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Characterization of marine *Streptomyces* spp. bacterial isolates from Tigris river sediments in Baghdad city with Lc-ms and 1 HNMR

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Abstract

The aim of this study is to characterize bioactive compounds from different *Streptomyces* spp. isolated from previous study from Tigris river sediment soil samples, the analysis done by HPLCs through using the crude extract compared to standards, which indicates the presence of four active compounds (Tetracycline, Clindamycin, Chloramphenicol and Rifampicin). While NMR data analysis performed by using the intracellular (biomass) extract due to more antagonism efficiency than that observed in extracellular crude extract, which revealed the presence of a clear peak for benzene ring at 7.24 ppm in sample N⁻, D⁻ and M5. Most peaks between 1.56-1.58ppm in sample D related to the first Alkyle group CH₃, while peaks between 1.68-1.89ppm are related to Alkane R₃CH, RCH₂R, RCH₃ appeared in sample 21 and M5. Alkyle double bond or nitrogen or oxygen binding between peak 4.99-5.01ppm found in sample M5, D and 21. Sample N⁻ showed signals of Vinylic which represented in signals between 4.78-5.17 ppm.

Keywords: Tigris river; actinomycetes; bioactive compounds; Lc-ms; NMR

Introduction

The biochemistry of Streptomycetes is highly notable, they produce half of all known antibiotics. The secondary metabolites of Streptomycetes have benefits in human medicine, may work as antibacterial, antifungal, antiparasitic and antitumor, also in agriculture as plant protection agents, growth promoters and herbicides. [1, 2, 21, 23] Antibiotic production of cultured *Streptomyces* on agar is usually corresponds with the early stages of morphological differentiation. [2, 3] The Actinomycetes have high ability to produce antibiotics. The metabolic variety of the Actinomycetes is due to encoding hundreds of transcriptional factors in their genome permitting them to control gene expression in response to specific needs. They are widely distributed in nature, living in cold and tropical areas, in addition to their ability to live in a very hard condition like that in desert. *Streptomyces* strains are also found to occur in fresh water and marine environment. [4, 5, 6] Techniques like high performance liquid chromatography used to analyze and characterize the active compounds from *Streptomyces* strains. [7] It is an equipment that used to identify components in a mixture like hydrocarbons, essential oils and solvents. The electron capture detector and a flame ionization detector can quantitatively determine the materials even very low concentrations. It is widely used especially in biochemistry because of its simplicity, sensitivity, and effectiveness in separating components of mixtures quantitatively and qualitatively to fix thermo chemical standards as heats of solution and vaporization, vapor pressure, activity coefficients and for purification of compounds [8, 9]. In addition to Nuclear magnetic resonance spectroscopy (NMR) or called magnetic resonance spectroscopy (MRS), it is an analytical chemistry used for determining the content, purity and molecular structure of a sample. By NMR, the mixtures containing known compounds analyzed quantitatively, determining the molecular conformation in solution, while the unknown compounds detected through matching against spectral libraries or by estimating the basic structure directly. The basis of NMR is that nuclei have spin and electrically charged. The signal is produced by the nuclei excitation with radio waves (the radio waves is the source of energy in NMR, which have more than 10⁷ nm long wavelengths, so low energy is produced by interaction of low wavelengths with a molecule) into NMR, then a sensitive radio receiver detects the signal. External magnetic field is applied to the sample, then the energy transfer from base energy to higher energy forming single energy gap. [10, 11, 12] The increasing in the antibiotic resistant pathogens and the pharmacological study limitation in antibiotics is an exigency to find a new antibiotic. [20] Therefore, the present study aimed to explore the bioactive compounds by NMR and HPLCs.

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Materials and methods

Sample collection

Samples were collected from a previous study, [13] about 4 *Streptomyces* spp. were selected from 42 isolates by primary and secondary screening. These optimum condition for these isolates was ISP2 broth, pH: 7.8, Temperature 30°C and best NaCl tolerance was 1%. The Intracellular extract was chosen for Bioactive compounds characterization because their antagonism activity and zone of inhibition were more perfect than that with extracellular extract.

Extraction and characterization of antimicrobial metabolites

a- Intracellular extract

The separation of the intracellular crude from the extracellular crude were done by centrifugation. The bacterial pellets in the tube contained intracellular antimicrobial metabolites. The intracellular antimicrobial activities were determined by agar well diffusion as follows: the pelleted cells were re-suspended in the test tube containing lysis buffer 1ml TE buffer Tris 200 ml and 50 ml EDTA, 60 µl of 10% SDS and 6 µl of proteinase K, with a gentle shaking, the mixture incubated at 37°C for 60 minutes.

The intracellular metabolites liberated after bacterial cell walls disruption. Six hundred µl of the intracellular crude metabolites was taken and mixed with 600 µl of methanol. The mixture was gently mixed and left for 60 minutes. Then the tubes were spun at 1000rpm for 10 minutes at room temperature. The mixture was separated into two phases, the upper phase methanolic phase containing dissolved metabolites, was collected and transferred to the sterilized petri dish, then kept in a hot air oven 45°C for 24 hours to dry the dissolved intracellular crude extract. Finally, the dried intracellular crude extracts were dissolved in double volume of sterilized distal water 1200 µl. Agar well diffusion procedures as mentioned previously were used to determine the antimicrobial activity against tested microbial pathogens. [14]

b- Preparation of Standards and Sample for HPLC Analysis

Standard Preparation

A weight of 10mg of standards (Ceftriaxone, Chloramphenicol, Rifampicin, Tetracycline and Clindamycin) were dissolved in 50ml of distilled water to get 200ppm which was further diluted by dissolving 1ml of this solution in 50ml distilled water. [15]

Sample Preparation

A volume of 10 µl from each sample (21, M5, N⁻ and D⁻) were dissolved in 1000 µl of deionized water. Using 20 µl of this solution to injection. [15]

High-Performance Liquid Chromatography (HPLC) Analysis for extracellular extract of *Streptomyces*

A volume of 20 µl of the standard and 20 µl of the sample were injected to HPLC and record the chromatogram, calculated the content of the sample in comparison with standard. The concentrations were calculated according to the following equation: Concentration = [area of sample/area of standard] × sample concentration × dilution factor. [16]

Analytical and semi-preparative HPLC was carried out using Waters system, controlled by Peak simple software, consisting of a Waters 515 HPLC pump in combination with a Waters 2487 dual absorbance detector. Pursuit XFs 3µC-18, 250 X 4.6 mm (analytical) and 250 X 10 mm (semi-preparative) columns were used for HPLC separation of semi

pure compounds. All the solvents used were HPLC grade. Deionized water was used for preparation of solvent systems for best separation of compounds.

All solvents were degassed prior to use. Mobile phase was run using different constitutions of acetonitrile: water solvent system 50:50, to get best possible separation in analytical HPLC before moving on to semi-preparative HPLC for collection of compounds. The temperature 35C, wavelength 280n, with flow rate 1ml\min.

c- Nuclear magnetic resonance spectroscopy (NMR)

Nuclear magnetic resonance spectroscopy (NMR) 1D and 2D NMR spectra were recorded 60 MHz (Varian) spectrometer using. Chemical shifts were expressed in δ (ppm) and coupling constants (J) are reported in hertz (Hz).

Nuclear magnetic resonance spectroscopy sample data set

The sample data set was acquired on Nanalysis 60 MHz NMR spectrometer. The compounds were dissolved in DMSO-d₆ and transferred into a 5 mm NMR tube. The residual proton signal from the DMSO-d₆ was referenced to ν 2.5 ppm. All samples were acquired with 16 transients, 1 s relaxation time, 12 ppm sweep width and an acquisition time of 0.3 s.

Partially purified extracts

The data were acquired on a Nanalysis 60 MHz NMR spectrometer using VAST. The partially purified samples were dissolved in 2% H₂O–DMSO-h₆ (protonated) and were placed in a 96-well microtiter plate (200 µl). The extracts (100 µl) were injected into the probe using a Gilson 215 liquid handler with a pusher liquid of 2% H₂O–DMSO-h₆ (320 µl). The spectra were referenced to the singlet from DMSO-h₆ at ν 2.6 ppm and the transmitter offset was set on resonance of the DMSO-h₆ and with a sweep width of 30 ppm. [17]

Results and discussion

The *Streptomyces* isolates (D⁻, N⁻, M5, 21), had been selected to characterize their bioactive compounds by HPLCs by using the crude extract, and by GC and NMR using the intracellular (biomass) extraction due to more antagonism efficiency than that observed in extracellular crude. This disagree with a study done by Al-rubaye, whos found that the supernatant had more antagonism against pathogenes. [18]

Purification of Intracellular Crude Extract from *Streptomyces* isolates

Semi purified intracellular crude extract was analyzed to determine the bioactive compounds through using the GC-Mass and NMR. While the crude was analyzed by High Performance Liquid chromatography (HPLC) through comparing the unknown sample with standard antibiotic.

High Performance Liquid Chromatography (HPLC)

As known the *Streptomyces* produces many antimicrobial compounds, which detected by the antagonism reaction against pathogenic microorganisms. [24] HPLC is very important tool which used to detect the concentration of the active compounds being produced by *Streptomyces*. For identification of bioactive compound (antimicrobials) in crude of four isolates (D⁻, N⁻, M5, 21), the unknown compounds were analyzed against the standard antibiotics (Tetracycline, Clindamycin, Chloramphenicol, Ceftriaxone, Rifampicin). HPLC columns were used with flow rate 1 ml/min and injection volume 20µl. Peaks of tested four samples present in

the same retention time in comparing with a standard but with different area. Figure (1a, b, c, d, e) revealed the HPLC of different peaks of standard antibiotic.

Peaks of sample D (figure 2) were observed at retention time of 3.340, 3.498, 6.698 minutes which indicate Chloramphenicol, Tetracyclin and Rifampicin respectively.

Peaks of sample M5 (figure 3) were observed at retention time of 3.432, and 4.182 minutes which indicate Tetracyclin and Clindamycin respectively.

Sample 21 (figure 4) shows no peak in compare with standard antibiotics, their antibacterial activity of this sample may be due to presence of bioactive compounds other than antibiotic which explained by GC-MS and NMR.

Peak of sample N (figure 5) was observed at retention time of 3.507 minutes which indicated Tetracyclin.

HPLC analysis of the crude of *Streptomyces* indicate the presence four active compounds (Tetracycline, Clindamycin, Chloramphenicol and Rifampicin). The R_f of the bioactive compound crude determined by the response factor of standard antibiotic (R_f). Table (1) showed the response factor, area for the standard antibiotic and the antibiotic concentration which produced from *Streptomyces* spp. This investigation could explain the ability of the *Streptomyces* spp. to produce antibiotics, also antimicrobial activity from different isolates can be evaluated by HPLC. Antibiotic production of four isolates, it might be cited that *Streptomyces* potential in antibacterial production could possibly meet the demand and need for antibacterial to use through industries. Using thin HPLC have been described by Awais *et al.*, (2007) to analyze and characterize antimicrobial compounds. [19]

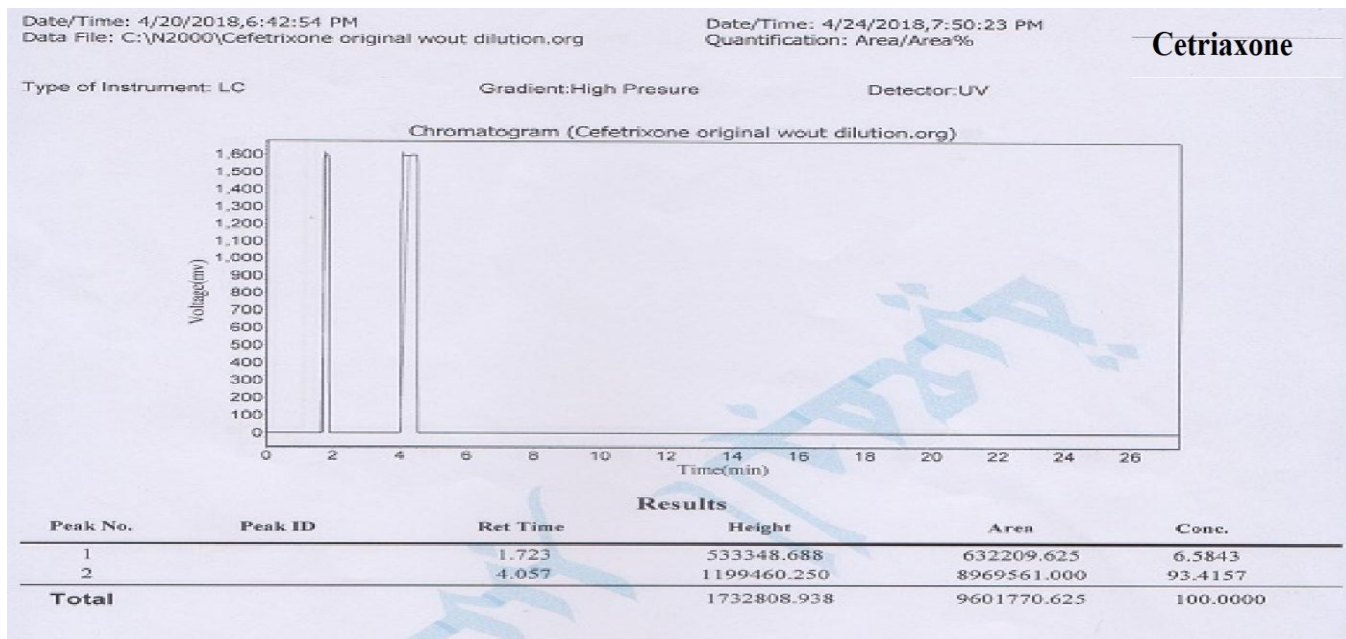


Fig (1a): High Performance Liquid Chromatography of standard antibiotic (Ceftriaxone)

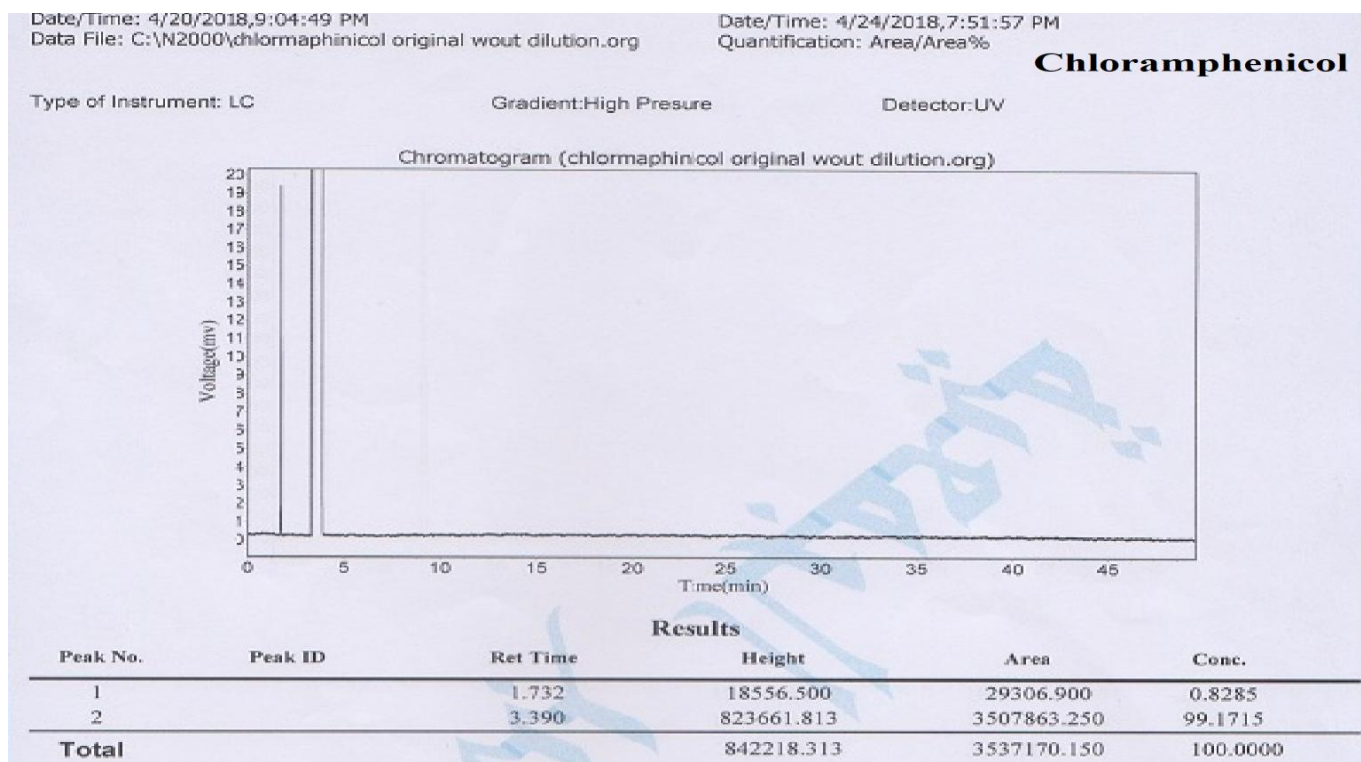


Fig (1b): High Performance Liquid Chromatography of standard antibiotic (Chloramphenicol)

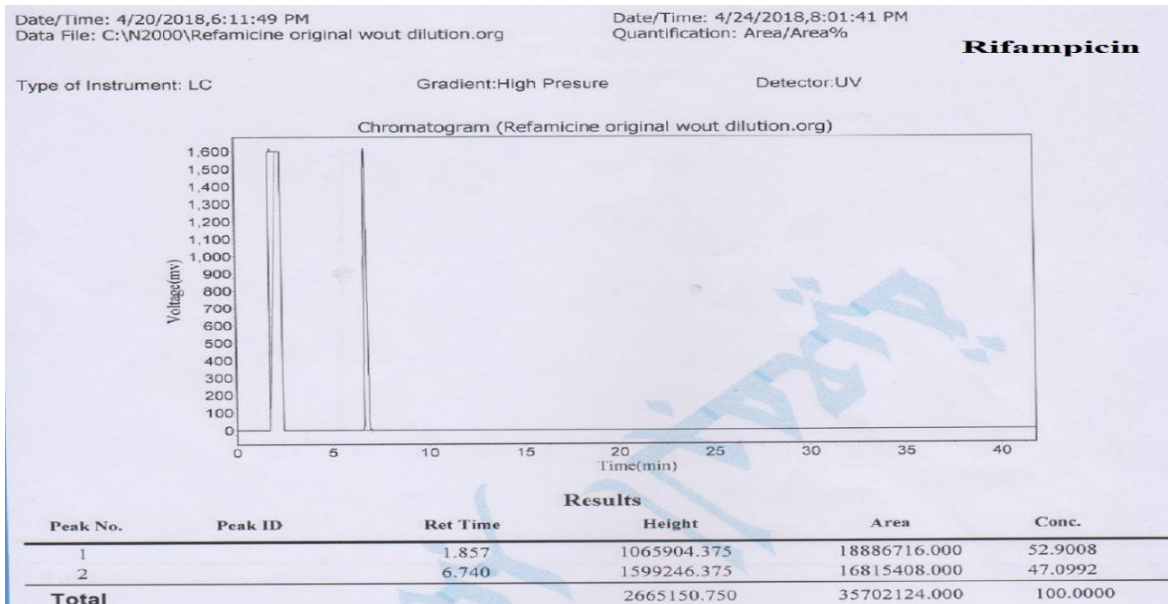


Fig (1c): High Performance Liquid Chromatography of standard antibiotic (Rifampicin)

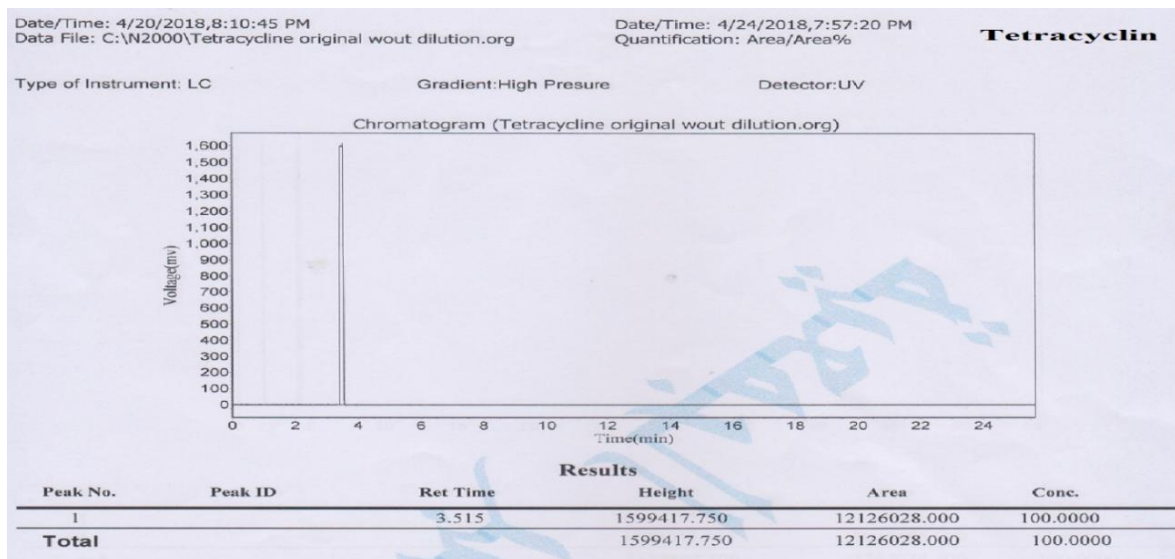


Fig (1d): High Performance Liquid Chromatography of standard antibiotic (Tetracycline)

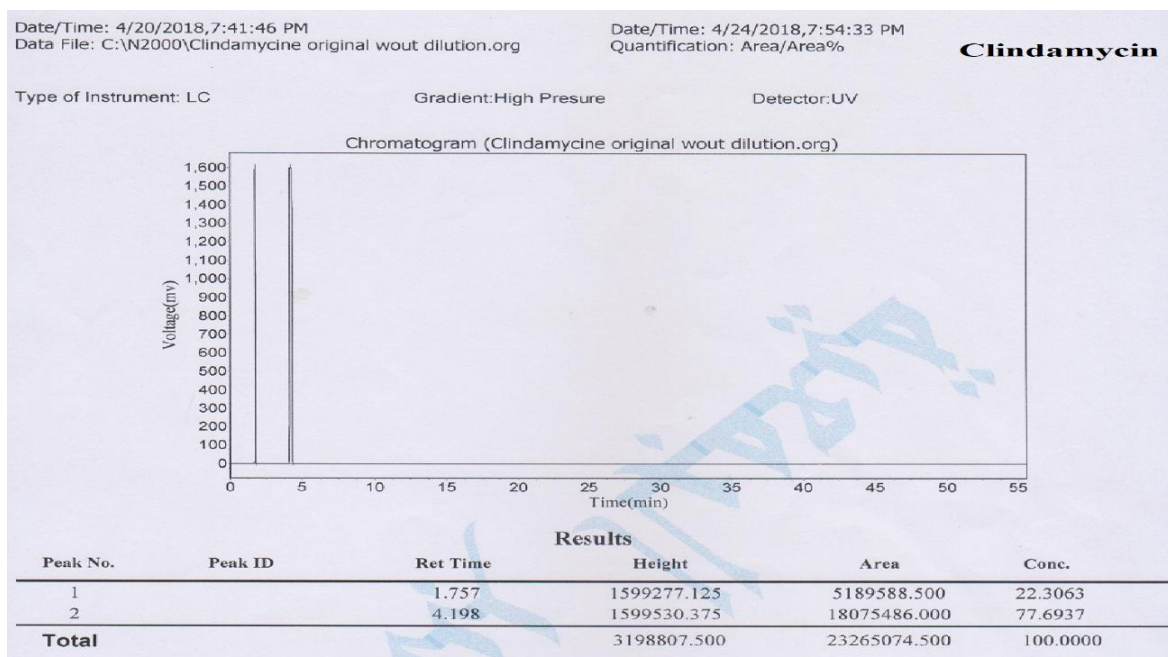
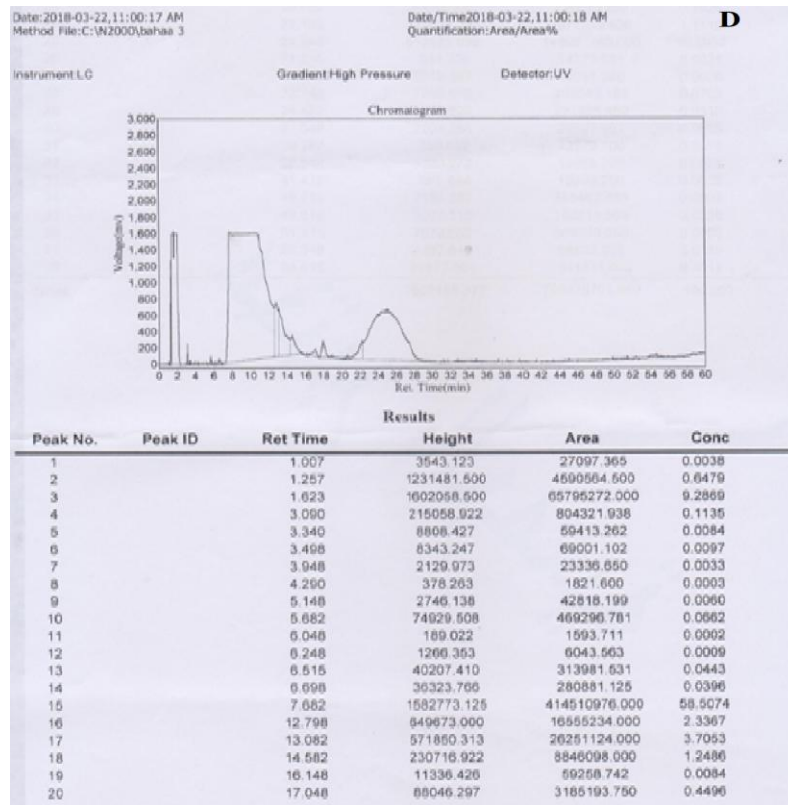
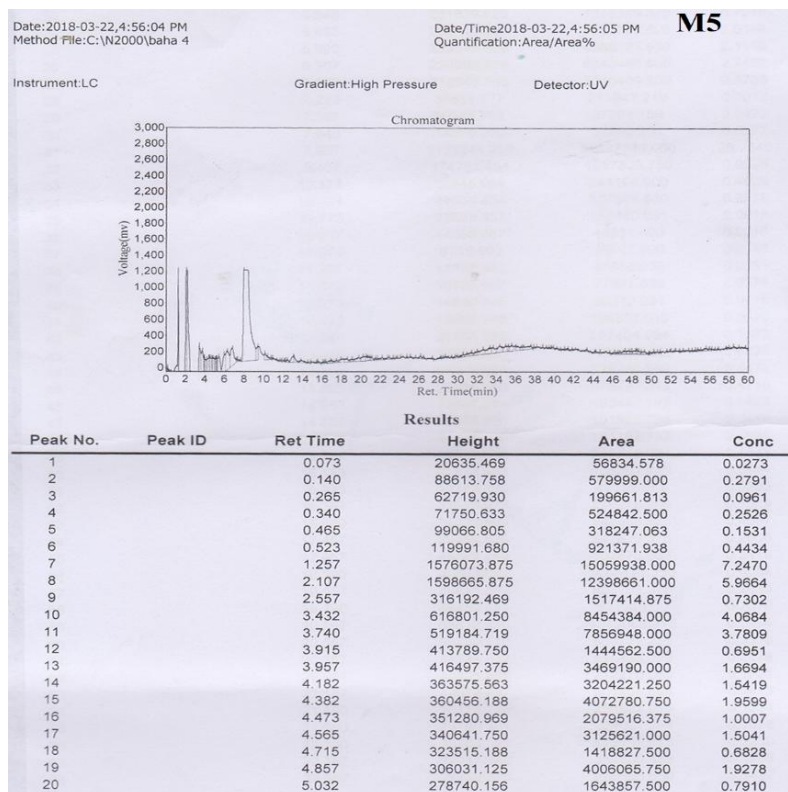


Fig (1e): High Performance Liquid Chromatography of standard antibiotic (Clindamycin).

Table 1: R_f values and area of each standard antibiotic in correlation to the concentration of each antibiotic in *Streptomyces* spp.

No	Subject	RT\ minute	Area	Concentration µg/ml	sample
1	Tetracycline	3.515	12126028	1.5169	N
				4.0684	M5
				0.0097	D
2	Rifampicin	6.740	16815408	0.0396	D
3	Chloramphenicol	3.390	3507863	0.0084	D
4	Clindamycin	4.198	18075486	1.5419	M5
5	Ceftriaxone	4.057	8969561	-	-

**Fig (2):** High Performance Liquid Chromatography for crude of *Streptomyces* sample D.**Fig (3):** High Performance Liquid Chromatography for crude of *Streptomyces* sample M5

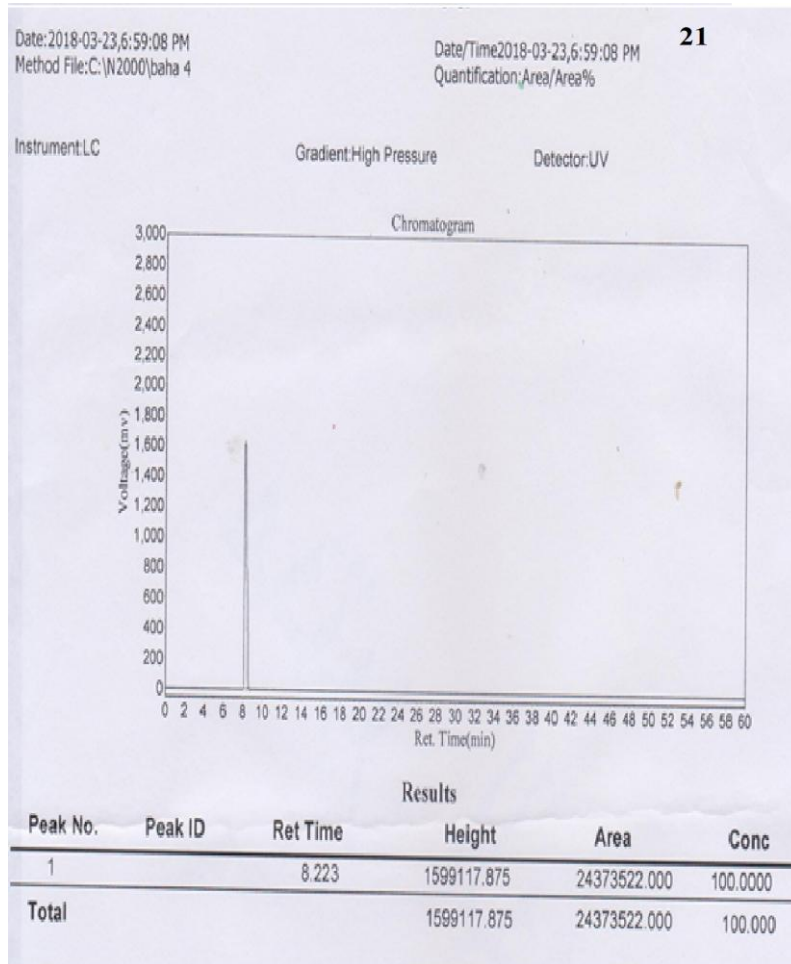


Fig (4): High Performance Liquid Chromatography for crude of *Streptomyces* sample 21

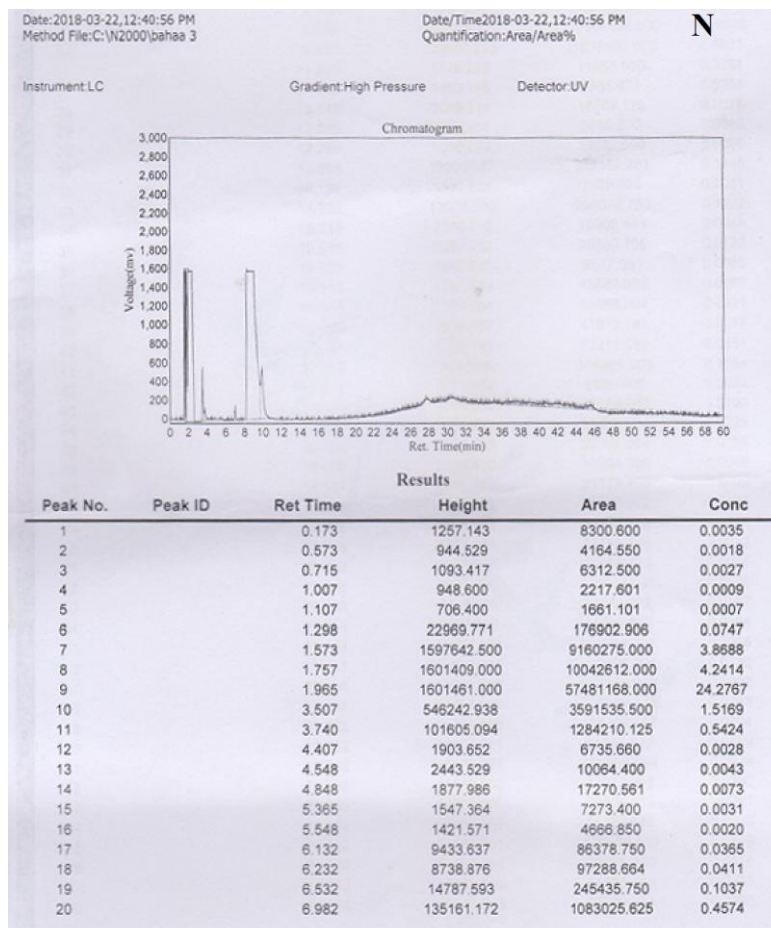


Fig (5): High Performance Liquid Chromatography for crude of *Streptomyces* sample N-

Nuclear Magnetic Resonance Spectroscopy

A sample data set was used to find a procedure that needs to recognize the compound at different concentrations and mixtures of compounds. The compounds were easily interpretable, according to the standard. Nuclear Magnetic Resonance Spectroscopy data of sample N: ^1H NMR spectrum showed signals of Vinylic which represented in signals between 4.78 -5.17 ppm which has a molecular formula $\text{C}_8\text{H}_{14}\text{O}_2$ with no NIST matching value with antimicrobial activities, but an important compounds in this sample which give a clear benzene signal is related to benzene at 7.24 ppm, which represented in more valuable compound with high antibacterial activity and appeared in two compounds like 1,2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester; 1, 2-Benzenedicarboxylic acid bis(2-ethylhexyl) phthalate; 1,2-Benzenedicarboxylic acid, diisooctyl ester (figure 6). Nuclear Magnetic Resonance Spectroscopy of sample M5: As appeared in figure (7), most peaks between 1.68-1.89ppm, they related to Alkane R_3CH , RCH_2R , RCH_3 , in addition to Alkyle double bond or nitrogen or oxygen binding at peak 5.01ppm. Clear benzene peak at 7.23 ppm, which may refer to vitamin E with a molecular formula $\text{C}_{29}\text{H}_{50}\text{O}_2$. Nuclear Magnetic Resonance Spectroscopy of sample 21: As appeared in figure (8), most peaks between 1.68-1.89ppm, they related to Alkane R_3CH , RCH_2R , RCH_3 , in addition to Alkyle double bond or nitrogen or oxygen binding at peak 5.01ppm. Nuclear Magnetic Resonance Spectroscopy of sample D: As appeared in figure (9), most peaks between 1.56-1.58ppm, they related to first Alkyle group CH_3 , which appear as a basic unit in Azetidine, 1- (1,1-dimethylethyl)-3-methyl-, in addition to Alkyle double bond or nitrogen or oxygen binding at peak 4.99ppm. In addition to a clear peak for benzene at 7.24 ppm, which appear as aromatic ether in Triadimenol.

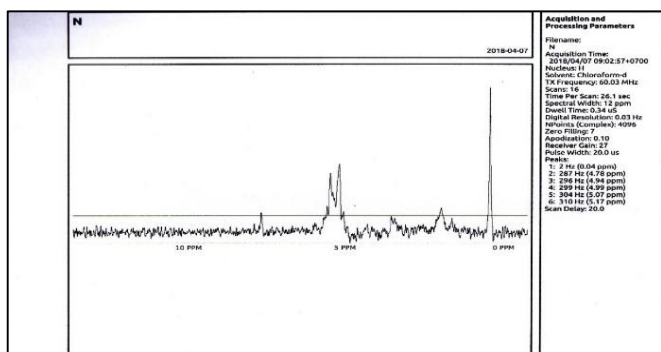


Fig (6): ^1H NMR spectrum of *Streptomyces* spp. intracellular extract, sample N.

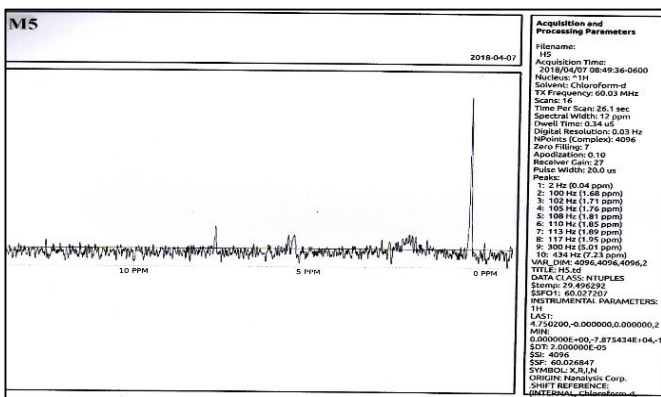


Fig (7): ^1H NMR spectrum of *Streptomyces* spp. intracellular extract, sample M5

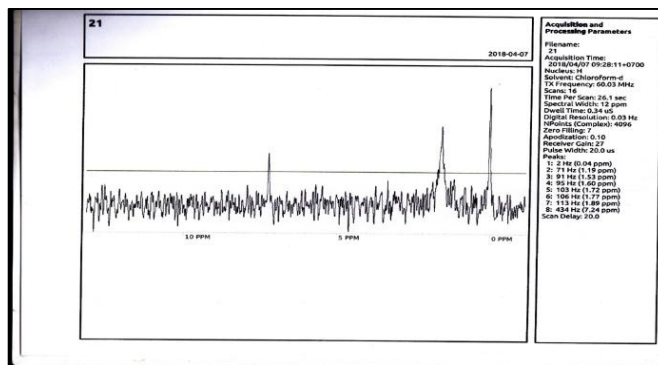


Fig (8): ^1H NMR spectrum of *Streptomyces* spp. intracellular extract, sample 21.

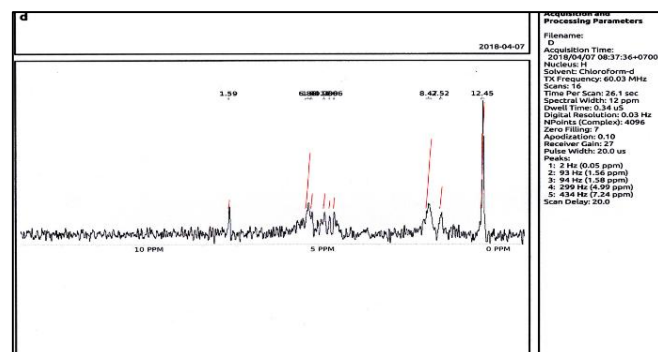


Fig (9): ^1H NMR spectrum of *Streptomyces* spp. intracellular extract, sample D.

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