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Antimycin-A Antibiotic Biosynthesis Produced by *Streptomyces* Sp. AZ-AR-262: Taxonomy, Fermentation, Purification and Biological Activities

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Abstract: An actinomycete culture was isolated from a soil sample collected from Alam Alroom districted, Marsa Matrouh governorate, Egypt. This isolate AZ-AR-262 was found to be active against unicellular and filamentous fungi. The nucleotide sequence of the 16s RNA gene (1.5 Kb) of the most potent strain evidenced an 83% similarity with *Streptomyces tsukiyonensis*, *Streptomyces xanthocidicus* and *Streptomyces misakiensis*. From the taxonomic features, the actinomycetes isolate AZ-AR-262 matches with *Streptomyces olivaceiscleroticus* in the morphological, physiological and biochemical characters. Thus, it was given the suggested name *Streptomyces olivaceiscleroticus,* AZ-AR-262. The active metabolite was extracted using n-Butanol (1:1, v/v) at pH 7.0. The separation of the active ingredient and its purification was performed using both thin layer chromatography (TLC) and column chromatography (CC) techniques. The physico-chemical characteristics of the purified antibiotic viz. color, melting point, solubility, elemental analysis, spectroscopic characteristics and chemical reactions have been investigated. This analysis indicates a suggested empirical formula of $C_{28}H_{40}N_2O_9$. The minimum inhibition concentrations "MICs" of the purified antifungal agent were also determined. The purified antifungal agent was suggestive of being belonging to Antimycin-A antibiotic produced by *Streptomyces olivaceiscleroticus*, AZ-AR-262.

Key words: Antimycin-A, *Streptomyces olivaceiscleroticus*, 16s RNA, Taxonomy, Production, Purification and Biological activities.

INTRODUCTION

Actinomycetes are prolific producers of antibiotics and other industrially useful secondary metabolites such as antibiotics, herbicides, pesticides and anti-parasitic (Osada, 1998 and Saadoun and Gharaibeh, 2003). Over 6000 of these compounds are produced by *Streptomyces* species and many are commercially important medicinal products used therapeutically as anti-infective (antibiotic, antifungal and antiparasitic), anticancer or immunosuppressant agents (Takahashi and Mura, 2003). The antimycin-A antibiotics are a series of nine-membered dilactones, which were isolated from a number of *Streptomyces* strains over many decades, (2{R},3(S},6{S},7{R},8{R})-3-[(3-formamido-2-hydroxybenzoyl)amino]-8-hexyl-2,6 dimethyl-4,9-dioxo- 1,5-dioxonan-7-yl 3-methylbutanoate) (Hosotani *et al.,* 2005). The antimycins have also other biological properties such as antifungal activity, inhibition of enzymatic activity as well as the ability to induce the death of cancer cells Shiomi *et al.,* 2005). Antimycin A functions inhibit electron transfer activity of ubiquinol-cytochrome c oxidoreductase (cytochrome bc_1 complex) (Barrow *et al.,* 1999) a membrane-bound complex comprised of multiple subunits, generally inhibiting mitochondrial respiration (Fujita, *et al.,* 2004). Antimycin blocks the flow of electrons from semiquinone to ubiquinone in the Q-cycle of complex III in oxidative phosphorylation. By doing so it inhibits the electron transport pathway (Kim *et al.,* 1999) thus preventing the consumption of oxygen (which occurs at Complex IV) and disrupting the proton gradient across the inner membrane (Barrow *et al.,* 1999). It is the disruption of the proton gradient that prevents the production of ATP as protons are unable to flow through the ATP synthase complex (Barrow *et al.,* 2000 and Hosotani *et al.,* 2005). The molecular formula of Antimycin antibiotic is $C_{28}H_{40}N_2O_9$ and molecular weight at 574 (Umezawa, 1977).

In the present work were describe the isolation of an actinomycete strain from Egyptian soil, which generates an antifungal compound. The identification of this strain, based on the cultural, morphology,

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physiology and biochemical characteristics, as well as 16s rRNA methodology, is also reported. The primary bioactive substance was isolated, purified and biological activities were determined.

MATERIALS AND METHODS

Microorganism:

The actinomycete AZ-AR-262 was isolated from soil sample collected from Alam Alroom districted, Marsa Matrouh governorate. It was purified using the soil dilution plate technique described by Williams and Davis (1965).

Screening for Antimicrobial Activity:

The anti- microbial activity was determined by cup method assay according to Kavanagh (1972).

Taxonomic Studies of Actinomycete Isolate:

Morphological characteristics of the most potent produce strain AZ-AR-262 grown on starch nitrate agar medium at 30 ºC for 8 days was examined under light and scanning electron microscopy (JEOL Technics Ltd.,). Physiological and biochemical characteristics: Lecithinase was conducted on egg–yolk medium according to the method of (Nitsh and Kutzner, 1969); Lipase (Elwan *et al.,* 1977); Protease (Chapman, 1952); Pectinase (Hankin *et al*., 1971); α-amylase (Cowan, 1974) and Catalase test (Jones, 1949). Melanin pigment (Pridham, *et al.,* 1957). Degradation of Esculin and xanthine (Gordon *et al*., 1974). Nitrate reduction (Gordon, 1966). Hydrogen sulphide production and oxidase test (Cowan, 1974). The utilization of different carbon and nitrogen sources (Pridham and Gottlieb, 1948). Cell wall was performed by the method of (Becker *et al*., 1964 and Lechevalier and Lechevaier, 1968). The cultural characteristics were studied in accordance with the guidelines established by the International *Streptomyces* Project (Shirling and Gottlieb, 1966). Colors characteristics were assessed on the scale developed by (Kenneth and Deane, 1955).

DNA Isolation and Manipulation:

The locally isolated actinomycete strain was grown for 7 days on a starch agar slant at 30°C. Two ml 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 preparation of total genomic DNA was conducted in accordance with the methods described by (Sambrook *et al*., 1989).

Amplification and Sequencing of the 16s rRNA Gene:

PCR amplification of the 16S rRNA gene of the local actinomycete strain was conducted using two primers, StrepF; 5.-ACGTGTGCAGCCCAAGACA-3. and StrepR; 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, 200µM dNTPs, and 2.5 units of Taq polymerase, in 50µl of polymerase buffer. Amplification was conducted for 30 cycles of 1 min at 94°C, 1 min of annealing at 53°C, and 2 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 rRNA gene was sequenced on both strands via the dideoxy chain termination method, as described by (Sanger *et al*. 1977). The 16S rRNA gene (1.5 kb) sequence of the PCR product was acquired using a Terminator Cycle Sequencing kit (ABI Prism 310 Genetic Analyzer, Applied Biosystems, USA). The sequence data were deposited in the GenBank database, under the accession number DQ386119.

Sequence Similarities and Phylogenetic Analysis:

The BLAST program (www.ncbi.nlm.nih. gov/blst) was employed in order to assess the degree of DNA similarity. Multiple sequence alignment and molecular phylogeny were evaluate using BioEdit software (Hall, 1999). The phylogenetic tree was displayed using the TREE VIEW program (Page, 1996).

Fermentation:

The isolate, AZ-AR-262 was inoculated into 250 ml Erlenmeyer flasks containing 75 ml of liquid starch nitrate medium (seven flasks). The flasks were incubated on a rotary shaker (200 rpm) at 30 ^{0}C for 6 days. A twenty liter total volume was filtered through Whatman No.1 filter paper and followed by centrifugation at 5000 rpm for 20 minutes.

Extraction:

The clear filtrate was adjusted at different pH values (4 to 9) and extraction process was carried out using different solvents separately at the level of 1:1 (v/v). The organic phase was concentrated to dryness under vacuum using a rotary evaporator at a temperature not exceeding 50°C.

Precipitation:

The precipitation process of the crude compound was carried out using petroleum ether (b.p 60-80 °C) followed by centrifugation at 5000 rpm for 15 min.

Purification by TLC: Separation of the antimicrobial compound into its individual components was conducted by thin layer chromatography using chloroform and methanol (24: 1, v/v) as a solvent system.

Purification by Column Chromatography:

The purification of the antimicrobial compound was carried out using silica gel column (2.5 X 50) chromatography. Chloroform and Methanol 95**:**5 (v/v) (Guangying *et al.*, 2005) was used as an eluting solvent. The column was left overnight until the silica gel (Prolabo) was completely settled. One-ml crude extract to be fractionated was added on the silica gel column surface and the extract was adsorbed on top of silica gel. Fifty fractions were collected (each of 5 ml) and tested for their antifungal activities.

Physico-chemical Properties:

1- Elemental analysis: The elemental analysis C, H, O, N, and S was carried out at the microanalytical center, Cairo University, Egypt.
2- Spectroscopic analysis: The IR, UV, Mass spectrum and NMR spectrum were determined at the micro analytical center of Cairo University, Egypt.
3- Reaction of the antifungal agent with certain chemical test:
   For this purpose, the following reactions were carried out: Molish's, Fehling, Sakaguchi, ninhydrin, Ehrlish, Nitroprusside, Ferric chloride and Mayer reactions (Atta, 1999).
4- Biological activity of the antifungal agent:
   The minimum inhibitory concentration (MIC) has been determined by the cup method assay (Kavanagh, 1972).
5- Characterization of the antifungal agent:
   The antibiotic produced by *Streptomyces olivaceiscleroticus*, AZ-AR-262 was identified according to the recommended international references of (Umezawa, 1977 and Berdy (1974, 1980a, b, c).

RESULTS AND DISCUSSION

Screening for the Antimicrobial Activities:

The active metabolites produced by actinomycete culture, AZ-AR-262 exhibited various degrees of activities against unicellular and filamentous fungi.

Identification of the Actinomycete Isolate:

The vegetative mycelia grew abundantly on both synthetic and complex media, and show fragmentation into bacillary elements. The aerial mycelia grew abundantly on Starch- nitrate agar medium; Oatmeal agar medium (ISP-3) and Glycerol – Asparagine agar medium (ISP-5). The Spore chains were spiral, and had a smooth surface (Fig. 1). Sclerotic granules are formed on some media, whereas sporangia are not observed. The cell wall hydrolysate contains LL-diaminopimelic acid (LL-DAP) and sugar pattern not detected the physiological and biochemical characteristics of actinomycete isolate AZ-AR-262 are summarized in (Table 2).

Color and Culture Characteristics:

The isolate AZ-AR-262 shows the aerial mycelium is gray; substrate mycelium is grayish yellow and the diffusible pigment not produced (Table 3).

Taxonomy of Actinomycete Isolate:

This was performed basically according to the recommended international Key's viz. (Buchanan and Gibsons, 1974; Williams, 1989; and Hensyl, 1994) and Numerical taxonomy of *Streptomyces* species program. On the basis of the previously collected data and in view of the comparative study of the recorded properties of AZ-AR-262 in relation to the most closest reference strain, viz. *Streptomyces olivaceiscleroticus*, it could be stated that actinomycetes isolate, AZ-AR-262 is suggestive of being likely belonging to *Streptomyces olivaceiscleroticus,* AZ-AR-262.

Table 1: Mean diameters of inhibition zones (mm) caused by 100µl of the antimicrobial activities produced by actinomycete isolate AZ-AR-262 in the agar plate diffusion assay (The diameter of the used cup assay was 10 mm).

Test organism		Mean diameters of inhibition zone (mm)
A-Bacteria		
1-Gram Positive		
Staphylococcus aureus,	NCTC 7447	0.0
Bacillus subtilis,	NCTC 1040	0.0
Bacillus pumilus,	NCTC 8214	0.0
Sarcina maxima,	ATCC 33910.	0.0
2-Gram Negative		
Escherichia coli,	NCTC 10416	0.0
Klebsiella pneumonia,	NCIMB 9111	0.0
Pseudomonas aeruginosa,	ATCC 10145	0.0
B- Fungi		
1-Unicellular fungi		
Candida albicans,	IMRU 3669	29.0
Saccharomyces cervicea	ATCC 9763	30.0
2-Filamentous fungi		
Asp. niger,	IMI 31276	28.0
Asp. fumigatus,	ATCC 16424	25.0
Aspergillus flavus,		27.0
Fusarium oxysporum		27.0
Botrytis fabae		21.0
Penicillium chrysogenum		22.0
Rhizoctonia solani		24.0

Table 2: The morphological, physiological and biochemical characteristics of the actinomycete isolate AZ-AR-262.

Characteristic	Result
Spore mass	Gray
Spore surface	Smooth
Spore chain	Spiral
color of substrate mycelium	Grayish yellow
Diffusible pigment	Not produced
Diaminopimelic acid (DAP)	LL-DAP
Sugar Pattern	Not detected
Hydrolysis of:	
Protein and Starch	+
Lipid	+
Egg-yolk (lecithin)	+
Catalase test	-
Production of melanin pigment	-
Degradation of: Esculin	+
Xanthin	+
H_2S Production	-
Nitrate reduction	+
Citrate utilization	+
Urea test	-
Coagulation of milk	-
Utilization of:	
D-Xylose	+
D- Mannose	+
D- Glucose	+
D- Galactose	+
Rhamnose	+
Raffinose	+
Mannitol	+
L- Arabinose	+
meso-Inositol	+
Lactose	-
Maltose	-
Trehalose	+
D-fructose	+
Sucrose	+
Starch	+++
L-Cycteine	+
L-Valine	-
L-Histidine	+
L-Phenylalanine	+
L-Hydroxproline	+
L-Lysine	+
L-Arginine	+
L-Serine	+
L-Tyrosine	+

Table 2:Continue

Growth with:	
Thallous acetate (0.001)	+
Sodium azide (0.01)	+
Phenol (0.1)	+
Growth temperature:	30 ºC (20-50 ºC)
Optimum pH:	7.0 (6.0-8.5)
Resistance to antibiotics:	
Ampicillin (10 ug); Ceplalexin (30 ug); Colistin (10 ug); Erthromycin (15 ug), Rifampicin (5 ug) and Amphotericin B(100 ug)	+
Antimicrobial activity against:	
Bacillus subtilis, NCTC 1040	+
Staph. aureus , NCTC 7447	+
Saccharomyces cerevisiae, ATCC 9763	+
Asp. niger, IMI 31276	+

=+Positive , - = Negative and ++ = moderate growth, +++= good growth results.

Table 3: Culture characteristics of the actinomycete isolate AZ-AR-262.

Medium	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigment
1-Starch nitrate agar medium	Good	264-L .Gray Light gray	90-gy-y grayish yellow	76-1-y-br Light yellowish brown
2-Tryptone yeast extract broth (ISP-1)	No growth	-	-	-
3-Yeast extract malt extract agar medium (ISP-2)	No growth	-	-	-
4- Oat-meal agar medium (ISP-3)	moderate	264-L .Gray Light gray	90-gy-y grayish yellow	-
5-Inorganic salts starch agar medium (ISP-4)	Good	264-L .Gray Light gray	90-gy-y grayish yellow	-
6-Glycerol – Asparagine agar medium (ISP-5)	Good	264-L .Gray Light gray	90-gy-y grayish yellow	-
7-Peptone yeast extract iron agar medium (ISP-6)	moderate	264-L .Gray Light gray	57-1.br light brown	58 m-br moderate brown
8-Tyrosine agar medium (ISP-7)	Poor	264-L .Gray Light gray	57-1.br light brown	58 m-br moderate brown
9- Potato dextrose agar medium	Good	264-L .Gray Light gray	90-gy-y grayish yellow	-
10- Glucose casein agar medium	moderate	264-L .Gray Light gray	90-gy-y grayish yellow	-

**The color of the organism under investigation was consulted with the ISCC-NBS color –name charts illustrated with centroid color.*

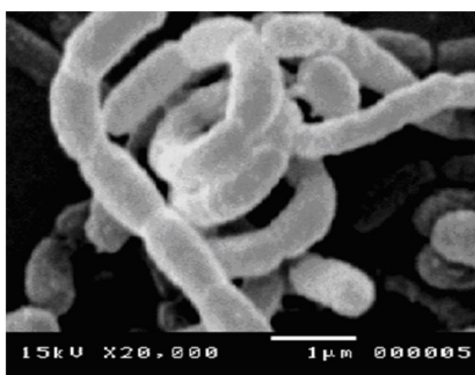

Fig. 1: Scanning electron micrographs of the actinomycete isolate AZ-AR-262 growing on starch nitrate agar medium, showing spore chain spiral and spore surfaces smooth (X20, 000).

A. Amplification of the 16s rDNA Gene:

The 16S rDNA gene was amplified by polymerase chain reaction (PCR) using the universal primers. The primers that was used to 16S rDNA sequencing were 16F357 of the sequencestrepF; 5'-ACGTGTGCAGCCCAAGACA-3' and strpR; 5'-ACAAGCCCTGGAAACGGGGT-3', the product of the PCR was analyzed on 1.5% ethidium bromide gel (Fig. 2).

B-Molecular Phylogeny of the Selected Isolate:

The 16S rDNA sequence of the local isolate was compared to the sequences of *Streptomyces* spp. In order to determine the relatedness of the local isolate to these *Streptomyces* strains. The phylogenetic tree (as displayed by the Tree View program) revealed that the locally isolated strain is closely related to *Streptomyces* sp., the most potent strain evidenced an 83% similarity with *Streptomyces tsukiyonensis*, *Streptomyces xanthocidicus* and *Streptomyces misakiensis* (Fig. 2). Multiple sequence alignment was conducted the sequences of the 16_S rDNA gene of the most potent strain evidenced an 83% similarity with *Streptomyces tsukiyonensis*, *Streptomyces xanthocidicus* and *Streptomyces misakiensis* (CHR and SNG). Computer assisted DNA searches against bacterial database similarly revealed that the 16_S rDNA sequence was 83% identical between *Streptomyces tsukiyonensis*, *Streptomyces xanthocidicus* and *Streptomyces misakiensis* (Fig. 2).

Fermentation, Extraction and Purification:

The fermentation process was carried out for six days at 30°C using liquid starch nitrate as production medium. Twenty-liter total volume filtered was conducted followed by centrifugation at 5000 r.p.m. for 20 minutes The clear filtrates containing the active metabolite (20 liters), was adjusted to pH 7.0 then extraction process was carried out using n-Butanol at the level of 1:1 (v/v) (Guangying *et al.,* 2005 and Khalifa, 2008). The organic phase was collected, and evaporated under reduced pressure using rotary evaporator. The antifungal compound was precipitated by petroleum ether (b.p. 60-80°C) and centrifuged at 5000 rpm for 15 minute. Its color is pale yellow. Separation of antifungal agent into individual components was carried out by thin-layer chromatography using a solvent system composed of chloroform and methanol (24: 1, v/v) (Atta, 1999). Only one band at R_f = 0.60 showed antifungal activity. The purification process through column chromatography packed with silica gel, revealed that the most active fractions against the tested organisms ranged between 25 to 32 (Fujita *et al.,* 2004 and Sekiguchi, *et al.,* 2007).

100% 95% 90% 85% 80%

Isolate, AZ-AS-233
S._albospinus
S._propurpuratus
S._albulus
S._indonesiensis
S._javensis
S._flavofuscus
S._kaniharaensis
S._tsukiyonensis
S._xanthocidicus
S._misakiensis

Fig. 2: The phylogenetic position of the local *Streptomyces* sp. strain among neighboring species. The phylogenetic tree was based on the pairwise comparisons of 16_S rDNA sequences.

Physicochemical Characteristics:

The purified antifungal agent produced by *Streptomyces olivaceiscleroticus,* AZ-AR-262 are produces characteristic odour, their melting points are 150°C. The compound is freely soluble in chloroform, ethyl acetate, n-butanol, acetone, ethyl alcohol, methanol and 10 % isopropyl alcohol, but insoluble in petroleum ether, hexan and benzene (Nishii *et al.,* 2003).

Elemental Analysis:

The elemental analytical data of the antifungal agent produced by *Streptomyces olivaceiscleroticus,* AZ-AR-262 showed the following: C=61.11; H=7.32; N= 5.30., O= 27.52 and S=0.0. This analysis indicates a suggested empirical formula of $C_{28}H_{40}N_2O_9$ (Umezawa, 1977).

Spectroscopic Characteristics:

The spectroscopic analysis of the purified of antimicrobial compound produced by *Streptomyces olivaceiscleroticus,* AZ-AR-262, Maximal IR spectra were 1,748 (ester and lactose C=0), 1,695 (NHCHO), 1,642, and 1,524 cm-'(ArCONH) 1,695 (NHCHO) (Fig. 3). The ultraviolet (UV) absorption spectrum are recorded a maximum absorption peak at 225 and 321 nm (Fig. 4). The Mass spectrum showed that the molecular weight at 574 (Fig.5). The NMR ($CDC1_3$) spectra showed resonances at 6 0.8 to 2.8 (CH_3-, -CH,--- ,and -CH), 5.0 to 6.0 (CH-O and COCHN), 6.8 to 7.1 (aromatic protons and NH and CHO) 8.53, 8.60 and 8.76 this indicates that that the compound is a mixture of two diastereomers *t*-butyl *γ*-hydroxyvalerateand and 12.7 are expected of the lactone, where the lactone side chain is n-hexyl (Fig. 6) (Wu and Yang, 2006).

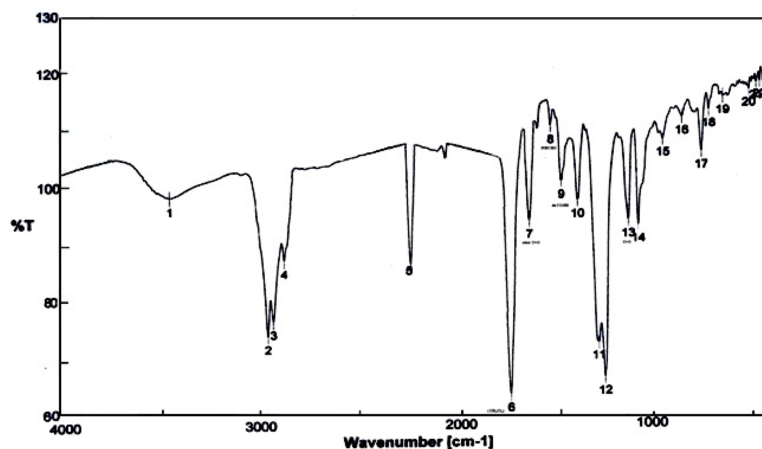

Fig. 3: I.R spectrum of antifungal agent produced by *Streptomyces olivaceiscleroticus,* AZ-AR-262

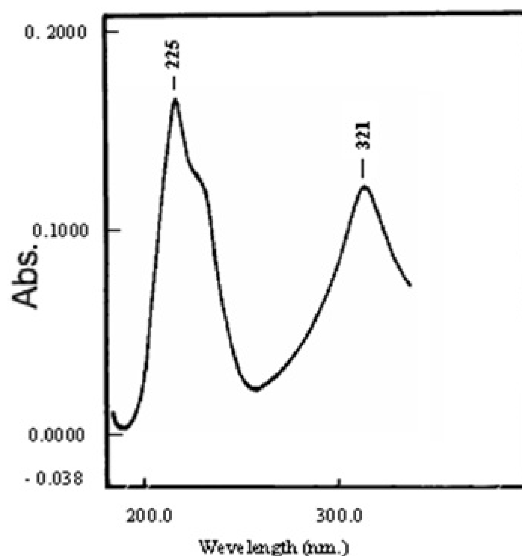

Fig. 4: Ultraviolet absorbance of antifungal agent produced by *Streptomyces olivaceiscleroticus,* AZ-AR-262.

Biochemical Reaction of the Antifungal Agent:

The reactions revealed the detection of certain groups in the investigated molecule. The antifungal agent exhibited positive results with ninhydrin, sakaguchi, ehrlish, ferric chloride and Mayer reactions; whereas negative results with molish's, fehling, and nitroprusside reactions (Atta, 1999).

Biological Activities of the Antifungal Agent:

Data of the antifungal agent spectrum indicated that the agent is active against unicellular and filamentous fungi strains (Guangying *et al.,* 2005; Hosotani *et al.,* 2005; Shiomi *et al.,* 2005 and Takeshi *et al.,* 2007) (Table 4).

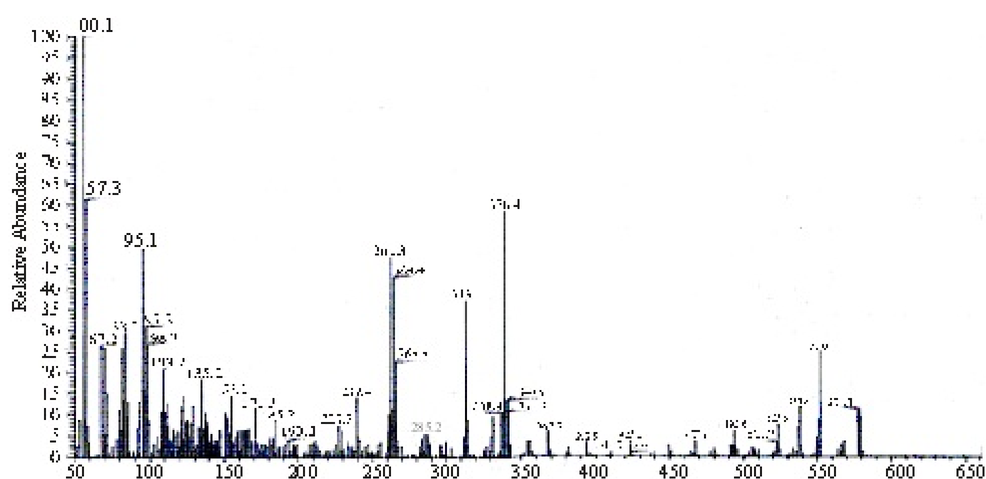

Fig. 5: Mass spectrum of antifungal agent produced by *Streptomyces olivaceiscleroticus,* AZ-AR-262

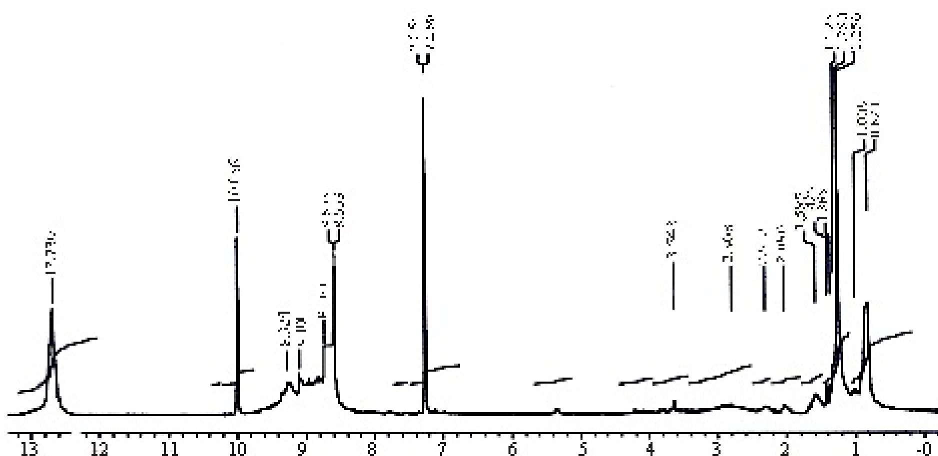

Fig. 6: NMR spectrum of antifungal agent produced by *Streptomyces olivaceiscleroticus,* AZ-AR-262.

Table 4: Biological activities (MIC) of the antifungal agent by cup method assay.

Test organisms	MIC (Mg/ml) concentration
1-Unicellular fungi:	
Candida albicans, IMRU 3669	31.25
Saccharomyces cervisiae ATCC 9763	23.4
2-Filamentous fungi:	
Aspergillus niger IMI 31276	31.25
Aspergillus fumigatus ATCC 16424	46.9
Aspergillus flavus	52.7
Fusarium oxysporum	31.25
Rhizoctonia solani.	41.6
Botrytis fabae	52.7
Penicillium chrysogenium	>100

Table 5: A comparative study of the characteristic properties of the antifungal agent produced by *Streptomyces rimosus,* AZ-AR-262 in relation to Reference antibiotic (Antimycin-A).

Characteristic	Purified Antifungal agent	Antimycin-A
1-Melting point	150°C	149-150°C
2-Molecular weight	574.7	574
3-Chemical analysis:		
C	61.11	61.11 (61.36)
H	7.32	7.32
N	5.30	5.32
O	27.52	27.50
S	0.0	0.0
4-Ultra violet	225 and 321	225 and 321
5-Formula	$C_{28}H_{40}N_2O_9$	$C_{28}H_{40}N_2O_9$
6-Active against	Active against unicellular and filamentous fungi.	Active against unicellular and filamentous fungi.

Identification of the Antifungal Agent:

On the basis of the recommended keys for the identification of antibiotics and in view of the comparative study of the recorded properties of the antimicrobial agent, it could be stated that the antimicrobial compound is suggestive of being belonging to Antimycin-A antibiotic (Umezawa, 1977; Berdy, 1974, 1980a, b, c; Guangying *et al.,* 2005 and Takeshi *et al.,* 2007) (Table 5).

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