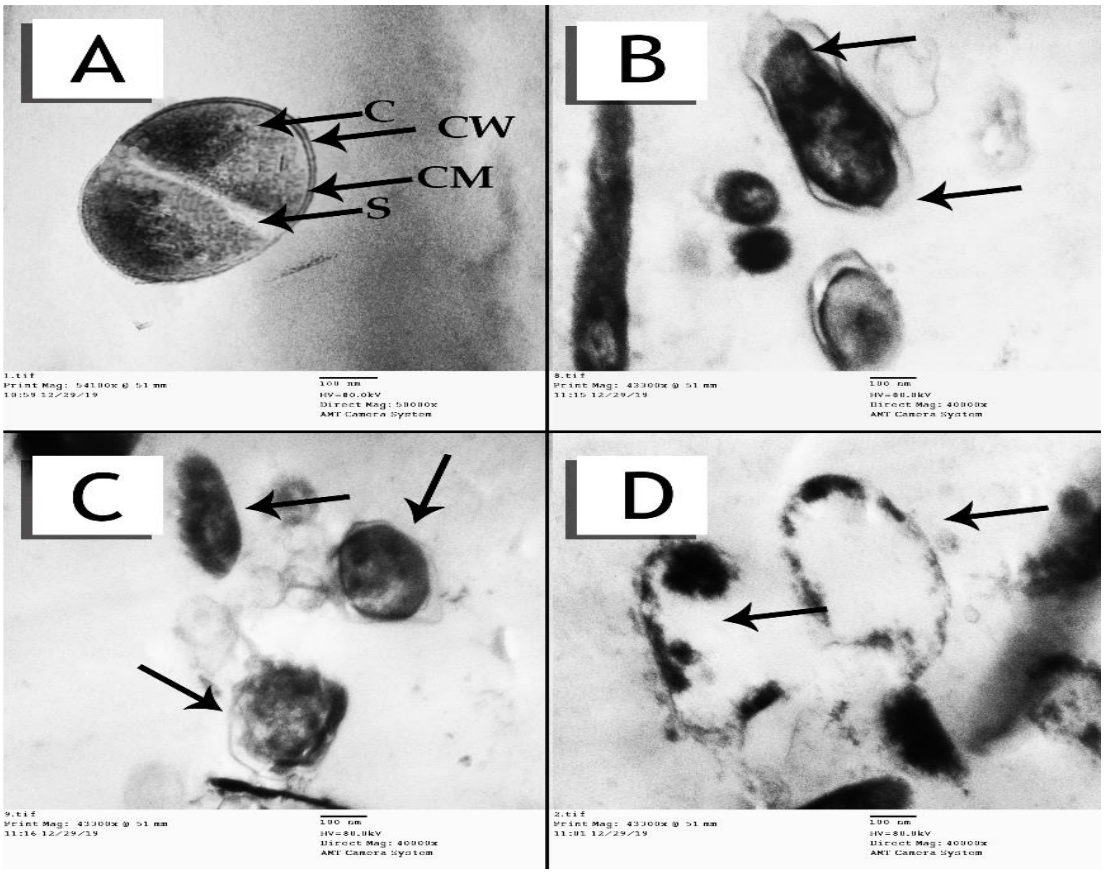

Figure (14): TEM microphotographs of *Staphylococcus aureus*. **A**: without treatment. **B, C&D**: treated with purified antimicrobial compound with concentration 62.5 µg/ml. Where, S: septum, CW: cell wall, CM: cell membrane, C: cytoplasm.

Escherichia coli ATCC 25922appeared short rods in TEM micrograph of untreated cells and showed a continuous thin smooth cell wall, cell membrane and nuclear material (Fig. 15). When subjected of *Escherichia coli* cells to purified antimicrobial compound, bacterial cells lysed rapidly, so cells appeared as very long threads. Cytoplasm shrinked leaving cell wall, while other cells appeared metamorphosed, cytoplasm lost its even distribution and showed clumping of intracellular materials. Cell wall was lost uniformity and leading to cell wall rupture and even strong damage in many areas with thickened appearance more pronounced

at polar-regions (Fig. 15).

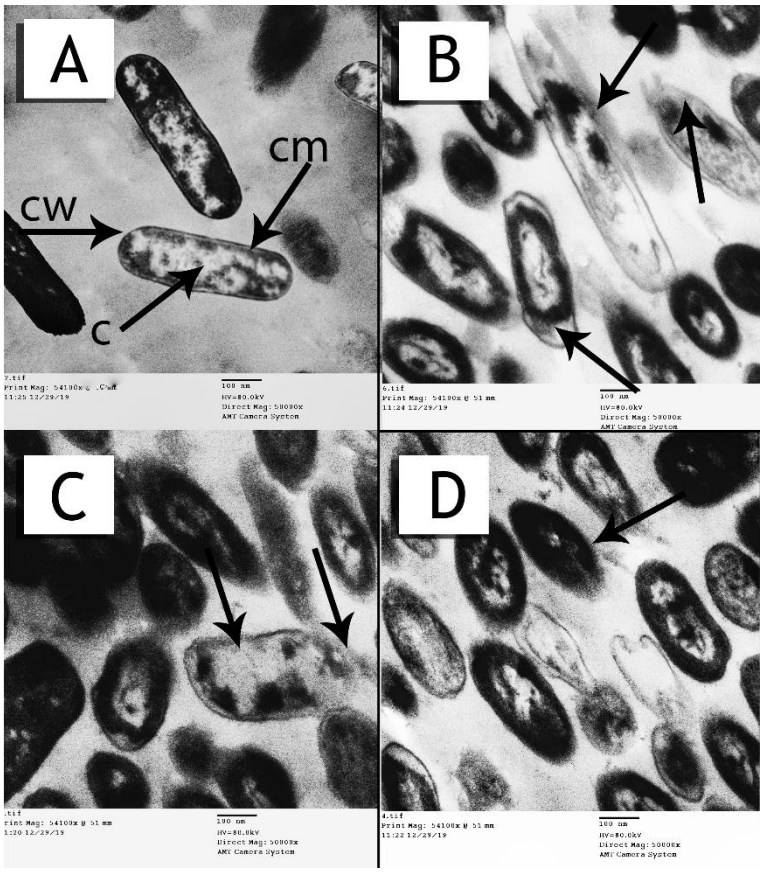

Figure (15): TEM microphotographs of *Escherichia coli*. **A**: without treatment. **B, C&D**: treated with purified antimicrobial compound with concentration 62.5 µg/ml. Where, CW: cell wall, CM: cell membrane, C: cytoplasm.

Candida albicans MTCC183in TEM micrograph of untreated cells and showed The cytoplasm of control cells appeared homogeneous containing a nucleus, vesicle and mitochondria, surrounded by a defined cell membrane and regular cell wall with a clear periplasm region(Fig. 16). When subjected of *Candida albicans* MTCC183 cells to purified antimicrobial compound, the inner organelles were completely discomposed and even cell membrane and wall were deeply affected and look like undulant. Yeast cells were found collapsed which followed by an outflow of the cytoplasmic component (Fig. 16).

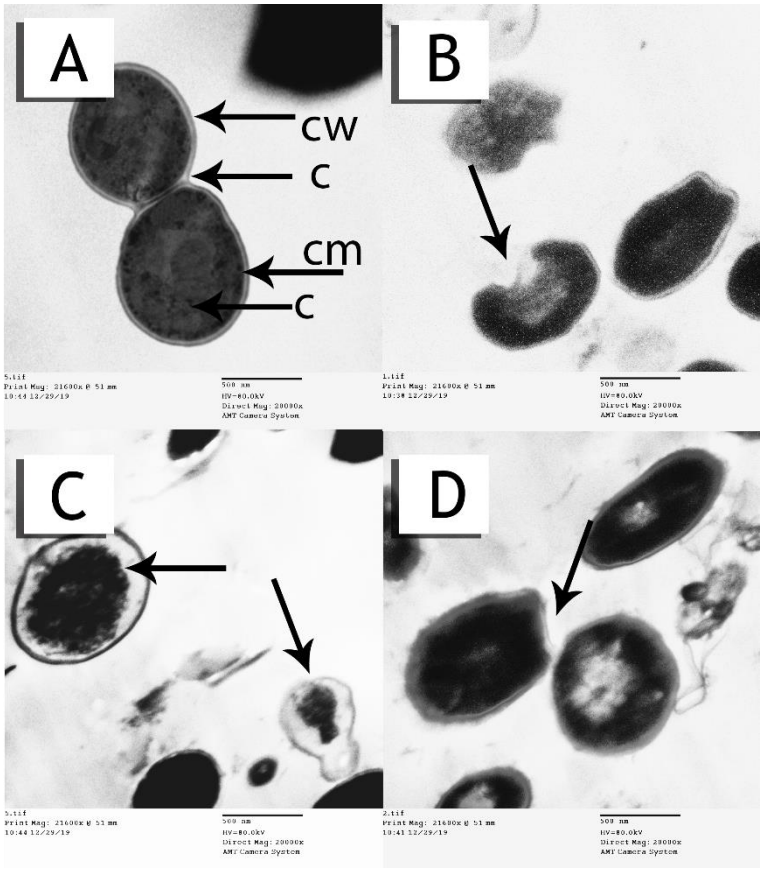

Figure (16): TEM microphotographs of *Candida albicans*. **A**: without treatment. **B, C&D**: treated with purified antimicrobial compound with concentration 62.5 µg/ml. Where, CW: cell wall, CM: cell membrane, C: cytoplasm.

Cytotoxic activity of the purified compounds:

The cytotoxic effect of the purified compounds was evaluated against normal lung fibroblast cell line (MRC-5) cells using MTT assay. The estimated CC_{50} was at 1000 µg/ml which was 15 more than the determined MIC. Interestingly, no cytotoxic effects were observed for the compound when tested even at high concentrations (Fig. 17).

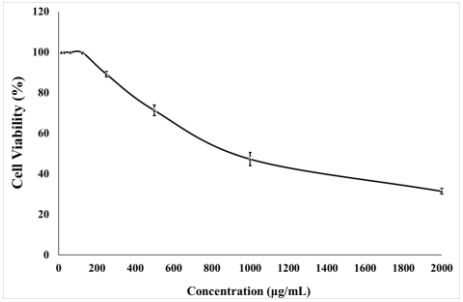

Figure (17): Dose response curve showing the cytotoxic activity of the purified compound obtained from *S. griseoplanus* S1SHA1 on the normal lung fibroblast cell line (MRC-5) cells using MTT assay.

Conclusion

The need for new, safe and more effective antimicrobial agent is a major challenge to the pharmaceutical industry today, especially with the increase in opportunistic infections in the immune compromised host. The result obtained in the present investigation indicated that *S. griseoplanus* S1SHA1 produced a non-protein, non toxic antimicrobial agent. Hence this antibiotic is a good drug against various microorganisms. We also further propose that the actinomycetes even today are a source for discovery of new antimicrobial antibiotics.

Acknowledgment

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نهج جديد للتكوين الحيوي لعامل مضاد للميكروبات يتحكم في الكائنات الحية الدقيقة المقاومة للأدوية المتعددة

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الملخص العربى :

الخلاصة: في هذا البحث ، أجريت تجربة للعثور على عامل جديد مضاد للميكروبات ينتج من الكائنات الحية الدقيقة في التربة من البيئة المحلية للسيطرة على مشكلة مقاومة الأدوية المتعددة. يستخدم مصطلح مقاومة مضادات الميكروبات (AMR) لوصف الكائنات الحية الدقيقة التي يمكن أن تقاوم آثار الأدوية والمواد الكيميائية المصممة لقتلها.. تم عزل ست وسبعين عزلة من خمسة عشر عينة تربة من مواقع مختلفة في مصر .تم اجراء الفحص الأولي للنشاط المضاد للميكروبات بواسطة طريقة انتشار سدادة آجار ضد ضد الكائنات الحية الدقيقة الممرضة. تم اختيار ستة عشر عزلة للفحص الثانوي في نظام التخمير المغمور على نطاق صغير وتم فحصها ضد الكائنات الحية الدقيقة المختبرة المسببة للأمراض باستخدام طريقة نشر الآبار. من بين هذه العزلات التي تم اختبارها ، أظهرت العزلة (S1SHA1) أعلى نشاط مضاد للميكروبات ضد كائنات الاختبار المسببة للأمراض. تم تحديد هذه العزلة على أنها متسلسلة الجريسوبلانوس من خلال الصفات المورفولوجية والفسيولوجية والكيميائية الحيوية وتسلسل جين rRNA 16s. تمت دراسة العوامل الفيزيائية والتغذوية التي تؤثر على نشاط العامل المضاد للميكروبات. أوضحت النتائج أن النشاط الأمثل للعامل المضاد للميكروبات تم تحقيقه مع الأس الهيدروجيني 7 ، ودرجة حرارة الحضانة 28 درجة مئوية ، لمدة 7 أيام ، عند 150 دورة في الدقيقة ، ونشا مصدر الكربون والنيتروجين 1.5٪ ونترات البوتاسيوم 0.4٪ ، وكذلك الفوسفور 2 جم / لتر و NaCl بتركيز 1٪. تم إخضاع العامل المضاد للميكروبات من مزرعة البيئة السائلة للاستخراج والتنقية باستخدام أسيتات إيثيل حيث تمت التنقية باستخدام كروماتوجرافيا الطبقة الرقيقة. تم دراسة تحديد الحد الأدنى من التركيزات المثبطة (MIC) وطريقة عمل العامل المضاد للميكروبات التي تنتجها متسلسلة الجريسوبلانوس على السلالات الميكروبية باستخدام المجهر الإلكتروني النافذ (TEM). أظهرت دراسات السامة للخلايا أنه لم يلاحظ أي تأثيرات سامة للخلايا على المركب عند اختباره حتى بتركيزات عالية

الكلمات المفتاحية:-

الكائنات الحية الدقيقة المسببة للأمراض ،عامل مضاد للميكروبات ،المقاومة للأدوية المتعددة , سمية الخلايا.