Microbiology Journal

ISSN 2153-0696

Academic
Journals Inc.

www.academicjournals.com

Microbiology Journal 2 (1): 1-22, 2012
ISSN 2153-0696 / DOI: 10.3923/mj.2012.1.22
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Identification of Luteomycin like Antibiotic Produced by *Streptomyces tanashiensis* AZ-C442 Isolated from Luxor Governorate at Upper Egypt

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ABSTRACT

A *Streptomyces* bacterium, designated AZ-C442 was isolated from a soil sample collected from Luxor governorate, Egypt. Phylogenetic analysis based on 16S rRNA gene sequences and culture characteristics demonstrated strain AZ-C442 affiliation to the genus *Streptomyces tanashiensis*. This strain exhibited inhibitory activity against gram-positive and gram-negative bacteria and unicellular and filamentous fungi by evaluation of optimum environmental and nutritional conditions. It was found that the *Streptomyces tanashiensis* AZ-C442 produced and excreted into the culture medium a large amount of the compound, which was isolated, purified and structurally characterized as known Luteomycin like antibiotic on the basis of combined spectral analyses which indicates a suggested impirical formula of $C_{15}H_{30}N_2O_{10}$. Luteomycin showed inhibitory effects against *Staphylococcus aureus*, *Klebsiella pneumonia*, *Escherichia coli* and *Salmonella typhi,* moreover, against *Aspergillus flavus*, *Alternaria alternate* and *Saccharomyces cerevisiae*.

Key words: Luteomycin antibiotic, *Streptomyces tanashiensis*, phylogeny, purification, identification, antimicrobial activity

INTRODUCTION

Luteomycin is similar to, Tautomycetin (TMC) is an unusual linear polyketide compound esterified with a cyclic anhydride. It exhibits novel activated T cell-specific immunosuppressant as well as anti-cancer activities. It was isolated and characterized the entire TMC biosynthetic gene cluster from *Streptomyces* sp. Since many antibiotics are able to suppress or retard the growth of tumors, an extensive effort has been made in many laboratories to find new antibiotics with antineoplastic properties either by screening new soil isolates or by chemical modification of the existing antibiotics such as our goal in this study. Because the Actinomycetes are the source of most clinically used antibiotics, as well as of several widely used drugs against common diseases, including cancer (Medema *et al.*, 2011) and their genome sequencing revealed that the potential of *Streptomyces* species for the production of valuable secondary metabolites is high. It is well documented that the biosynthesis of *Streptomyces* secondary metabolites is typically regulated via multiple regulatory pathways operating with several layers of complicated control systems (Chen *et al.*, 2010; Lee *et al.*, 2005).

The comparison of rRNA sequences is a particularly powerful tool in streptomycete taxonomy. rRNA sequence comparisons have also been useful for answering questions concerning the horizontal transfer of genes within the genus *Streptomyces* (Huddleston *et al.*, 1997). The nucleotide sequence of the 16S rRNA gene of *Streptomyces* sp. was determined (Edwards *et al.*, 1989; Wang *et al.*, 2011), the PCR product was analyzed by agarose gel electrophoresis and the DNA fragment with the expected size was purified and sequenced (Palaniappan *et al.*, 2009; Wang *et al.*, 2011).

Cultural criteria, such as growth on different media, the color of aerial and substrate mycelia and the formation of soluble pigments among others and ecological properties has also been used for the classification of *Streptomyces* sp. (Shirling and Gottlieb, 1966; Lechevalier and Lechevalier, 1980). Colour names were assigned to the mycelial and diffusible pigments on the basis of the Inter-Society Color Council-National Bureu of Standards (ISCC-NBS) centroid color charts (Burres *et al.*, 1995). Many investigators have attempted to obtain optimal liquid culture requirements for obtaining a maximum yield of antibiotic production by different *Streptomyces* species (Yoshimoto *et al.*, 2000; Momose *et al.*, 2001; Kurosawa *et al.*, 2001; Fang, 2002; Huang *et al.*, 2003; Xu and Yun, 2003; Atta *et al.*, 2011).

In the course of antibiotic purification, different solvents were used and tested for the extraction of the antibiotic, in case of *S. albidoflavus* PU 23, maximum antibiotic yield was observed in residue, which was extracted by using n-hexane and purified by column chromatography using silica gel (Augustine *et al.*, 2005). On the same context, El-Naggar *et al.* (2006) analysed Meroparamycin antibiotic produced by newly isolated *Streptomyces* sp. by elemental analysis method such as IR (infrared spectrophotometer), UV absorption, high and low-resolution mass spectra and using the Electron Impact (EI) method to elucidate its structure.

Also, UV or MS detection are the most popular techniques in antibiotics analysis, however, fluorescence has also been used successfully (Diaz-Cruz *et al.*, 2003). Moreover, the actinomycins are characterized using a variety of analytical methods including ultraviolet visible spectroscopy system, Infrared (IR), electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometry (MS-MS) as well as various NMR techniques (Singh and Gurusiddaiah, 1984; Kurosawa *et al.*, 2006). The absorption spectrum of active extracts in methanol were recorded in the UV region (210-400 nm) by using a UV-vis spectrophotometer (Cintra 40) and compared with those of known polyenic antifungal antibiotics (Thakur *et al.*, 2007). Many authors have synthesized antibiotics which possess biological activities including, antimicrobial, antifungal, anti-tumor, antimalarial and antiparasitic effects (Laursen and Nielsen, 2004; Mavrodi *et al.*, 2010, Wang *et al.*, 2011; Atta *et al.*, 2011).

This investigation aimed to isolate and identify the antimicrobial producing *Sterptomycete* sp. from the soil in Upper Egypt and to isolate, purify and identify the produced antimicrobial agent as well as evaluate its MIC.

MATERIALS AND METHODS

Microorganism: The actinomycete AZ-C442 was isolated from soil sample collected from Luxor governorate. It was purified using the soil dilution plate technique described by Williams and Davis (1965).

Taxonomic studies of actinomycete isolate (AZ-C442)

Morphological characteristics: Morphological characteristics of aerial hyphae, spore mass, spore surface, color of aerial and substrate mycelia and soluble pigments production were conducted by growing the organism on starch-nitrate agar medium and yeast extract-malt extract agar medium.

Physiological and biochemical characteristics: Lecithinase was detected using egg-yolk medium according to the method of Nitsch and Kutzner (1969), lipase (Elwan *et al.*, 1977), protease (Chapman, 1952), pectinase (Hankin *et al.*, 1971), α-amylase (Ammar *et al.*, 1998) and catalase test (Jones, 1949). Melanin pigment (Pridham *et al.*, 1957), esculin broth and xanthine have been done according to Gordon *et al.* (1974). Nitrate reduction was performed according to the method of Gordon (1966). Hydrogen sulphide production was carried out according to Cowan and Steel (1974). The utilization of different carbon and nitrogen sources was carried out according to Pridham and Gottlieb (1948).

Determination of Diaminopimelic acid (DAP) and sugar pattern were carried out according to Becker *et al.* (1964) and Lechevalier and Lechevalier (1970).

Color characteristics: The ISCC-NBS color-name charts illustrated with centroid detection of the aerial, substrate mycelia and soluble pigments (Kenneth and Deane, 1955) was used.

DNA isolation and manipulation: The locally isolated actinomycete strain was grown for 6 days on a starch agar slant at 30°C. Two milliliter of a spore suspension were inoculated into the starch-nitrate broth and incubated for 4 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 as described by Fritsch *et al.* (1989).

PCR 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 Strep R; 5.ACAAGCCCTGGAAACGGGGT-3 (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, in 50 μL 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 (Sanger *et al.*, 1977).

Sequence similarities and phylogenetic analysis: The BLAST program (Altschul *et al.*, 1997) was employed in order to assess the degree of DNA similarity. Multiple sequence alignment and molecular phylogeny were evaluated using BioEdit software (Hall, 1999). The phylogenetic tree was displayed using the TREE VIEW program.

Parameters controlling antimicrobial agent biosynthesis: These parameters included, incubation period and temperature, agitation and aeration, pH values, carbon source and nitrogen sources, vitamins, $MgSO_4.7H_2O$ and K_2HPO_4 concentrations, inoculum age and size, amino acids and the medium kinds. All these parameters have been determined by the standard methods.

Purification of antimicrobial agent

Fermentation: *Streptomyces tanashiensis,* AZ-C442 inoculum was introduced aseptically into each sterile flasks containing the following ingredients (g L^{-1}): Glycerol, 20; $NaNO_3$, 2.0; K_2HPO_4, 0.6; $MgSO_4.7H_2O$, 0.5; Vitamin B_{12}, 200 (ppm) and KCl, 0.5. The pH was adjusted at 8.0 before sterilization. After 10 days of incubation at 35°C filtration was carried out through filter paper Whatman No. 1 and followed by centrifugation at 5000 rpm for 15 min. Only clear filtrates were tested for their antimicrobial activities (Eladly, 2009).

Extraction: The clear filtrates were 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.

Precipitation: The precipitation process of the crude compound was carried out using petroleum ether (bp 60-80°C) followed by centrifugation at 5000 rpm for 15 min. The precipitate was tested for its antimicrobial activities.

Separation: 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: The purification of the antimicrobial compound was carried out using silica gel column (2.5×50) chromatography. Chloroform and Methanol 10:2 (v/v) was used as an eluting solvent. The column was left overnight until the silica gel (Prolabo) was completely settled. One milliliter 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 antibacterial activities.

Physico-chemical properties of antimicrobial agent

Elemental analysis: The elemental analysis of C, H, O, N and S were carried out at the Microanalytical Center, Cairo University, Egypt.

Spectroscopic analysis: The IR, UV and Mass spectrum were determined at the Micro analytical Center, Cairo University, Egypt.

Biological activity: The Minimum Inhibitory Concentration (MIC) has been determined by the cup method assay (Kavanagh, 1972).

Characterization of the antimicrobial agent: The antibiotic produced by *Streptomyces tanashiensis* AZ-C442 was identified according to the recommended international references of Umezawa (1977) and Berdy (1974, 1980a-c).

RESULTS

Isolation, purification and bioactivity of actinomycete isolates: In a previous study, we did isolation and purification of actinomycete colonies (the broadest source of antibiotics) from 40 soil samples collected from various Egyptian localities and found that the highest number of isolates

(84) out of 194 (43.2%) were isolated on starch nitrate agar (SNA) medium followed by 55 isolates (28.3%) on both starch casein agar and glycerol asparagine agar. The screening test for 194 actinomycete isolates, against certain bacteria, fungi and yeast, confirmed that the highest percentage (38%) 74 active isolates was obtained against *Staphylococcus aureus* 90.5% (67) followed by *Aternaria alternata* 43.2% (32) and *klebsiella pneumoniae*, 41.8% (31), while the lowest percentage was obtained against *Fusarim verticillioides* 21.6% (16 isolates), *Salmonella typhi* 20.2% (15 isolates), *Escherichia coli* 9.4% (7 isolates), *Aspergillus fumigatus* 9.4% (7 isolates), *Saccharomyces cerevisiae* 8.1% (6 isolates) and *Aspergillus flavus* 6.7% (5 isolates). Consequently, the previous above screening test indicated two Actinomycete isolates (AZ151 and AZ-C442) showed high potencies against all microorganisms tested. Thus, all items in this investigation are concerned with the most active Actinomecete AZ-C442, isolated from Luxor governorate, for production of antimicrobial agent.

Characterizations of the actinomycete isolate AZ-C442

Morphological characteristics: Spore chains were rectiflexibiles, spore masses were gray, spore surfaces were smooth and the reverse color pale yellow-light yellowish brown, while, diffusible pigment not produced (Fig. 1).

Cell wall hydrolysate: The cell wall hydrolysate contains LL-diaminopimelic acid (LL-DAP) and sugar pattern not detected (Table 1).

Physiological and biochemical characteristics: The actinomycete isolate AZ-C442 could hydrolyze starch, protein, lipid and pectin, whereas catalase was negative. Melanin pigment production, degradation of xanthin, production of H_2S, decomposition of urea, nitrate reduction, utilization of citrate and KCN were positive but esculin decomposition is negative.

The isolate under study utilizes D-xylose, D-mannose, D-glucose, D-galactose, L-arabinose and trehalose but do not utilize sucrose, rhamnose, raffinose, mannitol, meso-inositol, lactose, maltose,

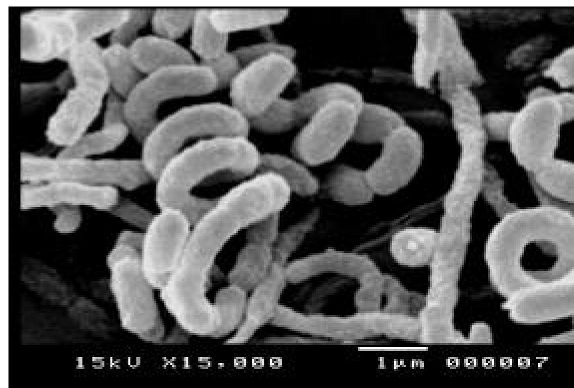

Fig. 1: Scanning electron micrograph of the actinomycete isolate AZ-C442 growing on starch nitrate agar medium showing spiral and rectiflexibiles spore chains and had a smooth surface. Neither sclerotic granules, sporangia nor flagellated spores were observed (X15, 000)

Table 1: The morphological, physiological and biochemical characteristics of the actinomycete isolate AZ151

Characteristic	Result	Characteristic	Result
Morphological characteristics		**Utilization of carbon sources**	
Spore chains	Rectiflexibiles	D- Xylose	+
Spore mass	Gray	D- Mannose	+
Spore surface	Smooth	D- Glucose	+
Color of substrate mycelium	Pale yellow- light yellowish brown	D- Galactose	+
Diffusible pigment	Not produced	Sucrose	-
Motility	Non-motile	L- Rhamnose	-
Cell wall hydrolysate		Raffinose	-
Diaminopimelic acid (DAP)	LL-DAP	-	-
Sugar Pattern	Not-detected	L- Arabinose	+
Physiological and biochemical properties		Meso-Inositol	-
Starch hydrolysis	+	-	-
Protein hydrolysis	+	Maltose	-
Lipid hydrolysis	+	Trehalose	++
Pectin hydrolysis	+	D- Ribose	-
Catalase test	-	D- Fructose	-
Production of melanin pigment on		**Utilization of amino acids**	
Peptone yeast- extract iron agar	+	L-Glycine	+
Tryptone – yeast extract broth	-	L-Valine and L-Leucine	-
Xanthin degradation	+	L-Histidine	+
Esculin degradation	-	L-Phenylalanine	-
H_2S Production	+	L-Asparagine	+
Nitrate reduction	+	L-Methionine	-
Citrate utilization	+	**Growth with (% w/v)**	
Urea test	+	Sodium azide (0.01)	-
KCN test	+	Phenol (0.1)	+
Growth at different concentration of NaCl (%)		**Growth at different temperatures (°C)**	
1	+	10	-
3	+	30	++
5	-	35	++
7	-	40	++
9	-	45	++
11	-	50	±
13	-	55	-
15	-		
Resistance to antibiotic			
Resistance to			
Nalidixic acid (30 µg mL^{-1})	+	Ampicillin (25 µg mL^{-1})	-
Cefoperazone (75 µg mL^{-1})		Polymyxin (30 µg mL^{-1})	
Fusidic acid (10 µg mL^{-1})		Gentamicin (10 µg mL^{-1})	
		Kanamycin (30 µg mL^{-1})	

-: Negative, +: Positive, ±: Doubtful results and ++: Moderate growth

D-fructose and ribose. It had good growth on L-glycine, L-histidine, L-asparagine, L-phenyl alanine and L-lysine and no growth on L-valine, L-leucine and L-methionine. Growth was inhibited in the presence of, up to 3% NaCl and of 0.01% (w/v) sodium azide but the growth was not inhibited in the presence of 0.1% (w/v) phenol and at 45°C. The actinomycete isolate was resistant to nalidixic acid (30 µg mL^{-1}), cefoperazone (75 µg mL^{-1}) and fusidic acid (10 µg mL^{-1}), whereas not resistant

Table 2: Culture characteristics of the actinomycete isolate AZ-C442

Medium	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigments
1-Starch- nitrate agar medium (SNA)	Good	263. L. Gray light gray	79.l.gy.yBr light gray yellowish brown	76-l.yBr light yellowish brown
2-Tryptone yeast extract broth (ISP-1)	No growth	-	-	-
3-Yeast extract malt extract agar medium (ISP-2)	Moderate	263. L. Gray light gray	76-l.yBr light yellowish brown	No
4-Oatmeal agar medium (ISP-3)	Moderate	263. L. Gray light gray	76-l.yBr light yellowish brown	No
5- Glycerol Asparagine agar medium (ISP-4)	Good	264.L. Gray light gray	73-p.oy pall orange yellow	No
6- Inorganic salts starch agar medium (ISP-5)	Moderate	263-L. Gray light gray	73-p.oy pall orange yellow	No
7-Peptone yeast extract- iron agar medium (ISP-6)	Good	263. L. Gray light gray	76-l.yBr light yellowish brown	59-d.Br deep brown
8-Tyrosine agar medium (ISP-7)	Good	263. L. Gray light gray	79.l.gy.yBr light gray yellowish brown	59-d.Br deep brown

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

to ampicillin (25 μg mL^{-1}), polymyxin (30 μg mL^{-1}), gentamicin (10 μg mL^{-1}) and kanamycin (30 μg mL^{-1}) (Table 1).

Color and culture characteristics: Data recorded in Table 2, declared that the growth on AZ-C442 strain was disappeared on ISP-1, moderate on ISP-2, 3 and 5 and good on SNA and ISP-4, 6 and 7. While, the aerial mycelium was light gray on almost all media used except ISP-1, substrate mycelium has been appeared, light gray yellowish brown on SNA and ISP-7, light yellowish brown on ISP-2, 3 and 6 and pall orange yellow on ISP- 4 and 5. Culture characteristics was not detected on ISP-1. Diffusible pigments were only detected on SNA and ISP- 6 and 7 (Table 2).

Identification of actinomycete isolate, AZ-C442: This was performed basically according to the recommended international Key's viz. (Buchanan and Gibsons, 1974; Holt and Williams, 1989; Hensyl, 1994). On the basis of above collected data and in view of the comparative study of the recorded properties of AZ-C442 in relation to the most closest reference strain, viz. *Streptomyces tanashiensis* (CHR and C442) (Table 3), it could be concluded that both isolates are identical on the basis of spore mass is gray, spore chain is retiflexibilies and spores are non motile. Cell wall hydrolysate contains LL-diaminopimelic acid and sugar pattern not detected. Melanin pigment not produced. They utilize D-xylose, D-glucose, D-galactose and L-arabinose but do not utilize sucrose, rhamnose, raffinose, mannitol, meso-inositol and D-fructose.

In view of all the previous characteristics of AZ-C442, it could be stated that it is suggestive of being belonging to *Streptomyces tanashiensis* and thus given the name *Streptomyces tanashiensis* AZ-C442.

Molecular phylogeny of the selected isolate: The 16S rDNA sequence of the local isolate was determined as 721 bp as shown in Table 4 and 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

Table 3: A comparative study of the characteristic properties of AZ-C442 in relation to reference strain, *Streptomyces tanashiensis*. (Bergey's 1989)

Characteristics	AZ-C442	*Streptomyces tanashiensis*
Morphological characteristics:		
- Spore mass	Gray	Gray
- Reverse color	Pall orange yellow	Pall yellow
- Spore chain	Rectiflexibles	Rectiflexibles
- Motility	Non-motile	Non-motile
Cell wall hydrolysate:		
-Diaminopimelic acid (DAP)	LL-DAP	LL-DAP
- Sugar pattern	Not-detected	Not-detected
Melanin pigment	+	+
Utilization of carbon sources		
L-Arabinose	+	+
D-Fructose	-	-
D-Galactose	+	+
D-Glucose	+	+
-Meso-Inositol	-	-
D-Mannitol	-	-
- Raffinose	-	-
- Sucrose	-	-
D-Xylose	+	+

-: Negative and +: Positive results

Table 4: Multiple sequence alignment was conducted the sequences of the 16S rDNA gene of *Streptomyces tanashiensis*

No.	Sequence
1	1 AACCATCTAG TCGTTCGATG AAGCGGTTCC GGGTGGATTA GTGGCGTTCG GGTGAGTTTC
2	61 ACGTGCCCAA TCTGGGCTTC ACTCTCCGAC AAGGGCTGGA TTCGCCGTCT TTTACCCCAT
3	121 ACGACACTCC GACCCATCTC CTGGACTCCA AAGCTCCCGC GGTGTTGGAT GAGGGCGCGC
4	181 CCTATCAGCT TGTTGGTGGG GTAATGGCCT ACCAAGGCGA CGACGGGTAG CCGGCCTGAG
5	241 AGCCCGACCG GCCACACTGG GACTGAGACA CGGCGGAGAC TCCTACGCCA GGCAGCAGTG
6	301 GGGAATATTG CACAATGGGC GAAAGCCTGA TGCAGGGACG CCGCGTGAGG GATGACGGCC
7	361 AACGGGTTGT TTTCCTCAAA CAGCAGGGTT GAAGCGTTTG TGACGGTACC TGCAGAAGTT
8	421 GCGCCGGCTA GCTACGTGGC AGCAGCCGCG GTTATACGTA GGGCGCAAGC GTTGTGGGGA
9	481 ATAATTCCGC GTTTAGAGCT CGTAGGCGGC TTGTCACGTC GGGTGTGATA GCCCGGGGCT
10	541 TAACGGCGGG TCTGCATCCG ATACGGGCAG GCTAGAGTGT GGTAGGGGAG ATCGGAATTC
11	601 CTGGTGTAGC GGTGAAATGC GCAGATATCA GGAGGAACAC CGGTGGCGAA GGCGGATCTC
12	661 TGGGCCATTA CTGACGCTGA GGAGCGAAAG CGTGGCCAGC GAACAGGATT AGATACCCTG
13	721 GTAGTCCACG CCGTAAACGT TGGGAAC

is closely related to *Streptomyces* sp., the most potent strain evidenced an 89% similarity with *Streptomyces tanashiensis* (Fig. 2).

Parameters controlling antimicrobial agent biosynthesis: The evaluation of different environmental and nutritional factors on the biosynthesized antimicrobial activity indicated that the maximum activity was exhibited in starch nitrate broth medium at, incubation period (8 days), incubation temperature (30°C), agitation and aeration (120 rpm), pH value (8.0), carbon source (glycerol), nitrogen source ($NaNO_3$), water soluble vitamin (Vitamin B12), inoculum age (15 days), inoculum size (10 %,v/v), amino acid (L-Asparatic acid), $MgSO_4.7H_2O$ concentration (0.5 g L^{-1}, w/v) and at K_2HPO_4 concentration (0.08 %,w/v) (Table 5).

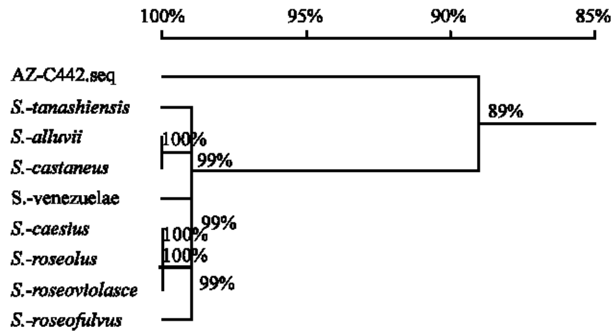

Fig. 2: The phylogenetic position of the local *Streptomyces* sp. strain among neighboring species. The phylogenetic tree was based on the pairwise comparisons of 16S rDNA sequences

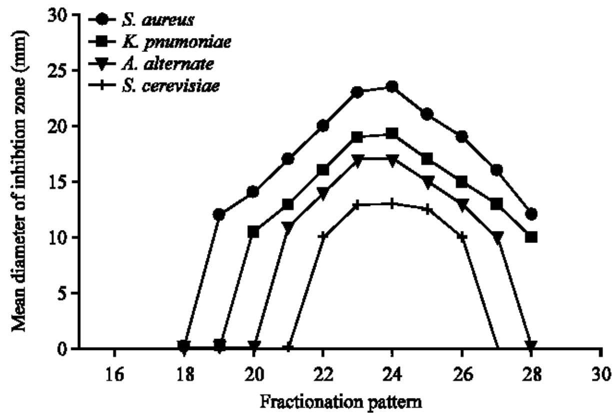

Fig. 3: Fractionationpattern of antimicrobial agent produced by *Streptomyces tanashiensis* AZ-C442

Fermentation and isolation of antimicrobial agent: The fermentation process was carried out, using the above parameters, of liquid starch nitrate medium as production medium. Filtration was conducted followed by centrifugation at 4000 rpm for 15 min. The clear filtrates containing the active metabolite, was adjusted to pH 8.0 then extraction process was carried out using Ethyl acetate at the level of 1:1 (v/v). The organic phase was collected and evaporated under reduced pressure using rotary evaporator. The residual material was dissolved in least amount of DMSO and filtered. The filtrates were tested for their antibacterial activities.

The antimicrobial agent was precipitated by petroleum ether (bp 60-80°C) and centrifuged at 4000 rpm for 15 min. The fraction was test for antimicrobial activities. Separation of antibacterial agent into individual components was carried out by thin-layer chromatography using a solvent system composed of chloroform and methanol (24:1, v/v). Among three bands developed, only one band at R_f 0.75 showed antibacterial activity. The purification process through column chromatography packed with silica gel indicated that the most active fractions against the tested organisms ranged between 19 and 28 (Fig. 3)

Physicochemical characteristics of the antimicrobial agent: The purified antimicrobial agent produced by *Streptomyces tanashiensis* AZ-C442 has a characteristic odour, its melting points is 190°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, hexane and benzene.

Table 5: The environmental conditions and nutritional requirements affecting the biosynthesis of antimicrobial agent by *Streptomyces tanashiensis* AZ-C442

	*Mean diameter of inhibition zone (mm) against					*Mean diameter of inhibition zone (mm) against			
Parameter	*S. cerevisiae*	*A. alternata*	*K. pnumoniae*	*S. aureus*	Parameter	*S. cerevisiae*	*A. alternata*	*K. pnumoniae*	*S. aureus*
Nitrogen source (2 g L^{-1})					**Incubation periods (day)**				
Ammonium nitrate	12.0±0.0	17.5±0.6	20.3±0.5	30.5±0.0	0	0.0	0.0	0.0	0.0
Casein	11.0±0.4	112.8±0.5	14.0±0.4	24.0±0.0	2	0.0	9.5±0.0	11.0±0.8	15.5±0.0
Sodium nitrate	16.0±0.8	22.5±0.0	25.5±0.4	35.0±0.0	4	9.8±0.0	12.0±0.8	13.5±0.3	20.5±0.0
Potassium nitrate	13.0±0.0	14.5±0.0	16.5±1.0	27.5±0.6	6	12.5±0.4	17.5±0.0	19.5±0.4	28.0±1.9
Magnesium nitrate	0.0	0.0	10.0±0.0	18.0±0.0	8	16.0±0.0	23.0±1.4	24.5±0.0	33.5±1.3
Ammonium sulphate	15.3±0.5	21.0±1.3	23.5±1.0	33.5±0.6	10	14.5±0.0	21.3±0.0	23.0±0.0	31.5±0.0
Ammonium carbonate	0.0	0.0	11.8±0.5	21.0±0.0	12	14.0±0.0	20.0±0.9	22.0±0.8	30.0±1.8
					14	12.0±0.0	18.0±0.0	21.0±0.0	28.5±0.3
					16	9.5±0.4	14.5±0.0	17.0±0.4	24.0±0.0
					18	0.0	0.0	12.0±0.0	18.0±0.2
					20	0.0	0.0	0.0	12.0±0.7
Amino acids (2 g L^{-1})					**Difference pH values**				
L-Phenylalanine	0.0	0.0	0.0	0.0	4	0.0	0.0	0.0	0.0
DL-Cystine	0.0	0	0.0	0.0	5	0.0	0.0	0.0	0.0
DL-Methonine	0.0	0.0	0.0	0.0	6	10.8±0.5	10.5±1.0	11.8±1.5	15.5±0.6
Lysine	12.8±0.4	17.5±0.3	20.8±0.5	27.5±0.2	7	13.5±0.0	18.3±0.5	20.0±0.0	27.5±5.0
L-Leucine	0.0	0.0	0.0	0.0	8	16.0±0.0	22.5±0.6	25.5±0.6	35.0±0.8
Glycine	0.0	13.0±0.2	15.5±0.4	22.0±0.3	9	17.5±1.0	18.0±0.0	20.5±0.0	28.5±0.6
Trptophane	12.0±0.0	17.5±1.0	20.5±0.6	28.0±0.5	10	13.8±0.5	14.5±0.6	15.5±1.0	22.0±0.0
L-Asparagine	12.8±1.0	17.0±0.0	20.8±0.5	27.5±1.0	11	0.0	0.0	0.0	0.0
L-Proline	14.0±0.0	19.0±0.8	21.0±0.0	29.5±2.4					
L-Asparatic acid	14.0±1.3	18.5±0.0	22.0±0.8	30.0±0.8					
L-Serine	0.0	0.0	0.0	0.0					
Sodium nitrate	15±0.0	20.8±1.0	24.0±0.0	32.5±1.0					
K_2HPO_4 conc. %(w/v)					**Temperature (°C)**				
0.00	10.5±0.4	14.5±0.0	15.0±0.0	19.5±0.0	15	0.0	0.0	0.0	0.0
0.02	11.5±0.0	16.0±0.0	17.0±0.0	22.5±0.4	20	0.0	13.0±0.4	15.0±0.0	22.0±0.0
0.04	13.0±0.4	18.5±0.0	18.5±0.9	25.0±0.8	25	12.0±0.0	16.3±0.5	19.5±0.0	28.3±0.5
0.06	19.5±0.0	23.0±0.0	21.0±0.0	29.5±0.0	30	16.5±1.0	21.5±0.0	25.3±0.5	35.5±0.6
0.08	14.0±0.0	20.5±0.6	26.0±0.0	37.0±1.9	35	15.0±1.2	14.5±1.0	17.8±1.0	26.5±1.0
0.0	12.8±0.8	18.0±0.0	22.0±0.0	31.5±0.8	40	13.5±1.0	12.0±0.0	15.3±0.5	22.5±1.7
0.2	9.0±0.0	13.8±0.7	16.5±0.4	24.5±0.0	45	11.0±0.0	9.3±0.5	12.0±0.0	11.5±1.0
0.3	0.0	10.3±0.0	12.5±0.4	19.0±0.0	50	0.0	0.0	0.0	11.5±1.0
					55	0.0	0.0	0.0	0.0

Table 5: Continued

	*Mean diameter of inhibition zone (mm) against					Mean diameter of inhibition zone (mm) against			
Parameter	*S. cerevisiae*	*A. alternata*	*K. pnumoniae*	*S. aureus*	Parameter	*S. cerevisiae*	*A. alternata*	*K. pnumoniae*	*S. aureus*
$MgSO_4$. $7H_2O$ Conc. ($G L^{-1}$, w/v)					**Different shaking speed (rpm)**				
0.0	0.0	0.0	11.5±0.6	18.5±0.6	0	0.0	13.0±0.6	15.8±1.5	22.0±3.0
0.0	10.5±0.0	12.5±0.6	14.8±1.0	22.5±1.0	40	9.5±0.0	14.5±0.0	17.0±0.0	24.0±0.0
0.3	14.0±0.0	17.3±0.5	19.0±0.0	28.8±1.5	80	12.3±0.5	17.8±1.0	19.5±0.4	27.5±1.0
0.5	16.8±1.5	22.5±0.0	25.5±0.0	35.5±1.9	120	17.5±0.6	21.5±0.0	24.5±1.0	34.0±0.0
0.7	14.0±0.8	19.3±0.5	21.5±0.0	30.0±2.4	160	14.0±0.6	19.0±0.0	21.0±0.0	29.3±0.0
1.0	11.0±0.0	15.8±1.0	17.5±0.0	24.5±1.0	200	13.0±0.0	19.0±0.0	21.0±0.0	29.0±0.5
2.0	0.0	0.0	0.0	12.5±0.0					
Media used**					**Inoculums age (days)**				
(SN)	16.5±0.4	21.0±0.6	24.0±5.8	33.5±0.0	3	0.0	10.5±1.0	12.0±0.0	16.0±0.0
(SC)	15.0±0.0	18.5±0.0	20.5±2.3	28.5±0.0	6	11.0±0.0	14.5±0.6	15.5±0.6	22.0±0.0
(YEME)	14.5±0.0	16.5±0.0	17.0±0.0	26.0±1.2	9	14.0±0.0	19.0±0.0	21.0±0.0	28.5±1.0
(GA)	13.0±0.8	15.5±0.4	17.5±0.7	24.5±1.0	12	17.0±0.8	22.5±0.0	24.0±0.0	32.5±1.0
(ISS)	±0.0	20.0±0.0	23.0±0.0	32.5±0.6	15	18.0±0.2	23.5±0.5	25.5±0.7	35.0±0.1
(TYE)	0.0	0.0	0.0	0.0	18	17.5±0.6	21.5±0.0	23.0±0.0	31.0±0.0
					21	14..0±0.4	16.5±0.6	18.5±0.0	26.5±1.7
					24	11.5±0.6	12.0±0.0	13.5±0.6	19.5±0.0
					27	0.0	0.0	10.5±0.6	13.0±0.0
					30	0.0	0.0	0.0	0.0
Different vitamins and their concentrations (ppm)					**Ioculum sizes % (v/v)**				
50 B12	18.3±0.5	23.5±0.6	26.0±0.8	35.5±0.6	2	13.0±0.0	18.0±0.0	21.0±0.0	29.5±1.0
100	20.0±1.4	26.0±0.4	29.5±1.0	39.5±1.0	4	14.5±1.0	20.0±0.8	23.5±0.6	33.0±0.0
200	21.5±0.6	28.5±1.0	30.5±1.0	41.0±1.6	6	16.0±0.0	22.3±1.3	26.5±1.7	37.5±1.9
50 D2	17.0±0.0	22.5±0.6	25.3±0.5	35.0±0.0	8	16.5±1.7	24.0±0.0	29.0±0.0	41.0±0.0
100	17.5±0.6	24.0±0.0	27.5±0.6	38.0±0.0	10	21.0±1.6	25.0±0.0	30.0±0.0	43.0±2.1
200	19.0±0.0	26.5±0.6	27.0±0.8	38.0±0.0	12	17.5±0.6	23.3±0.5	30.5±0.6	41.5±1.0
50 D-Pantothenic acid	17.5±0.6	23.0±1.6	26.3±0.5	36.0±1.4	14	14.0±0.0	20.0±1.2	25.5±1.7	40.0±0.0
100	19.0±0.8	25.0±0.8	27.8±1.0	38.5±1.0	16	10.5±0.6	16.0±0.0	21.0±0.8	34.5±1.0
200	19.5±1.0	26.0±0.0	29.0±0.8	40.5±1.9	18	0.0	12.5±0.6	16.5±0.6	30.5±1.7
50 Folic acid	18.0±0.0	24.3±0.5	27.5±0.6	37.5±0.0	20	0.0	0.0	13.0±0.0	26.5±1.5
100	18.0±0.0	24.0±0.0	27.0±0.0	37.5±0.0					
200	18.0±0.0	24.0±0.0	27.5±1.0	37.0±0.0					

Table 5: Continued

Parameter		*Mean diameter of inhibition zone (mm) against				Parameter	Mean diameter of inhibition zone (mm) against			
		S. cerevisiae	*A. alternata*	*K. pnumoniae*	*S. aureus*		*S. cerevisiae*	*A. alternata*	*K. pnumoniae*	*S. aureus*
						Carbon sources				
50	Vitamin H	16.0±0.8	22.3±0.3	25.0±1.6	34.5±0.6	Starch	17.3±0.5	22.0±0.0	25.0±0.0	35.5±1.9
100		18.0±0.8	24.5±1.0	28.0±0.0	38.0±0.0	Galactose	12.5±0.6	16.0±0.0	19.5±0.6	26.5±0.6
200		19.5±0.6	26.5±0.7	29.5±0.5	40.5±1.0	Glycerol	20.0±0.0	24.5±2.5	27.0±0.0	38.5±0.6
50	Riboflavine	18.0±0.8	24.0±0.8	26.8±1.5	36.5±0.6	Glucose	15.0±0.0	18.5±0.0	21.3±0.5	24.0±0.0
100		19.5±0.6	26.0±0.0	28.5±1.0	39.0±0.0	Cellulose	±1.0	20.5±0.6	22.0±0.0	32.5±0.6
200		19.0±0.9	26.0±0.4	28.5±1.0	39.0±0.0	Arabinose	15.5±0.6	17.5±0.4	23.5±0.6	33.0±0.0
50	Thiamin	17.0±0.0	23.3±1.3	25.5±0.0	35.0±0.0	Trehalose	15.0±0.0	18.5±0.6	20.3±0.5	28.5±0.6
100		18.0±0.0	25.0±0.0	27.3±0.5	38.5±2.1					
200		18.0±0.0	25.0±0.0	28.0±0.0	39.5±1.7					
Control (no vitamin)		16.0±0.8	21.0±1.4	24.5±1.0	33.5±0.6					

*Mean values of triplicate determinations were calculated. **SN: Starch nitrate medium, SC: Starch casein medium , YEME: Yeast extract malt extract medium, GA: Glycerol asparagine medium, ISS: Inorganic salt starch medium, TYE: Tryptone yeast extract medium

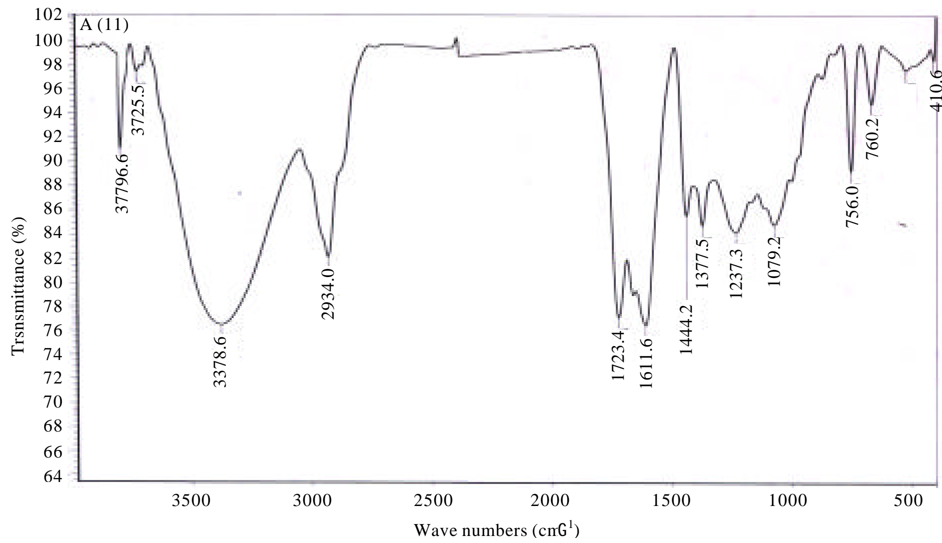

Fig. 4: Infrared spectrum of the antimicrobial agent

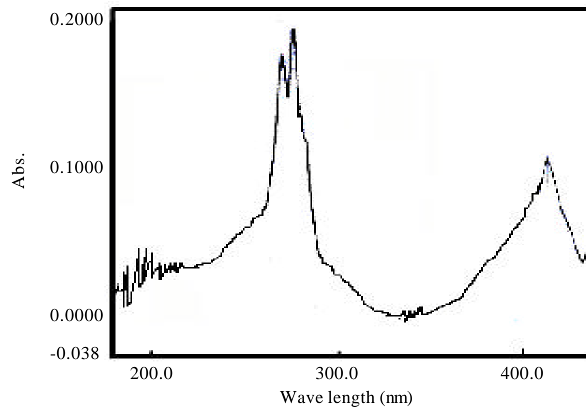

Fig. 5: Ultraviolet absorbance of antimicrobial agent

Elemental analysis: The elemental analytical data of the antibacterial agent revealed the following data: C = 59.61, H = 6.32, N = 2.97, O = 31.1 and S = 0. 0. This analysis indicates a suggested calculated empirical formula of $C_{26}H_{33}NO_{12}$.

Spectroscopic characteristics: The spectroscopic analysis of the purified of antimicrobial agent, produced by *Streptomyces tanashiensis* AZ-C442 indicated that, the Infrared (IR) spectrum of antimicrobial agent shows a characteristic band corresponding to 19 peaks (Fig. 4), the Ultraviolet (UV) spectrum record a maximum absorption peak at 280 and 420 nm (Fig. 5) and the Mass spectrum showed that the molecular weight is 552.52 (Fig. 6).

Biological activities of the antimicrobial agent: Data recorded in Table 6 indicated that the antimicrobial agent is fairly active against both Gram positive and Gram negative bacteria and

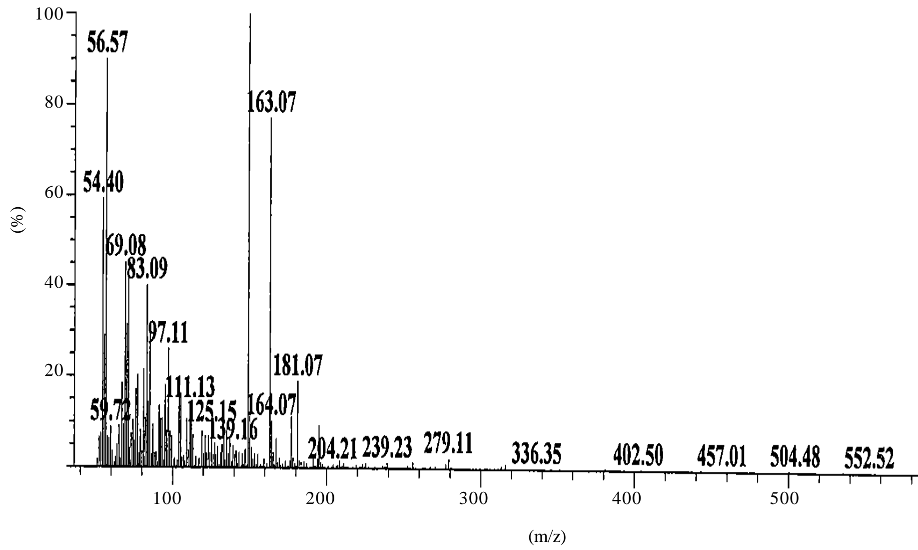

Fig. 6: Mass spectrum of antimicrobial agent.

Table 6: Mean diameters of inhibition zones (mm) caused by 100 μL of the antimicrobial activities produced by *Streptomyces tanashiensis* AZ-C442 in the agar plate diffusion assay (The diameter of the used cup assay was 10 mm)

	MIC (ug mL^{-1}) concentration
Test organism	Antimicrobial agent produced by AZ-C442
Staphylococcus aureus	1.73
Escherichia coli	7.8
Klebsiella pneumonia	3.9
Salmonella typhi	7.8
Saccharomyces cerevisiae	62.5
Aspergillus flavus	31.25
Alternaria alternate	31.25

unicellular and filamentous fungi. The Minimum Inhibitory Concentration (MIC) of antibiotic produced by *Streptomyces tanashiensis* AZ-C442 was determined and results showed that the MIC of antibiotic (μg mL^{-1}) against *S. aureus* (2.6), *K. pneumonia* (7.8), *E. coli* and *S. typhi* (15.6), *A. flavus* and *A. alternata* (46.87) and *S. cerevisiae* (62.5).

Identification of the antimicrobial 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 agents, it could be stated that the antimicrobial agent is suggestive of being belonging to Luteomycin antibiotic (Table 7).

DISCUSSION

Because a luteomycin is produced by microorganisms and is effective against some forms of cancer. Since a Streptomyces bacteria have long been appreciated as a rich source for the production of various secondary metabolites including many pharmaceutically valuable compounds such as antibiotics, anti-cancer agents, immunosuppressants and enzyme inhibitors

Table 7: A comparative study of characteristic properties of antimicrobial agent produced by *Streptomyces tanashiensis* AZ-C442 in relation to reference antibiotic (Luteomycin)

Characteristic	Purified antimicrobial agent	Luteomycin
Melting point	190°C	190-200°C
Molecular weight	552.52	551
Chemical analysis:		
C	59.61	59.60
H	6.32	6.30
N	2.97	3.0
O	31.1	31.1
Ultra violet	280 and 420	280 and 420-440
Formula	$C_{26}H_{33}NO_{12}$	$C_{26}H_{33}NO_{12}$
Active against	Active against Gram positive, Gram negative bacteria, unicellular and filamentous fungi	Active against Gram positive, Gram negative bacteria, unicellular and filamentous fungi

(Hindra and Elliot, 2010; Hranueli *et al.*, 2005; Myles, 2003). Which gives us the opportunity to announce the importance of this research in the medical field.

Given that there is an instance of this antibiotics, a studies have shown that, among them is a protein phosphatase PP1/PP2A inhibitor named tautomycetin (TMC), which is also, a pharmacokinetically-superior T cell-specific immunosuppressant produced by *Streptomyces* sp. CK4412 (Chae *et al.*, 2004; Shim *et al.*, 2002) and similar to our produced antibiotic.

However, there has been increasing interest in production of wide range of secondary metabolites by *Streptomyces* of clinical importance in the treatment of infectious disease or diseases caused by proliferation of malignant cells (Pelczar *et al.*, 1986; Innes and Allan, 2001). Nevertheless, there is a different type of regulatory system that affects only the corresponding target metabolite biosynthesis in *Streptomyces* species, which is commonly named the 'pathway-specific' regulatory system(Lombo *et al.*, 1999; Niraula *et al.*, 2010; Retzlaff and Distler, 1995; Tang *et al.*, 1996).

In a previous study, the most active actinomycete isolate AZ-C442, was isolated from Luxor governorate, Egypt, on the bases of its antimicrobial activities, thus this investigation is concerned with the production of antimicrobial agent by its growth on starch nitrate medium.

Identification process of this strain has been carried out according to (Buchanan and Gibsons (1974) Holt and Williams (1989) and Hensyl (1994). For the purpose of identification of this actinomycete isolate, the morphological characteristics and microscopic examination emphasized that the spore chain is spiral, spore mass is red, while spore surface is smooth, substrate mycelium is light yellow-brown and diffusible pigment grayish red orange. The results of physiological, biochemical characteristics and cell wall hydrolysate of actinomycetes isolate, exhibited that the cell wall containing LL-diaminopimelic acid (DAP) and sugar pattern of cell wall hydrolysate could not detected. These results emphasized that the actinomycetes isolate related to a group of *Streptomyces*.

Besides, computer assisted DNA searches against bacterial database similarly revealed that the 16S rDNA sequence was 89% identical with *Streptomyces tanashiensis* (CHR and SNG) (Augustine *et al.*, 2005; Thenmozhi and Kannabiran, 2010).

In view of all the previously recorded data, the identification of actinomycete isolate AZ-C442 was suggestive of being belonging to *Streptomyces tanashiensis* which can produce a broad-spectrum antibiotic. A 16S rRNA sequence data have proved invaluable in streptomycetes

systematics, in which they have been used to identify several newly isolated *Streptomyces* (Mehling *et al*., 1995). This finding is in agreement with antibiotic phenazine derivatives and their formation pathways in a new *Streptomyces* strain P510, where culture characteristics and 16S rRNA nucleotide analysis confirmed strain P510 as *Streptomyces griseoluteus* (Wang *et al*., 2011). Further, exploratory research into phenazine biosynthetic genes in strain P510 was carried out to provide the foundation for pathway engineering. One 3111 bp DNA fragment (NCBI Genbank Accession No. HM363127) was amplified from *S. griseoluteus* P510 through PCR amplification using primers A and B. GC content of the amplicon was 74.67%, this high G+C content was similar to that found in other phenazine-producing species (Mavrodi *et al*., 2006).

In this study we found the highest antimicrobial activity was achieved at optimum environmental and nutritional conditions in *Streptomyces tanashiensis* AZ-C442 culture. Many evaluations have been carried out on the optimum conditions controlling the biosynthesis of the antimicrobial substances (Vasavada *et al*., 2006; Boudjella *et al*., 2006, Kumar and Satyanarayana, 2007; Latifian *et al*., 2007; Gupta *et al*., 2008; Kagliwal *et al*., 2009; Atta *et al*., 2011). The active metabolites were extracted by n-Butanol at pH 8 as the results obtained by Puius *et al*. (2006), Criswell *et al*. (2006), Sekiguchi *et al*. (2007) and Atta *et al*. (2009). While, the organic phase was collected and evaporated under reduced pressure using rotary evaporator. The extract was concentrated and treated with petroleum ether (bp 60-80°C) for precipitation process, where only one fraction was obtained in the form of viscous syrup and then tested for its antimicrobial activities. The purification process through a column chromatography packed with silica gel and an eluting solvents composed of chloroform and methanol (10:2, v/v), indicated that maximum activity was recorded among fraction Nos. 19 and 28. Many workers used a column chromatography packed with silica gel and obtained a nearly similar results (Hitchens and Kell, 2003; Criswell *et al*., 2006; Sekiguchi *et al*., 2007; Atta *et al*., 2011).

A study of the physico-chemical characteristics and elemental analysis of the produced antibacterial agent lead to an empirical formula of $C_{26}H_{33}NO_{12}$. In this trend, many antibiotic produced by *Streptomyces* spp. and identified by elemental analysis methods to elucidate their structures (Singh and Gurusiddaiah, 1984; Diaz-Cruz *et al*., 2003; Augustine *et al*., 2005; El-Naggar *et al*., 2006; Kurosawa *et al*., 2006; Thakur *et al*., 2007; Arakawa *et al*., 2011).

The biological activities (MICs) of the antimicrobial agent are fairly active against all tested bacterial and fungal strains tested as conducted (Gourevitch *et al*., 1958; Misumi and Tanaka, 1980; Criswell *et al*., 2006; Sekiguchi *et al*., 2007; Laursen and Nielsen, 2004; Mavrodi *et al*., 2010) synthesized phenazine derivatives and found that almost all biosynthesized phenazine compounds possess biological activities, including antimicrobial, antifungal, anti-tumor, antimalarial and antiparasitic effects. Identification of our synthetics antibiotic, according to recommended international keys, indicated that the antibiotic is suggestive of being likely to Luteomycin antibiotic (Umezawa, 1977; Berdy, 1974; Berdy, 1980a, b and c). In similarity to our antibiotic, Tautomycetin as a structurally-unique ester bond linkage between a terminal cyclic C8 dialkylmaleic anhydride moiety and linear polyketide chain bearing an unusual terminal alkene and its chemical structure is identical to a previously reported antifungal compound produced by *S. griseochromogenes* (Cheng *et al*., 1989). Also, tautomycetin (TMC) was reported to possess anti-cancer activities against colorectal and thyroid cancer cells (Lee *et al*., 2006), implying that TMC could be a potentially-valuable immunosuppressive and anti-cancer drug lead compound.

The antimicrobial activities of 15-norlankamycin derivatives were determined by the agar dilution methods against *Micrococcus luteus*. The mixture of 2a and 2b exhibited moderate antimicrobial activity with a MIC value of 4.0 μg mL^{-1}, which was four-fold less than that of lankamycin 1 (MIC value of 1.0 μg mL^{-1}) (Arakawa *et al*., 2011).

Since many antibiotics are able to suppress or retard the growth of tumors, an extensive effort has been made in many laboratories to find new antibiotics with antineoplastic properties either by screening new soil isolates or by chemical modification of the existing antibiotics.

CONCLUSION

Strain AZ-C442 was confirmed to be *S. tanashiensis* based on 16S rRNA sequence analysis and culture characteristics. *S. tanashiensis* AZ-C442 produced a Leteomycin antibiotic. Which showed an inhibitory effects against many bacterial and fungal strains. From this study, we can suggest that the leteomycin biosynthesis in *S. tanashiensis* AZ-C442 might by applied in medical sector as antimicrobial agent as well as in anti-tumor (of its activity is known by previous studies as anti-cancer) after its investigation and evaluation.

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