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LINCOMYCIN ANTIBIOTIC BIOSYNTHESIS PRODUCED BY *STREPTOMYCES* sp. ISOLATED FROM SAUDI ARABIA SOIL II - EXTRACTION, SEPARATION AND PURIFICATION OF LINCOMYCIN

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ABSTRACT

The most potent actinomycete isolates which was previously identified as *Streptomyces* sp. MS-266 Dm4 was selected for the biosynthesis of the active metabolite having biodiversal activities. The active metabolite was extracted by diethyl ether at pH 7.0. The organic phase was collected and evaporated under reduced pressure using a rotary evaporator. The extract was concentrated and treated with petroleum ether (b.p. 60-80ºC) for precipitation process, where only one fraction was obtained in the form of yellowish brown viscous texture. The purification process was performed using both thin layer (TLC) and column chromatography (CC) techniques. The active compound under study was tested for its physicochemical characteristics, where the results revealed that the compound melting point is 155ºC; and soluble in chloroform, n-butanol, methanol, acetone, ethanol, ethyl acetate and isopropyl alcohol but insoluble in petroleum ether, hexane and water. The elemental analysis of the active compound suggested the empirical formula of: (C_{10} H_{20} N_2 O_{16}). The spectroscopic characteristics of active compound revealed the presence of the maximum absorption peak in UV at 269 nm, infrared absorption spectrum represented by nine peaks in addition to Mass- spectrum suggests the molecular weight of the active compound as 447 Dalton. The purified antimicrobial agent was suggestive of being belonging to Lincomycin antibiotic. The Minimum Inhibitory Concentration (MIC) of the antimicrobial agent was also determined which was found to have a bacteriostatic activity.

Keywords: *Streptomyces* sp., purification, lincomycin.

INTRODUCTION

Since almost antibiotics are made by aerobic fermentation processes, a number of similarities in the processes used in their production exist. The general outline of these methods is fairly well known although the industrial concerns producing antibiotics have been reluctant to publish details of their processes. Extraction, Separation, Purification and Identification of the antimicrobial agent which produced by different actinomycetes had been done by many researchers (Enomoto *et al*., 2000; Pandey *et al*., 2004; Ilić *et al*., 2005; Kim *et al*., 2005; Jeong *et al*., 2006; Ahmed, 2007; Xie *et al*., 2007; Igarashi *et al*., 2008; Malik *et al*., 2008).

The (MIC) of the active substance produces by different streptomycete isolates were investigated by many researchers. Pandey *et al*. (2004) estimated the MIC of the active substance produced by *Streptomyces* spp and *Saccharopolyspora* spp against *Staph aureus* and it was 5, 1.25 mg/ml respectively. Mukai *et al*. (2006) studied the MIC of transvalencin Z antibiotic and they estimated the MIC value against gram positive bacteria by less than (4.0mg/ml) and against gram negative bacteria by (0.25mg/ml). Xie *et al*. (2007) found that the MIC of sansanmycin antibiotic against the *Mycobacterium tuberculosis* H37 and *Pseudomonas aeruginosa* was (10.0 and 12.5mg/ml), respectively.

In the course of our continuing search for new antibiotics produced by microorganisms (Abd El- Aziz *et al*., 1997; Ghazal *et al*., 2001, 2002), a culture of streptomycetes which identified as *Streptomyces* sp. MS-266 Dm4 (Ababutain *et al*., 2012) was found to produce an lincomycin antibiotic. This antibiotic exhibited antibacterial and insecticidal activities against gram positive, gram negative bacteria and *Culex pipiens* mosquito. The objective of this study was to separate, purified and identifies the active compounds.

MATERIALS AND METHODS

Microorganism

Streptomyces sp. MS-266 Dm4 (Ababutain *et al*., 2012) isolated from soil sample collected from Dammam governorate, Saudi Arabia was used for antibiotic production.

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Fermentation
The isolate Dm4, was inoculated into yeast extract glucose broth at pH7, and incubated under aerobic conditions using incubator shaker at 200 rpm/ mint at 30°C for three days. One ml of these cultures has been used to inoculate the production medium (Starch-nitrate broth). The flasks were incubated on incubator shaker at 200rpm at 30°C for 7 days. A twenty liter total volume was filtered through Whatman No.1 filter paper and followed by centrifugation at 5000rpm for 20 minutes.

Extraction
The clear filtrate was adjusted at different pH values (4 to 9) and extraction process was carried out using diethyl ether solvent 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 yielding a yellowish brown viscous texture.

Precipitation
The precipitation process of the crude compound was carried out using petroleum ether (b.p 60-80°C) followed by centrifugation at 5000rpm 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 (2.5X50) 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 5ml) and tested for their antimicrobial activities.

Bioautography
It is conducted by preparing nutrient agar medium, seeded by *Bacillus cereus* as a test organism, flooded over the glass sheet of the tray and left to cool under aseptic conditions. The developed whatman No. 1 chromatographic strips containing the antibiotic material and flooded over the agar plate, left for half an hour in a refrigerator for diffusion and then incubated at 37°C for 18 hours (Weinstein and Wagman, 1978) which indicates the purity of the active substance under study.

Physicochemical Properties and Spectroscopic analysis
Physical and chemical properties of the purified active substance such as solubility in organic solvents, behavior towards acids and alkalis and melting point were studied.

1- Elemental analysis: The elemental analysis C, H, O, N, and S was carried out at the micro-analytical 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- Biological activity of the antimicrobial agent: The Minimum Inhibitory Concentration (MIC) has been determined by using the agar plate dilution technique (Betina, 1983).
4- Characterization of the antimicrobial agent: The antibiotic produced by *Streptomyces* sp. MS-266 Dm4 was identified according to the recommended international references of (Umezawa, 1977; Berdy 1980 a, b, c).

RESULTS

Fermentation, Extraction and Purification
The fermentation process was carried out for seven days at 30°C using liquid starch nitrate as production medium. Twenty-liter total volume filtered was conducted followed by centrifugation at 5000rpm for 20 minutes. The clear filtrates containing the active metabolite (20liters) was adjusted to pH 7.0 then extraction was carried out using diethyl ether at the level of 1:1 (v/v). The organic phase was collected and evaporated under reduced pressure using rotary evaporator. The extract was concentrated and treated with petroleum ether (b.p. 60-80°C) for precipitation process, where only one fraction was obtained in the form of yellowish brown viscous syrup. The purification process was carried out through a column chromatography packed with silica gel, where one definite inhibition zone was detected using *B. cereus* as a test organism, indicating that the metabolite under study is composed of one compound (Table 1, Fig. 1).

Table 1. Bioautographic mobility of the antimicrobial agent produced by *Streptomyces* sp. MS-266 Dm_4.

No.	Developing solvent	R_f
1	Diethyl ether	0.95
2	Ethyl acetate	0.85
3	Chloroform / Ethyl acetate (1:1)	0.85
4	Alkaline chloroform	0.84
5	Chloroform	0.80
6	Acetone	0.77
7	Acidic chloroform	0.75
8	Ethyl alcohol	0.69
9	Methyl alcohol	0.60
10	Alkaline diethyl ether	0.60
11	Acidic diethyl ether	0.50
12	Petroleum ether	0.0
13	n – Hexane	0.0
14	Water	0.0

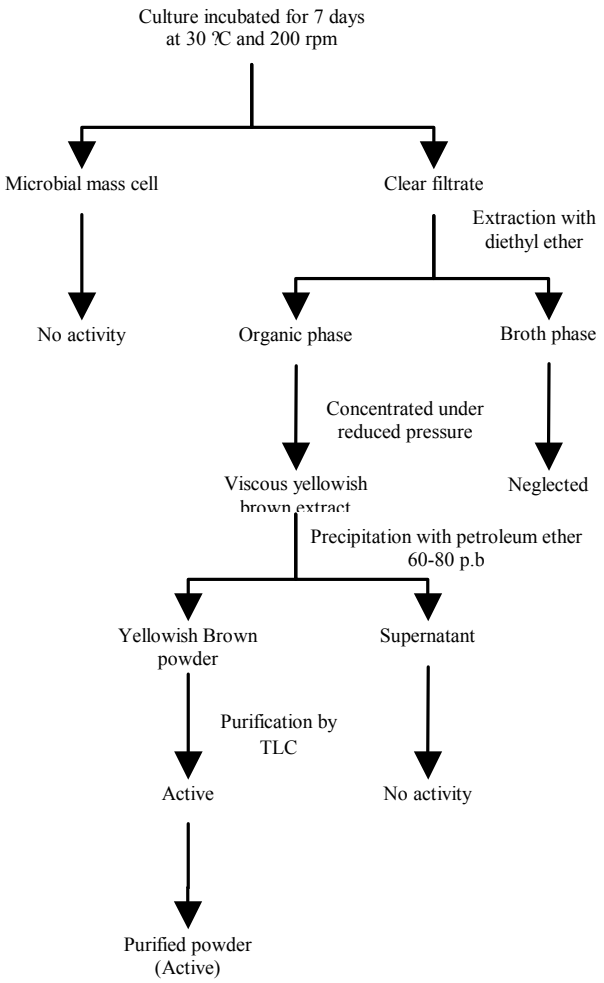


Fig. 1. Dichotomous scheme for production and purification of the antimicrobial agent produced by *Streptomyces* sp. MS-266 Dm_4.

Physicochemical Characteristics

The purified antimicrobial agent produced by *Streptomyces* sp. MS-266 Dm_4 are produces characteristic odor, their melting points are 155^{o}C. The compound is freely soluble in chloroform, ethyl acetate, acetone, ethyl alcohol, diethyl ether but insoluble in petroleum ether, n-hexane and water.

Elemental Analysis

The elemental analytical data of the antimicrobial agent produced by *Streptomyces* sp. MS-266 Dm_4 showed the following: C=29.46; H=4.42; N=3.86; and O=60.76, from which the empirical formula is calculated to be: $C_{10}H_{20}N_2O_{16}$.

Spectroscopic Characteristics

Mass spectrometry analysis of the active substance (Mass spectrum), gave an account for the molecular weight (447) Dalton (Fig. 2). The ultra violet absorption spectrum of the active substance exhibits maximum absorption band at 269 nm (Fig. 3). The infrared absorption spectrum of the active substance gave nine values of absorption (3414.4, 2924.5, 2858.0, 2355.6, 1742.4, 1634.4, 1451.2, 1028.8 and 607.5) nm (Fig. 4). H-Nuclear Magnetic Resonance (H-NMR) illustrated in (Fig. 5).

Identification of the purified active antimicrobial agent

The study concluded that the active substance contains the effective hard-core, which is a pyrrolidine with a percentage of similarity with lincomycin antibiotic (Fig. 6).

Screening for the Antimicrobial Activities

The active metabolite produced by actinomycete culture, *Streptomyces* sp. MS-266 Dm_4 exhibited various degrees of activities against gram positive and gram negative bacteria. The MIC was found to be 31.25µg/ml against the tested microorganisms, except for *E. coli* was found to be15.62µg/ml, (Table 2). The effect of the active substance on the organisms tested was bacteriostatic.

Table. 2. Minimum Inhibitory Concentration (MIC) of *Streptomyces* sp. MS-266 Dm_4 against test microorganisms

Test organisms	MIC (µg/ml) concentration
Bacillus cereus ATCC 14579	31.25
Bacillus subtilis ATCC 6633	31.25
Staphylococcus aureus ATCC6538P	31.25
E. coli ATCC 7839	15.62
Pseudomonas aeruginosa ATCC9027	31.25
Candida albicans ATCC 10231	31.25

DISCUSSION

The most potent actinomycete isolates which was identified as *Streptomyces* sp. MS-266 Dm4 (Ababutain *et al*., 2012) was selected for the biosynthesis of the active metabolite having biodiversal activities. For this reason *Streptomyces* sp. MS-266 Dm4 was inoculated in nutrient broth media under favorable environmental and nutritional conditions. At the end of the incubation time, the active metabolite was extracted by diethyl ether at pH 7.0. The organic phase was collected and evaporated under reduced pressure using a rotary evaporator. The extract was concentrated and treated with petroleum ether

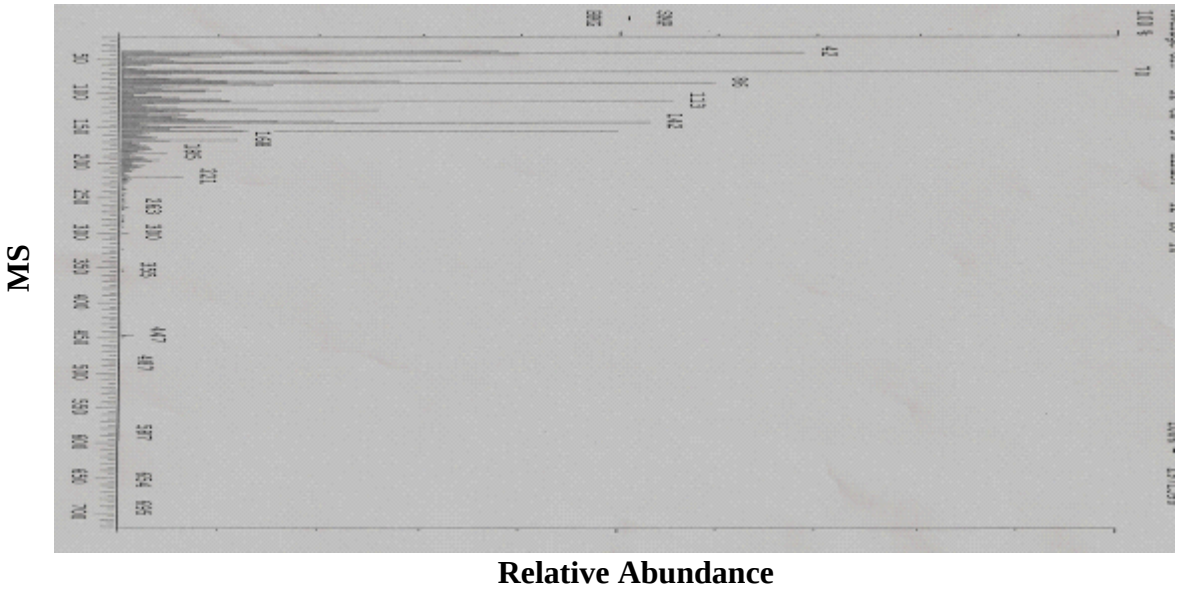


Fig. 2. Mass – spectrum of the purified active substance produced by *Streptomyces* sp. MS-266 Dm_4

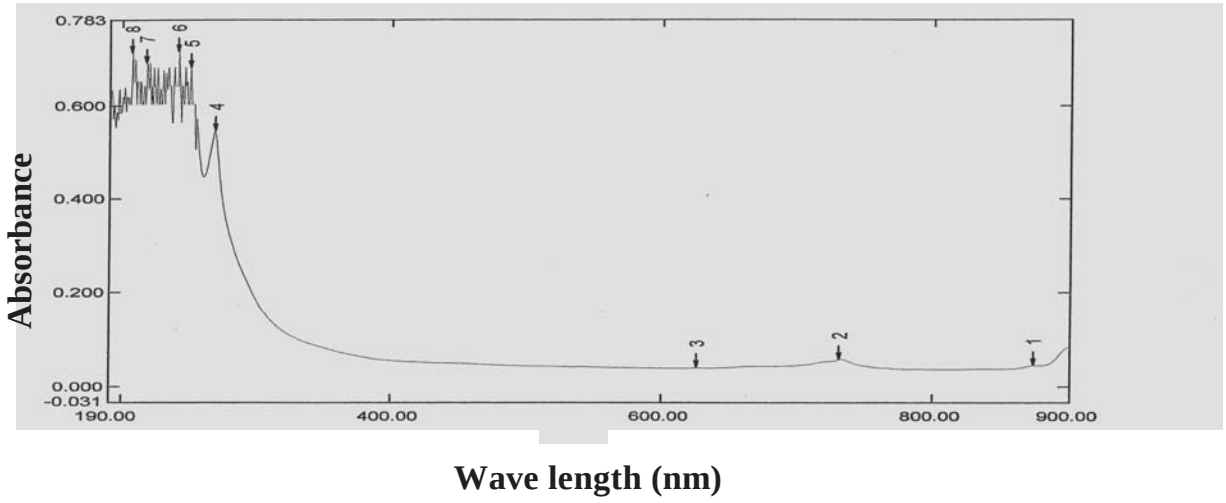


Fig. 3. The UV- spectrum of the purified active substance produced by *Streptomyces* sp. MS-266 Dm_4

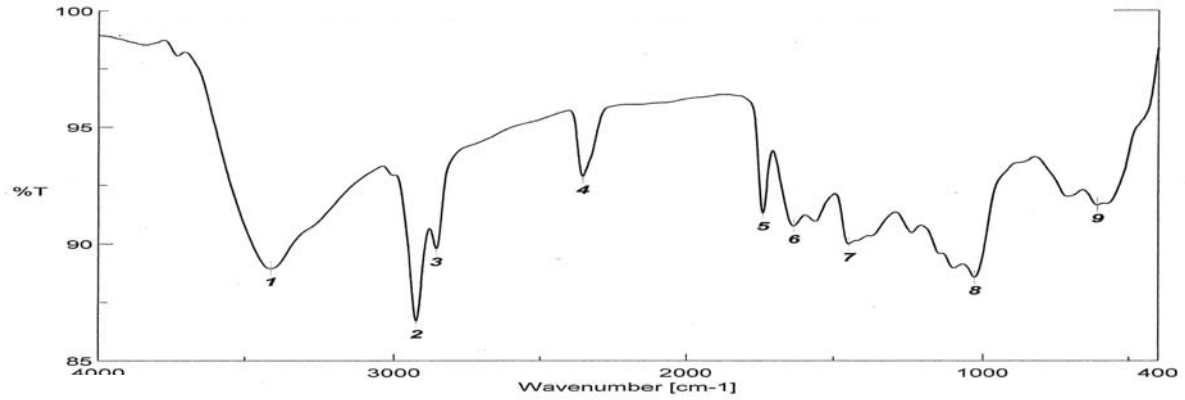


Fig. 4. IR – spectrum bands of the purified active substance produced by *Streptomyces* sp. MS-266 Dm_4

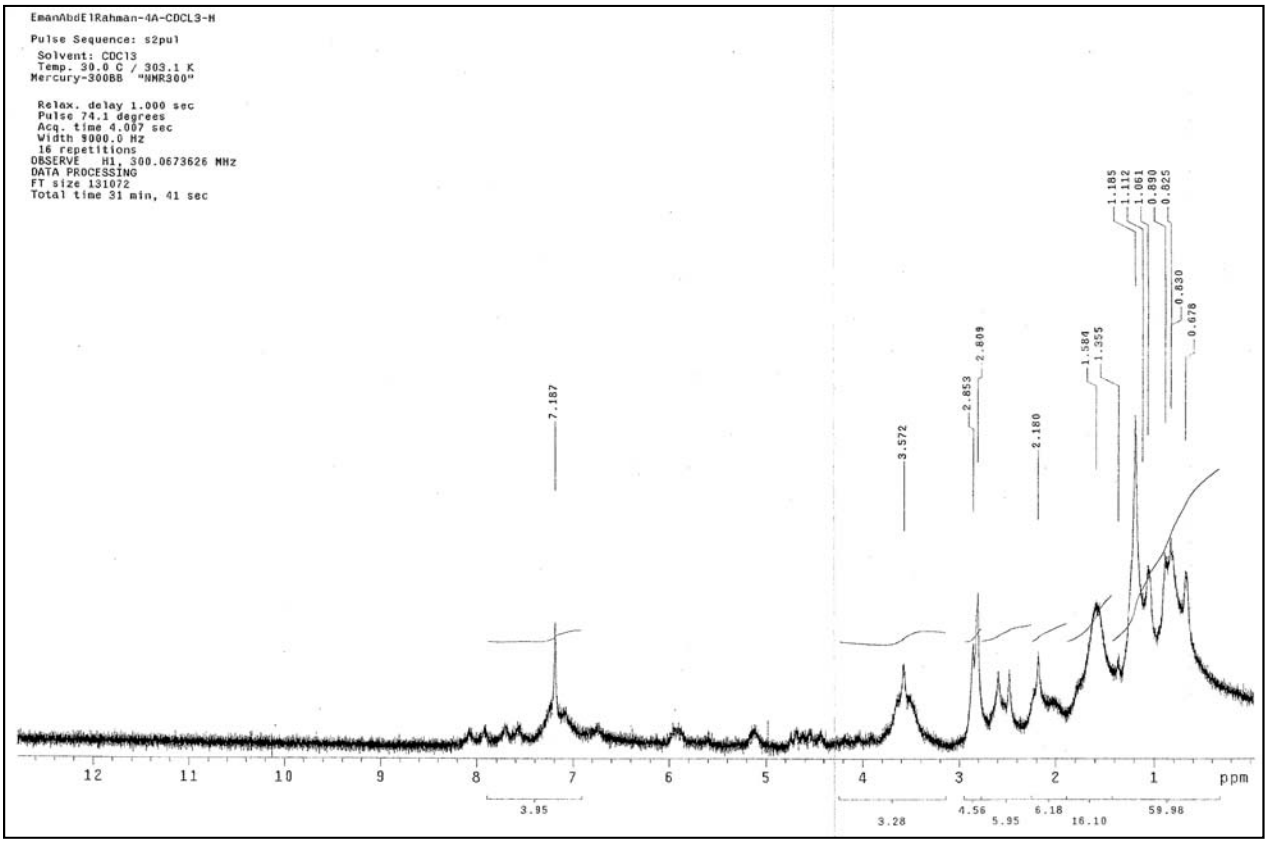
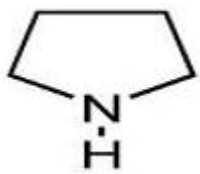


Fig. 5. H. NMR – spectrum peaks of the purified active substance produced by *Streptomyces* sp. MS-266 Dm_4

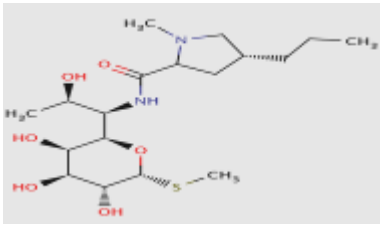
(b.p. 60-80^{o}C) for precipitation process, where only one fraction was obtained in the form of yellowish brown viscous texture. The purification process was carried out through a column chromatography packed with silica gel, where one definite inhibition zone was detected using *B. cereus* as a test organism, indicating that the metabolite under study is in pure form. The active compound under study was tested for its physical and chemical characteristics, where the results revealed that the compound melting point is 155^{o}C and soluble in chloroform, n-butanol, methanol, acetone, ethanol, ethyl acetate and isopropyl alcohol but insoluble in petroleum ether, n- hexane and water.

The elemental analysis of the active compound revealed the detection of the following elements (%): C, (29.46); H, (4.42); N, (3.86) and O, (60.76) which give the empirical formula of: (C10 H20 N2 O16). The spectroscopic characteristics of active compound revealed the presence of the maximum absorption peak in UV at 269 nm, infrared absorption spectrum represented by nine peaks in addition to Mass- spectrum suggests the molecular weight of the active compound as 447 Dalton. In addition, H-Nuclear Magnetic Resonance was determined (Pandey *et al*., 2004; Ilic` *et al*., 2005; Jeong *et al*., 2006; Ahmed, 2007; Xie *et al*., 2007).

On the basis of comparative study of the recorded chemical composition and physical properties of the active substance produced by *Streptomyces* sp. MS-266 Dm4 and by consulting the recommended identification keys of antibiotics such as (Umezawa, 1977; Berdy, 1980 a, b, c) it could be stated that the compound contains an effective hard-core Pyrrolidine and have high similarity with Lincomycin antibiotic. The obtained active substance was investigated for (MIC) by using various microbial test organisms; it was found that the active substance has antimicrobial activity against gram positive, gram negative bacteria this result agree with several researchers (Pandey *et al*., 2004; Mukai *et al*., 2006; Xie *et al*., 2007).



Pyrrolidine



Lincomycin

Fig. 6. The identification of the purified active antimicrobial agent produced by *Streptomyces* sp. MS-266 Dm_4

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