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Microbiological and molecular characterization of thermophilic *Streptomycesvariabilis* with antimicrobial activity

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Abstract

The rise of antibiotic resistance phenomena increased the interest for finding effective antimicrobial alternatives to control microbial pathogens. Actinomycetes represent a great reservoir for isolation and identification of antimicrobial compounds. In this study, various samples were randomly collected from the soil of different places in south Delta in Egypt. Two isolates of thermophilic *Streptomyces spp* were isolated on starch nitrate agar under thermophilic conditions, and were identified according to their morphological characteristics. Only, one isolate showed antifungal and antibacterial activity against various fungal and bacterial pathogens including *Staphylococcus aureus*, *Escherichia coli* and *Streptococcus*. Maximum antimicrobial activity was detected against *Aspergillus niger*. This isolate was identified as *Streptomyces variabilis*EG1on the base of the morphological characteristics and 16s rRNA gene sequencing. Optimization of some culture conditions was studied for attaining maximum antimicrobial agent production. This was achieved via adjusting carbon, nitrogen sources including starch and potassium nitrate, pH, time and temperature. Antimicrobial agent production by *S.variabilis* EG1 was observed on the 5th day of growth on the starch nitrate culture media (incubation at 50° C and pH 7.0) and was efficiently extracted by many solvents. This work confirmed the ability of thermophilic actinomycetes, *S. variabilis*EG1 to produce unpurified antimicrobial agents for pharmaceutical applications.

Key Words: Thermophilic actinomycetes, *Streptomyces*, antimicrobial, microbial pathogens
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1. Introduction

The discovery and development of antibiotics was one of the most significant advances in medicine in the 20th century. Nevertheless, many antimicrobial agents that were used to treat human infectious diseases are now ineffective [1]. Therefore, effective drugs need to be available in the future, to improve the antimicrobial use patterns and to devise strategies to identify new antibiotics through previously unexplored targets [2]. Microbial environment is an important source of novel active agents [3]. Many of these products currently used are produced by microbial fermentation, or are derived from chemical modification of a microbial product [4]. The fermentation process is an important tool for production of secondary metabolites that cannot be isolated from plants and animals, or synthesized by chemists because of the ease of increasing production by environmental and genetic manipulation [5].

Streptomyces have been, and remain, one of the most fruitful sources for all types of bioactive metabolites. These secondary metabolites have important applications inhuman medicine (antiviral and anticancer compounds), in agriculture such as herbicides, insecticides and antiparasitic compounds [6]. Invasive and disseminated microbial infections are serious clinical problems, difficult to diagnose, often lethal to patients, and increasing in occurrence [7]. Despite the development of newer antimicrobial drugs, the polyene antimicrobials continue to be the most potent broad-spectrum fungicides available for clinical use. As a result of the continuing search for new polyenes and non-polyenes, as well as the limitations of current production methods. In our present study, our aim was the isolation, characterization of thermophilic *Streptomyces* showing antimicrobial activity and improving the production and purification processes.

2. Material and method

Isolation of actinomycetes

Around 100 soil samples were collected from two of diverse habitats for the isolation of *Streptomyces*. These habitats consisted of plants rhizosphere and agricultural soil. After removing approximately 3 cm of the soil surface, the

samples were taken up to a depth of 20 cm. Isolation and screening of *Streptomyces* was carried out.

The collected soil samples were sieved to remove unwanted materials. The two soil samples were treated with CaCO3 (10:1 w/w) and incubated at 37°C for 5 days. They were then perched in sterile distilled water. Test tubes containing a 10^{-2} dilution of the samples were kept in a water bath at 45°C for 16 h to detach the spores from vegetative cells. These dilutions were spread on the surface of the starch nitrate medium. The composition of this medium was (g/l): starch 10.0, KNO_3(2.0), NaCl (2.0), K_2HPO_4(2.0), $MgSO_4.7H_2O$ (0.05), $CaCO_3$(0.02), $FeSO_4.7H_2O$ (0.01) and agar (20.00), the plates were incubated at 45°C until the appearance of *Streptomyces* colonies. Two colonies of *Streptomyces* were then purified, transferred to starch nitrate agar and preserved at 45°C. The two isolates were maintained as spore suspensions and mycelia fragments in 20% v/v glycerol. Primary screening was carried out.

Primary screening for antimicrobial activity

Primary screening was carried out. Antimicrobial activity was determined on starch nitrate agar media inoculated with test organisms. The tested organisms included different bacterial strains including *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pneumonia* and the fungus; *Aspergillus niger*. The *Streptomyces* isolates were cultured by dense streaking on starch nitrate medium plates and incubated at 45°C for seven days. The 6 mm agar discs were prepared using sterile cork borer from well grown cultures and placed on fresh lawn culture of test organisms and then incubated at their respective optimum temperature (37°C for bacteria and 30°C for the fungi). The zones of inhibition for bacteria were determined after 24 h for bacteria and for fungi after 3 days. One of the two isolates showing broad spectrum activity against tested organisms in primary screening was subjected to secondary screening by agar well diffusion method. The first identified isolate (*Streptomyces variabilis*) have shown great antimicrobial efficacy against all pathogenic bacteria and fungi and so was used for further work.

Isolation of antibacterial metabolites

The antimicrobial substance was produced under optimal production parameters. The cultural filtrate containing the antimicrobial activity was separated from biomass by solvent extraction method, following the process described by mixing the organic solvent with filtrate. Ethyl acetate was added to the filtrate in the ratio of 1:1(v/v) and shaken vigorously for 1 hour for complete extraction. The ethyl acetate phase that contains antibiotic was separated from the aqueous phase. It was evaporated to dryness in water bath at 80°- 90°C and the residue obtained was weighed. Thus, obtained compound was used to determine antimicrobial activity. The same steps repeated by different solvents; n-butanol, n-hexane, petroleum ether, chloroform, benzene and xylene then the obtained compound was used to determine antimicrobial activity with each solvent.

Determination of the antimicrobial activity of crude extract

The antimicrobial activity was determined by agar well method. The partially purified extract obtained by the ethyl acetate extract and with other solvents was dissolved in 1 ml 0.2 M phosphate buffer (pH 7.0). Then the partially purified extract was loaded into wells prepared in culture plates treated with tested organism *E. coli*, *S. aureus*, *S. pneumonia* and fungi (*Aspergillus niger*). The plates were incubated at 37°C for 24 h for bacteria and 7 days for fungi. The diameter of the zones of complete inhibition was measured to the nearest whole millimeter.

Thermal stability of the antimicrobial substance

Thermal stability determination is very important to facilitate the extraction, purification, and storing of antimicrobial substances. Thermal stability of the crude antimicrobial substance was investigated by storing it at various temperatures ranged from 0°C to 100 °C for 1 h; after that, the antimicrobial activity was assayed to determine the percentage of residual activity.

Morphological and cultural characteristics

Morphological and cultural characteristics of *S. variabilis*EG1 were studied in different media (obtained from Oxoid, UK) following the instructions given by the International *Streptomyces* Project (ISP) **[1].** The microscopic characterization was performed by the cover slip culture method. The spore chain morphology was also analyzed by Transmission electron microscope (TEM at The Regional Centre for Mycology and Biotechnology at Al Azhar University, Egypt). (Different media used were: Starch nitrate agar medium, tryptone yeast extract agar (ISP-1) (The composition is tryptone 5 g; yeast extract 3g; agar 20g; H_2O 1000 ml, pH 7.2), ISP-2 agar (The composition is yeast extract 4 g; malt extract 10g; glucose 4g; agar 20g; H_2O 1000 ml, pH 7.2), ISP-3 agar (The composition is oatmeal 20g; $MnCl_2.4H_2O$ 0.1g; $FeSO_4.7H_2O$ 0.1g; $ZnSO_4.7H_2O$ 0.1g; agar 18 g, H_20 1000 ml, pH 7.2), ISP-4 agar (The composition is L-asparagine 1g; glycerol 10g; K_2HPO_4 1g; $MnCl_2$. $4H_2O$ 0.1g; $FeSO_4.7H_2O$ 0.1g; $ZnSO_4.7H_2O$ 0.1g; agar 20g; H_2O 1000 ml, pH 7.2), ISP-5 (The composition is starch soluble 10g; K_2HPO_4 1g; $MgSO_4.7H_2O$ 1g; NaCl 1g; $(NH_4)_2SO_4$ 2g; $CaCO_3$ 2g; agar 20g; H_20 1000 ml, pH 7.2), ISP-6 (peptone yeast extract iron agar) and ISP-7 (tyrosine agar medium) at 28°C for 7 to 14 days (Shirling and Gottlieb, 1966b).

Physiological and biochemical studies

Catalase activity was checked by the method of Jones, [8]. Melanin pigment production was studied according to the method described by [9]. Nitrate reduction, hydrogen sulphide production and oxidase test was also performed. The cultural behaviors were studied according to the guidelines available in International *Streptomyces* Project [1]. Colors characteristics of colonies, mycelia and pigments among others were studied by the method of Kenneth and Deane, [10]. The use of different carbon and nitrogen

sources by these isolates was investigated with the help of method described by Shirling and Gottlieb, [1].

Molecular Characterization of *S. variabilis* EG1

DNA isolation and manipulation

The locally isolated *Streptomyces* strain was grown for 5 days on a starch agar slant at 35 ° C. Two milliliters of a spore suspension were inoculated into the starch–nitrate broth and incubated for 3 days on a shaker incubator at 200 rpm and 30° C to form a pellet of vegetative cells (pre-sporulation). The extraction of total genomic DNA was conducted in accordance with the methods described by Sambrook [11].

Amplification and sequencing of the 16S rDNA gene

PCR amplification of the 16S rDNA gene of the local *Streptomyces* strain was conducted using two primers, StrepF;5'-ACGTGTGCAGCCCAAGACA-3' and Strep R; 5'-ACAAGCCCTGGAAACGGGGT-3', in accordance with the method described by Edwards et al., (1989). The PCR mixture consisted of 30 pmol of each primer, 100 ng of chromosomal DNA, 200 µM dNTPs, and 2.5 units of Taq polymerase, in50 µl of polymerase buffer. Amplification was conducted for30 cycles of 1 min at 94 °C, 1 min of annealing at 53° C, and2 min of extension at 72° C. The PCR reaction mixture was then analyzed via agarose gel electrophoresis, and the remaining mixture was purified using QIA quick PCR purification reagents (Qiagen, USA). The 16S rDNA gene was sequenced on both strands via the dideoxy chain termination method, as described by Sanger et al. (1977). The 16S rDNA gene (1.5 kb) sequence of the PCR product was obtained using a Terminator Cycle Sequencing (ABI Prism 310 Genetic Analyzer, Applied Biosystems, USA). The sequence analysis was performed using BLAST search in the NCBI database (http://www.ncbi.nlm.nih.gov/blast). Published 16S rRNA gene sequences along with *S. variabilis*EG1 used in this study were then aligned and a Neighbor-joining tree was generated using ClustalW software and performed by using MEGA 6.0 software.

Antibiotic purification

Recovery of the Bioactive Metabolite from Methanol Extract of the brown colored crude extract obtained was subjected for isolation and purification of components by analytical methods namely Thin Layer Chromatography and Column chromatography. The methanol extract was spotted on TLC plate and developed with solvent systems that consisted of different ratios of ethanol:water, methanol:water and ethanol:methanol:water. The chromatograms were allowed to air dry and were exposed to iodine vapours for the detection of resolved components. The recovery of bioactive principle from the methanol extract was carried out by Column chromatography. Column chromatography was performed by employing silica gel 80-120 mesh size of chromatography grade in a sintered filter column (40x4cm). The column was treated with chromic acid followed by water and then rinsed with acetone. The column was packed well with activated silica using chloroform solvent. The methanol extract of isolate was loaded at the top of the column. The column was eluted using solvent systems. Fractions were collected at regular intervals, loaded on TLC plates, developed with solvent system and observed for spots in iodine chamber. The fractions exhibiting same Rf values were pooled and the solvent was evaporated at 40°C to get concentrated fraction. Two pure fractions (A and B) were obtained previous studies, in the present study, we describe characterization and antibacterial activity of a glycoside antibiotic obtained from methanol extract *S. variabilis*.

Spectral Analysis of Bioactive Compound

The purity and molecular weight of the bioactive fraction B was assessed by Liquid Chromatography and Mass Spectrum. The fraction B was dissolved in methanol and 5µl of the compound was injected into a C-18 HPLC column with methanol: water (90:10) at a flow rate of 0.2ml/min. The UV-VIS detection was carried out. Mass spectral studies were carried out by atmospheric pressure chemical ionization probe for the analysis of nonpolar compounds and Electron Spray Ionization probe for the analysis of polar compounds. The Infra red (IR) spectra was recorded on Shimadzu IR - model. The fraction B was scanned from 450-4000cm/range. The fraction was dissolved in DMSO and was subjected to 1H-NMR (Nuclear Magnetic Resonance) and 13C-NMR in Bruker BioSpin model. The nature and structure of the bioactive fraction was elucidated by analyzing the chromatograms and spectra with pertinent literature.

3. Results

Isolation and screening of *Streptomyces*:

Thermophilic *Streptomyces* were reported to occur predominantly in soil. In the present study, two thermophilic isolates were recovered from soil samples collected from different locations in Benha and Gharbia. Only one survived sub-culturing at high temperatures, showed antimicrobial activity and was used in this work. Figure 1 shows the *Streptomyces* isolate grown on starch nitrate agar.

Fig (1): *Streptomyces* sp isolated from soil and grown on starch nitrateagar.

Primary screening for antimicrobial activity

Primary antimicrobial screening was carried out. Antimicrobial activity was determined on starch nitrate agar media inoculated with tested organisms. The test organisms included *E. coli*, *S. aureus*, *Streptococcus* and *Aspergillus niger*. Inhibition zones were 18 mm, 15 mm, 11 mm and 21mm against *S. aureus*, *Streptococcus*,*E. coli* and *Aspergillus niger*, respectively (Table 1).

Table (1): Inhibition zones diameters (mm) shown by *Streptomyces* isolate on the growth of four pathogenic microorganisms

Microorganisms	Diameter of clear zone (mm)
S. aureus	18
Streptococcus	15
E. coli	11
Aspergillus niger	21

Extraction of antibiotics produced by *Streptomyces* isolates using different solvents

Isolates showing activity against any of the tested microorganisms were grown in 500 ml flasks containing 100 ml of liquid starch nitrate agar. Fermentation broths were extracted with n- hexane and n-butanol. The extracts were bio-assayed for their antimicrobial activities. The exhibited inhibition zones, against the tested fungus and bacteria are shown Table (2). The n-hexane extracts of *Streptomyces* isolate have shown activity against one or more of the tested organisms. However, the activities were strong against *Aspergillus niger*, and bacteria*; Staphylococcus aureus, Streptococcus,* and *E. coli.* The greatest zone of inhibition (45mm) was exhibited by *S. variabilis* isolate against *Aspergillus nige r*followed by *S. aureus* (30mm), *Streptococcus* (20mm) and *E. coli* (15mm) (Table 2 and Fig. 2). N-butanol extracts have higher activities against *Aspergillus* (40 mm) than the tested bacteria (Table 3).

Table (2): Inhibition zone Diameters(mm) shown by N-hexane extracts against tested microorganisms.

Pathogenic micro organisms	Clear zone diameter (mm)
S. aureus	30
Streptococcus	20
E. coli	15
A. niger	45

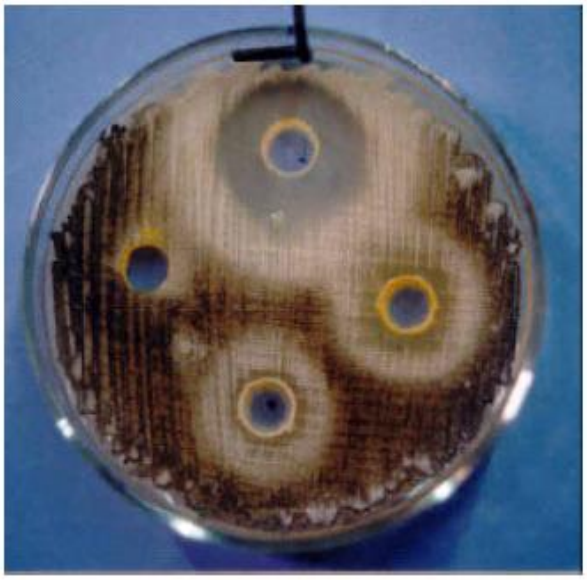


Fig (2): Antifungal activity by crude extract with n-hexane as a solvent.

Table (3)**:** Inhibition zone diameters(mm) shown by n-butanol extracts against tested microorganisms.

Pathogenic micro organisms	Clear zone diameter (mm)
S.aureus	25
Streptococcus	16
E. coli	10
A. niger	40

Thermal stability of the antimicrobial substance

Thermal stability of the crude antimicrobial substance was investigated by storing it at various temperatures for 1 h. The antifungal activity was noticeable at all temperatures but decreased by increasing temperature (Table 4).

Table (4): Thermal stability of the crude antimicrobial substance against *Aspergillus niger*

Temperature ° C	Clear zone (mm)
40	18
60	14
80	9
100	8

Cultural characteristics

The cultural characteristics of thermophilic *S. variabilis* are shown in Figure 3 and Table (5). In addition, the growth characteristics are summarized in table 6.

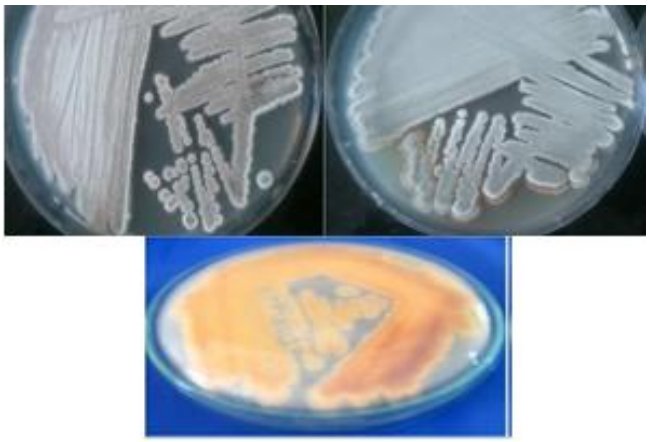


Fig (3): Growth characteristics of the thermophilic *Streptomyces variabilis*EG1.

Table (5): The morphological and cultural characteristics of the *Streptomyces variabilis* EG1

Morphological (Starch nitrate agar medium)	**Results**
Vegetative Growth	+++
Aerial Mycelium	Grey
Substrate Mycelium	Brown
Soluble pigment	Brown
Motility	Non-motile
Cultural	
Growth on Yeast extract-malt extract agar medium	+++
Growth on Oatmeal agar medium	++
Growth on Inorganic salts starch agar medium	+++
Growth on Peptone yeast extract iron agar	+++
Hydrolysis activities	
Starch	+++
Xylene	+++
Catalase test	+++
Tryptone yeast extract agar medium (ISP-1)	++
Yeast extract-malt extract agar medium (ISP-2)	+++
Oatmeal agar medium (ISP-3)	++
Glycerol asparagine agar medium (ISP-4)	+++
Inorganic salts starch agar medium (ISP-5)	++

Table (6): The growth characteristics of the *Streptomyces variabilis*EG1

Parameter	Features
Shape	Filamentous
Chromogensis	Cream
Edge	Filamentous
Opacity	Opaque
Elevation	Flat
Surface	Powdery
Consistency	Dry

Biochemical characteristics

Biochemical characterization of the isolated thermophilic *Streptomyces* was performed as recommended by International *Streptomyces* Project (ISP) (Table 7).

Table (7): Carbohydrates utilization by the thermophilic *Streptomyces variabilis*EG1

Sugar	Result
Arabinose	+
Rhamnose	+
Glucose	+
Galactose	+
Fructose	+
Sucrose	+
Maltose	+
Lactose	+
Mannitol	+

Microscopic Characteristics

Results of microscopic examination are shown in Table (8). The vegetative hyphae were branched and were non-fragmented. The non-fragmentation of the mycelium is generally accepted as a diagnostic characteristic of the *Streptomyces*.

Table (8): Microscopic characteristics of the *Streptomyces variabilis*EG1

Parameter	Results
Gram Stain	+ve
Acid-fast Stain	-ve
Motility Aerial	-ve
mycelium	Present
Conidia	Present

The transmission electron microscope showed long straight chains of spores. The spores appeared as cylindrical immotile with smooth, warty or hairy appearance as shown in Figure 4.

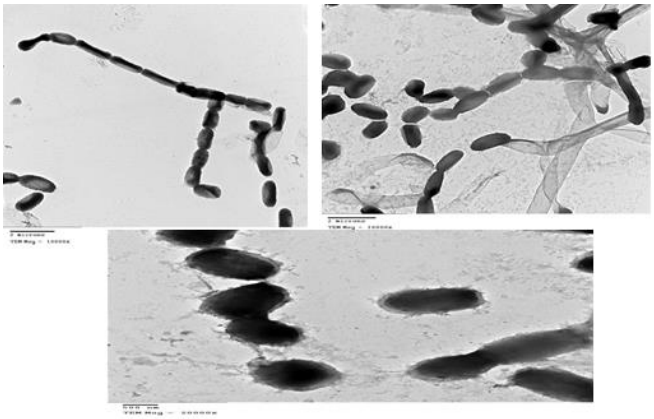


Fig (4): The electron micrographs of *Streptomyces* spores showing their morphological characteristics

Molecular identification of *Streptomyces* using 16 S RNA gene sequencing

The molecular identification of thermophilic *Streptomyces* isolate using 16s rRNA gene was performed. The small subunit of rRNA (16S) was amplified and sequenced to yield a partial nucleotide sequences for the *Streptomyces* isolate. Comparison of this isolate with published reference sequences on GenBank Database using BLASTn tool indicated that the thermophilic isolate belongs to the genus, *Streptomyces* with greatest homology to *S. variabilis* published sequences. Therefore, this studied isolate were designated as *S. variabilis* EG1. A multiple sequence alignment was constructed between the studied isolate and reference strains. Phylogenetic analysis was performed by construction of phylogenetic tree using a neighbor joining method to unravel the relationships among all *Streptomyces* isolates (Fig. 5). The phylogenetic tree resulted in three clades in which *S. variabilis* EG1was in one separate cluster along with *S. variabilis* (accession number; KR909307.1). Thus, the molecular identification based on sequence homology of the 16s rRNA gene confirmed the identity and phylogeny of the studied *Streptomyces* isolate.

Fig (5): Phylogeny of the two studied *S. variabilis*EG1 as compared to reference published strains on GenBank. Minimum bootstrap frequency used during analysis was 1000.

Spectral Analysis of Purified Bioactive Compound

The Figure (6) shown that the compound is pure as the LC chromatogram showed a single prominent peak. The peak observed at Mass Speak 513 confirms the molecular weight of the compound i.e. 514.

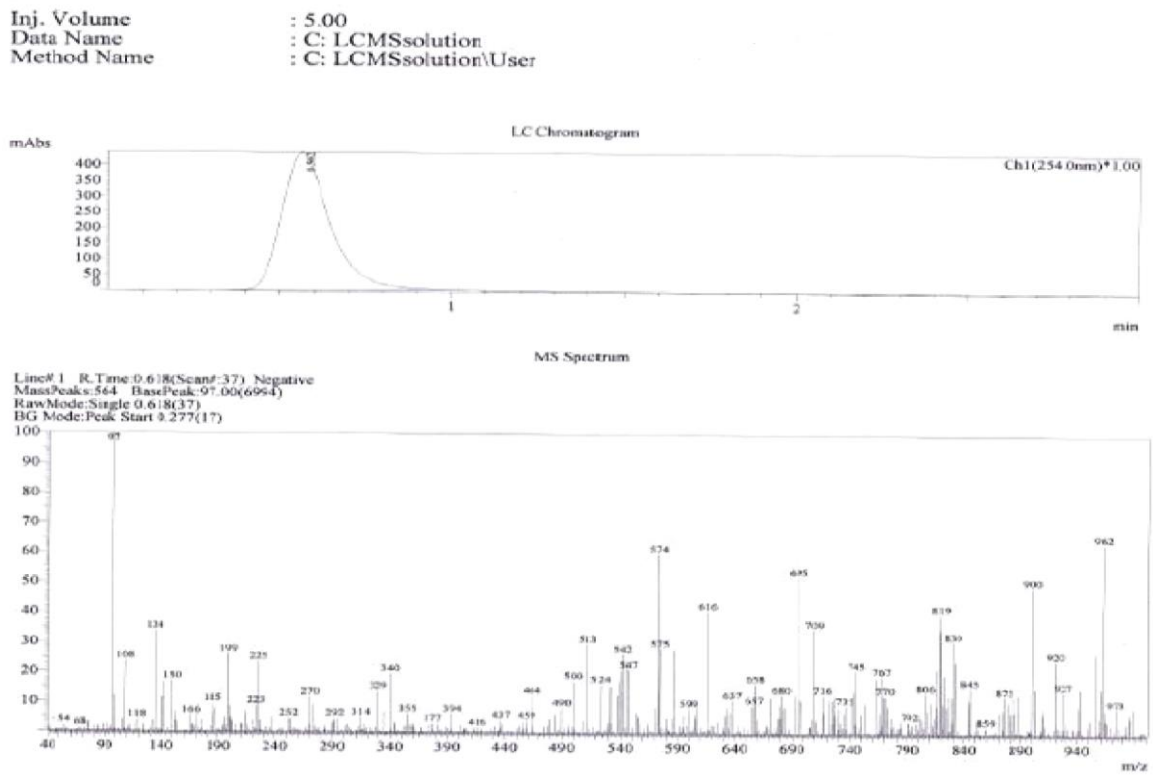
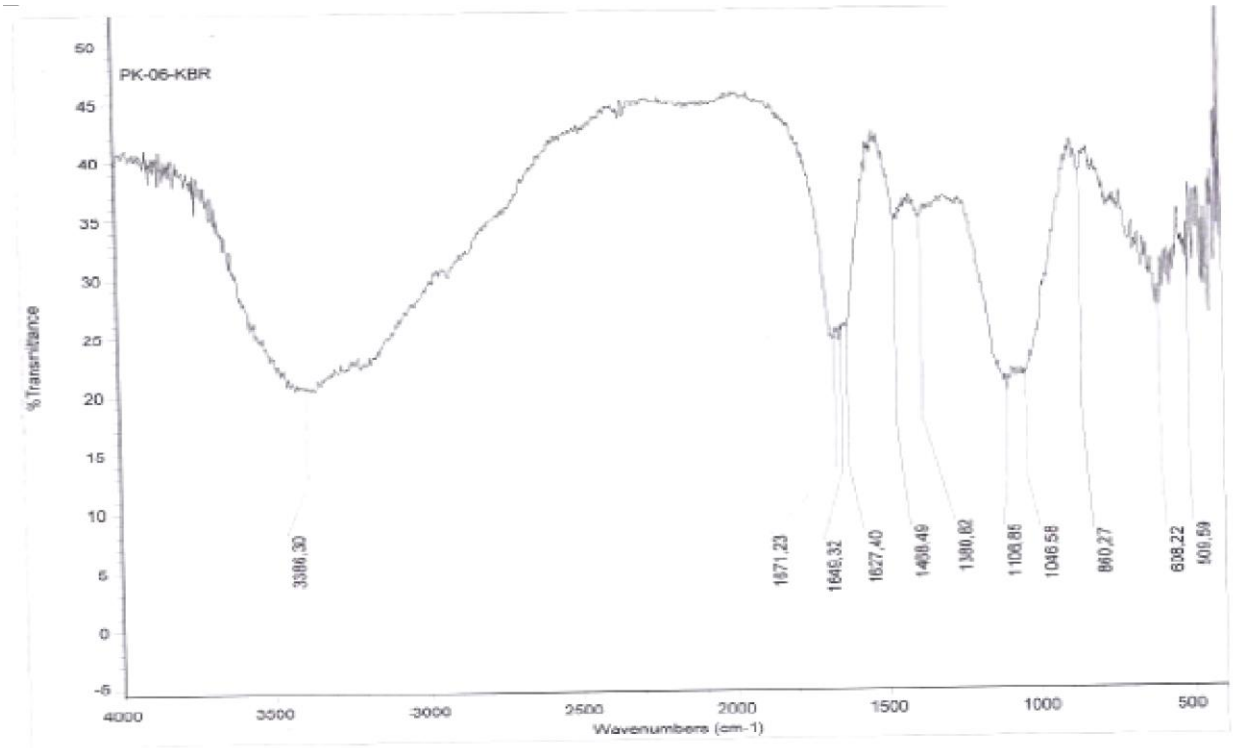
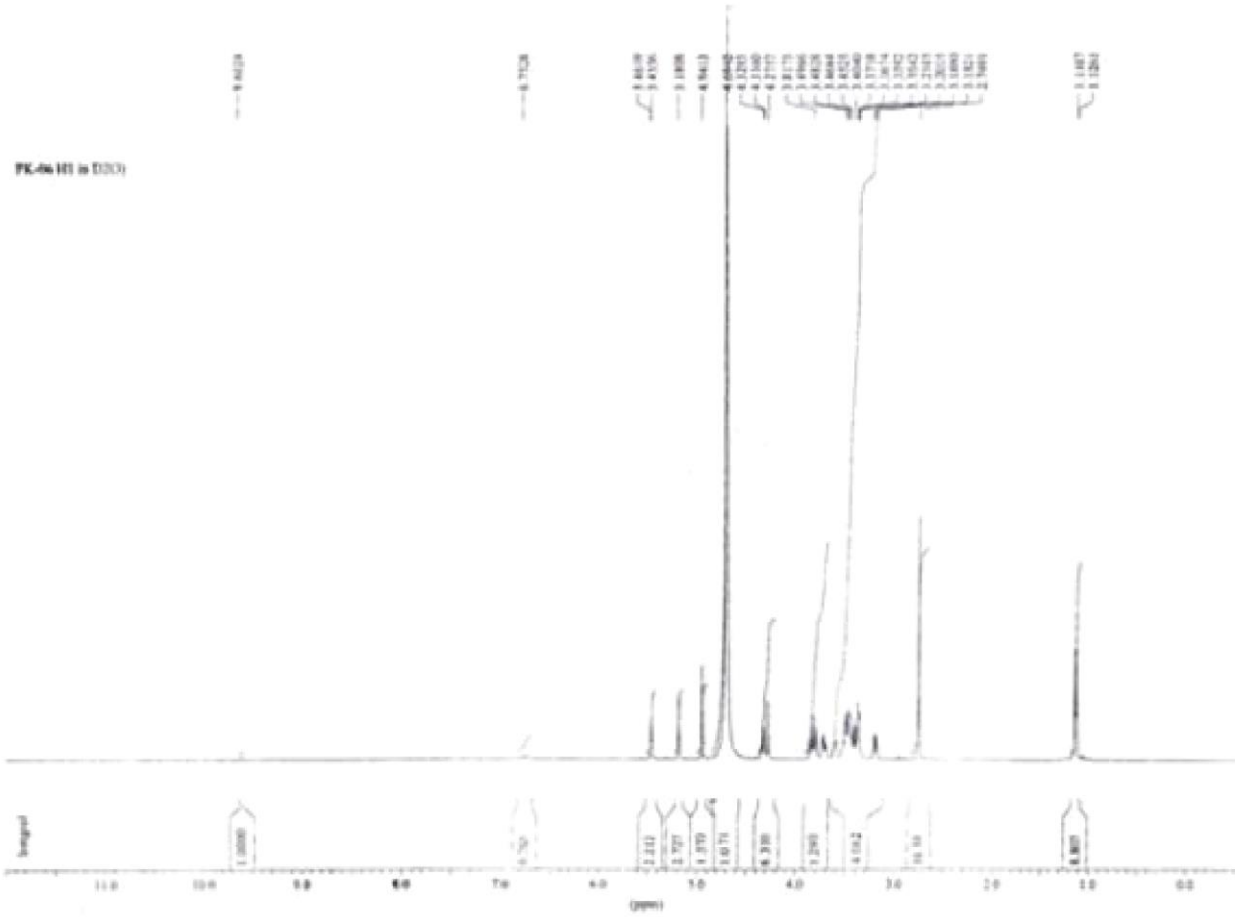


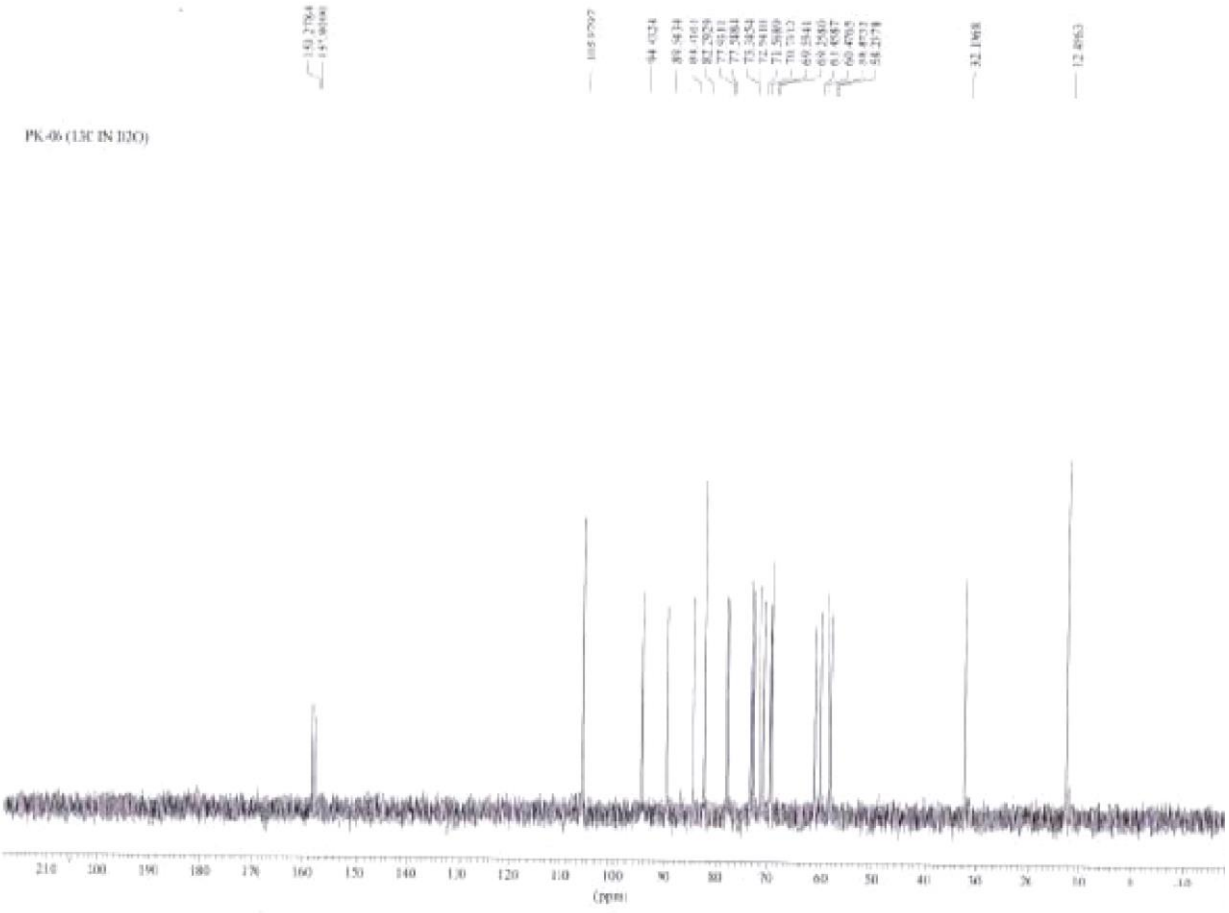
Fig (6): shows the IR spectra of the purified compound. Strong absorbance frequency observed at 3387cm-1 and 1672cm-1 indicated the presence of hydroxyl (-OH) and carbonyl (-C=O) groups respectively.



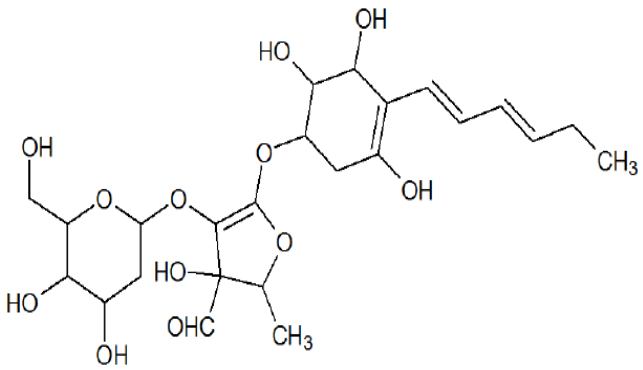
1H-NMR spectra of purified compound are shown in Figure (7). The singlet appeared at the region of 9.6δ value is confirmed the presence of aldehyde proton. The singlet appeared at the region of 5.1δ value is for –OH group present in α position to aldehyde group. The two doublets for –CH=CH appeared at the region of 5.4 to 5.6δ values. The 6 singlets for 6 –OH groups appeared at the region of 4.2 to 4.9δ values. The singlet appeared at the region of 1.1 δ value is for – CH3 proton and the multiplets present at the region of 2.7 to 3.8 is for –CH and –CH2 protons and the multiplets present at the region of 2.7 to 3.8 is for –CH and –CH2 protons.



13C-NMR spectra spectral analysis of purified in Figure (8). The peak appeared at the region of 158δ value is confirmed the presence of aldehyde carbon. The peak appeared at 105δ value confirmed the presence of furon ring carbon. The peak appeared at 82-94δ value confirmed the presence of –C-OH carbons. The peak appeared at 32δ value confirms the presence of aliphatic (-CH) carbons.



On the basis of obtained spectral data as shown in figure (9), the purified compound was named 5-(4-(1*E*,3*E*)-hexa-1,3-dienyl)-3,5,6-trihydroxycyclohex-3-enyloxy)-2,3-dihydro-3-hydroxy-2-methyl-4(tetrahydro-4,5-dihydroxy-6-hydroxymethyl)-2*H*-pyron-yloxy)furan-3-carbaldehyde. The molecular formula of the compound is C24H34O12 as shown in figure (10)



4. Discussion

Actinomycetes have been recognized as the potential producers of metabolites such as antibiotics, growth promoting substances for plants and animals, immunomodifiers, enzyme inhibitors and many other compounds of use to man. They have provided about two-thirds of the naturally occurring antibiotics discovered, including many of those important in medicine, such as aminoglycosides, anthracyclines, chloramphenicol, *b*-lactams, macrolides, tetracyclines etc. [12]. Microbial pathogens are responsible for large number of infections and deaths worldwide. In this

work, the isolated antimicrobial compounds exhibited great antifungal activity as compared to the same effect against pathogenic bacteria. Fungi are eukaryotic and have machinery for protein and nucleic acid synthesis similar to that of higher animals. It is, therefore, very difficult to find out compounds that selectively inhibit fungal metabolism without toxicity to humans [13]. Recently presented a mathematical model which estimated the total number of antimicrobial compounds that *Streptomyces* is capable of producing to be in the order of a 100,000-a tiny fraction of this has been unearthed so far. This means that if the screening efforts are maintained, novel antibiotics are expected to be discovered regularly. It should be emphasized that, the search for a metabolite of pharmaceutical interest requires a large number of isolates [14]. The search will be more promising if diverse actinomycetes are sampled and screened (Porter and Wilhelm, 1960). In this study, soils were specifically collected from different agricultural locations in Egypt based on the assumption that these bacteria grow profusely in the humus and leaf litter layers [15].

Based on performed morphological and physiological characterization of the isolate, it was classified in the order, actinomycetes; family *Streptomycetaceae* and genus *Streptomyces*. The isolates showed varying degrees of retarding effect against one or more of the tested microorganisms. The absence or the presence of mild activity may be due to the low concentration of the crude preparation used. A shortcoming of our study was the use of a fixed concentration in the antimicrobial activity experiments. If higher concentrations of the crude preparations could be used, stronger inhibition might have been noted. Nevertheless, the antifungal activities shown by the isolates highlighted them as candidates for further *in vitro* and *in vivo* investigations. In this respect our results came in agreement with those of Chamness [16]. Also, our results demonstrated that the isolation of thermophilic *Streptomyces* species may present significant capacity for antifungal production. Therefore, we recommend a systematic approach to evaluate and optimize production under different fermentation conditions using locally available substrates. Most of the antibiotics, in use today, are produced by microorganisms specifically by *Streptomyces* species that are isolated from soils. Egyptian soils have different physical and chemical properties according to the specific location and environment, which play an important role in the diversity of the soil populations. However, these soils have not been fully explored. This study shows the great potentiality of a thermophilic *Streptomyces* species that can produce novel antimicrobial agents.

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