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Characterization of antibacterial agent produced by *Streptomyces* spp. isolated from soil samples

توصيف العامل المضاد للبكتريا المنتج بواسطة الستربتومايسس المعزولة من عينات تربة

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Abstract:

Thirty soil samples were collected from Hilla City. Twelve actinomycetes spp. were isolated. Five isolates of *Streptomyces* spp. were detected according to morphological and biochemical testes. Antibacterial activity of these isolates were tested against *S.aureus* .*Streptomyces* spp.4 was most active against *S.aureus*. *Streptomyces* spp.4 was selected for isolation of antibacterial agent. Antibacterial agent was active against *S.aureus* and *E.coli* with inhibition zone(22,14)mm subsequently. Characterization of antibacterial agent was studied. Antibacterial agent was brown in color and having melting point equals to 110 ^{O}C. Thin layer chromatography (TLC) for antibacterial agent showed single spot for agent had RF equals to 0.88. Spectroscopic analysis were made. UV spectrum for antibacterial agent showed that single peak with maximum absorption(λ max) at 289 nm. IR (KBr) (v-, cm-1) spectrum for agent had a peak about 3400 cm-1 which indicate presence of OH group absorption, and a peak appearing at 1635 cm-1 that indicated the presence Carbonyl group(C=O). The peak at 1410 and 1450 indicates to presence of CH3 group. Anther peak at 1076 indicated to presence of carbonyl (C=O) fuction of aster or an amide group. Proton magnetic resonance spectrum (NMR) of antibacterial agent indicated to presence a sharp singlet at δ4 may attributed to OH group. Another singlet at δ1.2 indicated to presence of peripheral CH3 group. Also Singlet at δ2.4 may be assigned to presence of CH2 group surrounded by carbonyl bond (C=O).

Key words: *Streptomyces* spp. antibacterial agent, Characterization

الخلاصة:

جمعت ثلاثون عينة تربة من مدينة الحلة.عزلت اثنتا عشر عزلة من الاكتينومايستات.اكتشفت خمسة عزلات من الستربتومايسس حسب الصفات الشكلية والبايوكيمياوية.فحصت الفعالية المضادة البكتيرية للعزلات ضد البكتريا العنقودية.كانت العزلة الستربتومايسس 4 اكثر فعالية .اختيرت العزلة 4 لعزل العامل المضاد البكتيري. لوحظ ان العامل المضاد البكتيري فعال ضد البكتريا العنقودية وبكتريا الاشيريشيا القولنية مع قطر تثبيط (22,14)بالتتابع.درست خصائص العامل المضاد حيث ان العامل المضاد بني اللون ويمتلك درجة انصهار 110 سيلزية. اظهرت تقنية الطبقة الرقيقة وجود بقعة واحدة للعامل المضاد يمتلك قيمة Rf مساوية الى 0.88. اظهر طيف امتصاص الاشعة فوق البنفسجية للعامل المضاد اقصى امتصاص مساوي الى 289 نانوميتر. اظهر طيف امتصاص الاشعة تحت الحمراء للعامل المضاد وجود قمة في 3400 سم $^{-1}$ والتي تشير الى وجود مجموعة هيدروكسيل(OH) ووجود قمة الى 1635 سم $^{-1}$ التي تشير الى وجود مجموعة كاربونيل(C=O) والقمة الى 1410 و 1450 تشير الى وجود مجموعة مثيل (CH_3) وقمة اخرى الى 1067 تشير الى وجود مجموعة (C=O) كمجموعة استر او امايد . اظهر طيف الرنين المغناطيسي النووي للعامل المضاد وجود قمة حادة مفردة الى δ4 والتي تشير الى وجود مجوعة هايدروكسيل (OH). وجود قمة أخرى إلى δ1.2 والتي تشير الى وجود مجموعة مثيل (CH_3) وايضا وجود δ2.4 يشير الى وجود مجموعة (CH2) محاطة بواسطة اصرة كاربونيلية.

Introduction :

Actinomycetes are dominant group of soil population together with bacteria and fungi and are originally considered as an intermediate group between bacteria and fungi. They are free living saprophytic bacteria and a major source for production of antibiotics and its play a major role in recycling of organic matter production of novel pharmaceuticals, nutritional materials, enzymes, antitumor agents, enzyme inhibitors, immune-modifiers and vitamins [1]. Around 80% of the total antibiotic production has been obtained from *Streptomces* [2].

Sreptomyces are the most economically valuable prokaryotes which are well known to produce chemically diverse metabolites with wide range of biological activity which used as pharmaceuticals and agrochemicals [3].These filamentous bacteria produce about 75 % of the commercially and medically useful antibiotics [4], and approximately 60 % of antibiotics developed for agricultural use were isolated from *Streptomyces* species as well [5].

Actinomycetes especially *Streptomyces* are the most economically and biotechnologically valuable prokaryotes [6]. They are responsible for the production of about half of the discovered bioactive secondary metabolites [7], antitumor agents [8].

Antibiotics of actinomycete origin evidence a wide variety of chemical structures, including aminoglycosides, anthracyclines, glycopeptides, β-lactams, macrolides, nucleosides, peptides, polyenes, polyketides, actinomycins, and tetracyclines [9].

The well known antibiotics derived from *Streptomyces* species included erythromycin, tetracycline, streptomycin, chloramphenicol, neomycin, nystatin, amphotericin, kanamycin and cycloheximide [10].

The aim of this study for isolation of *Streptomyces* spp. with antibacterial activity and study characterization of antibacterial agent.

Materials and Methods:

Isolation and characterization of actinomycetes colonies from the soil:

Soil samples were collected from Hilla City. These samples were pretreated with calcium carbonate and dried in hot air oven at 45^{o}C for 1 hr. in order to reduce the incidence of bacteria and molds. Soil dilution plate technique was maked for isolate on yeast malt dextrose (YMD) agar medium and pH adjusted to7.2and the plates were incubated at30^{o}C for10days [11].Characterization of *Streptomyces* was tested. A assimilation of carbon sources like, glucose , fructose, xylose, sucrose, were tested on phenol red broth supplemented with 1% carbon source [12].

Isolation of bioactive compounds:

Streptomyces spp.2 was grown in ISP – 2 medium (yeast malt extract agar) as a production media for the extraction of crude compound. The active isolate was inoculated in ISP-2 (International Streptomyces project – 2) broth and incubated for 7 days in shaker incubator at 28°C. It was centrifuged for 15mins at 8,000 rpm and the supernatant collected was mixed with an equal volume of ethyl acetate 1:1 (v/v). The crude compound were extracted by using ethyl acetate extraction method [13].

Antibacterial activity of agent:

The crude extract were screened for antibacterial activity against *Stapylococous aureus* and *E.coli* by well diffusion method. 100 µl of the crude was placed in wells made on Muller Hinton agar plates seeded with the test bacterial pathogen cultures. The plates were incubated at 37°C and observed for inhibition zone after 24 h. [14].

Method of agar blocks:

Cylindrical pieces of agar were cut out from well grown and sporulated culture of the actinomycete strain on solid nutrition medium. The blocks were placed on the Petri dishes deep inoculated with a fixed amount of test-microorganisms (10^8 cells/ml). The antibacterial activity was measured after incubation 24hr. at 37^{o}C [15].

Thin layer chromatography (TLC) for antibacterial agent:

The crude extract was purified by Thin Layer Chromatography (TLC) using silica gel coated plates. The extract was dissolved in 200µl of methanol. Sample was spotted by (using capillary tube), at the bottom of silica gel coated plate and placed in the developing chamber which contains solvent mobile phase (Ethyl acetate and Methanol), covered with the glass in order to prevent the evaporation of the solvents. Solvent was allowed to run till it reaches about half a centimeter below the top of the plate. After running, the plates were kept in room temperature for the complete drying of the plate. The plates were kept in a TLC chamber containing iodine pellets. Spots were observed Rf value of the spot on the TLC plate was determined.

Rf value= Movement of solute / Movement of solvent [16].

Physico-chemical properties of antibacterial agent:

The physical properties of antibacterial agent produced by *Streptomyces* spp.4 was studied to determine their physical properties like color, melting point and solubility.

Melting point:

The melting point of antibacterial agent was measured by melting point apparatus (Stuart ,meting point SMP30).

Solubility:

Solubility of antibacterial agent was tested in many solvent such as ethyl acteate, methanol, DMSO and distil water.

Spectroscopic analysis of antibacterial agent of antibacterial agent:

In order to characterize the antibacterial metabolites produced by *Streptomyces* spp.4 spectral studies such as Ultra-Violet (UV) , Infra–Red (IR), in College of Science Babylon University Chemistry department.

Ultraviolet (UV) and Fourier transform infrared spectra(FT-IR):

Ultraviolet (UV) spectrum of antibacterial agent was recorded on Shimadzu UV- VIS 1650 spectrophotometer. One milligram of sample was dissolved in 10 ml ethyl acetate and the spectra were recorded at 200–400 nm range.Infrared spectrum was recorded on Shimadzu FTIR-8400 model. The spectrum was scanned in the 400 to 4000 cm–1 range. The spectra were obtained using potassium bromide pellet technique. Potassium bromide (AR grade) was dried under vacuum at 100°C for 48 h and 100 mg of KBr with 1 mg of sample was taken to prepare KBr pellet. The spectra were plotted as intensity versus wave number[17].

Nuclear Magnetic Resonance (NMR):

Nuclear Magnetic Resonance for antibacterial agent were recorded on NMR apparatus (Bruker 300MHz) in University of Jordan, College of Science, Chemistry department.

Results and Discussion:

Isolation of *Streptomyces* spp.:

Twelve actinomycetes spp were isolated. Five isolates of *Streptomyces* spp. were detected according to morphological and biochemical testes(Table 1). These isolates were tested for antibacterial activity against *S.aureus*. The results showed that *Streptomyces* spp.was most active against *S.aureus*. *Streptomyces* are the group of gram positive filamentous bacteria and it having ability for producing bioactive compound isolated from soil samples.[18].

Table (1): Characterization of *Streptomyces* spp. isolates

Characteristics	*Strep* spp.1	*Strep* spp.2	*Strep* spp.3	*Strep* spp.4	*Strep* spp.5
Gram stain	+	+	+	+	+
Aerial mycelium	grey	grey	grey	grey	grey
Substrate mycelium	Yellow-brown	Yellow-brown	Yellow-brown	Yellow-brown	Yellow-brown
catalase	+	+	-	+	-
oxidaes	+	-	+	+	+
Sugar fermentation					
glucose	+	+	+	-	+
mannitol	-	+	+	+	+
sucrose	+	-	-	+	-
mannose	-	-	+	-	+

Screening for antibacterial activity:

The antibacterial activity of *Streptomyces* spp. isolates was tested by using agar block method. The results showed that five isolates were active against *S.aureus* (Table 2).

Strep spp.4 had high activity against *S.aureus* with (20 mm) inhibition zone. According this results these isolate was selected for isolation of antibacterial agent and study of characteristics. Our results agreed with results obtained by [19]who founded that the antibacterial activity of extract produced by *Streptomyces* Species Isolated from soil samples was active against *S. aureus* with 19 mm inhibition zone .

Table(2):Antibacterial activity of *Streptomyces* spp. isolates against *S.aureus*

Streptomyces spp.	Inhibition zone (mm)
Strep spp.1	8
Strep spp.2	10
Strep spp.3	12
Strep spp.4	20
Strep spp.5	14

Streptomyces species have the ability to synthesis many active secondary metabolites such as antibiotics, herbicides , pesticides , anti parasitic and enzyme inhibitors. These compounds antibiotics are much more important therapeutically, commercially and approximately one third of known antibiotics have been isolated from *Streptomyces*. [20].Actinomycetes are producers for many antimicrobial agents [21].

Extraction of antibacterial agent:

The antibacterial agent was extracted from *Streptomyces* spp.4. Antibacterial activity of agent was tested against *S.aureus* and *E.coli*, Results showed that the antibacterial agent was most active against *S.aureus* with inhibition zone 22mm and 14 mm against *E.coli*. Our results agreed with results obtained by [22], who found that *Actinomycetes* isolated from soil samples had antibacterial activity against *S.aureus and E.coli.*

Thin layer chromatography (TLC) of antibacterial agent :

The results of thin layer chromatography showed that , the antibacterial agent have single spot with with Rf value equal to 0.88.

Physicochemical properties of antibacterial agent:

Physicochemical properties of antibacterial agent was studied. The results showed that the antibacterial agent was brown in color and the melting point is 110 C.The solubility of antibacterial agent was tested with many solvent such as DMSO, methanol, Ethyl acetate and distil water (Table 3).

Table(3): Characteristics of antibacterial agent

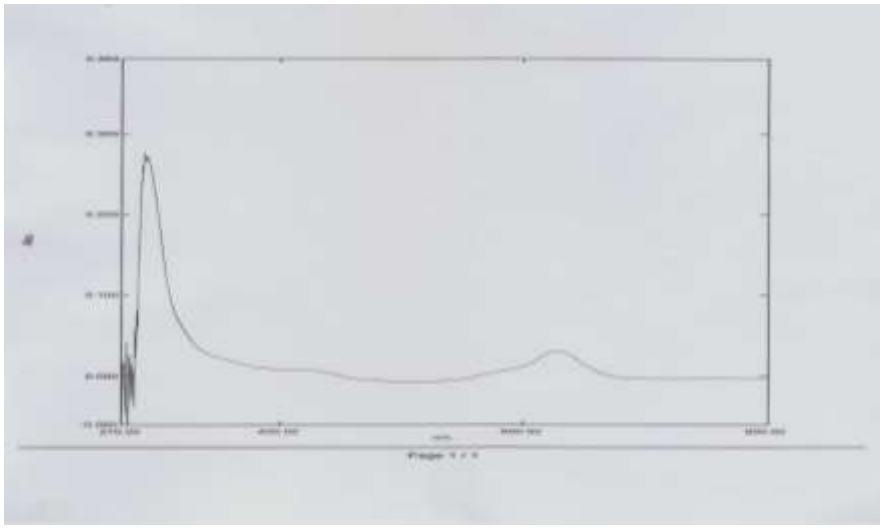
Character	Result
Color	brown
Melting point	110 ^{O}C
Solubility:	
Ethyl acetate	Soluble
Di-methyl-sulfo oxide (DMSO)	Soluble
Methanol	Soluble
Distil water	Soluble

Spectroscopic analysis:

Spectroscopic analysis of antibacterial agent was studied. These analysis includes :Ultraviolet spectrum (UV), Fourier transform infrared spectra(FT-IR) and Nuclear magnetic resonance (NMR).

Ultraviolet spectrum (UV) of antibacterial agent:

Ultraviolet spectrum (UV) of antibacterial agent produced by *Streptomyces* spp.4 showed that it have peak with maximum absorption at λ max 289nm Figure (1).Our results agreed with results obtained by [23] who found that The UV spectral data for the ethyl acetate extract of the selected strains from fermented broth have a maximum absorbance peaks range between 215 to 320.Ultraviolet (UV) absorption spectrum of the Sparsomycin Produced by *Streptomyces* Sp. AZ-NIOFD1 recorded a maximum absorption peak at 270 and 302 nm [24].



Figure(1): Ultra violet spectrum of antibacterial agent produced by *Streptomyces* spp.4

Fourier transform infrared spectra(FT-IR):

The IR (KBr) (v-, cm-1) spectrum Figure (2) had a peak about 3400 cm-1 which indicate presence of OH group absorption, and a peak appearing at 1635 cm-1 that indicated the presence Carbonyl group(C=O). The peak at 1410 and 1450 indicates to presence of CH3 group. Anther peak at 1076 indicated to presence of carbonyl (C=O) fuction of aster or an amide group. Our results was read according to [25].IR spectrum, of Natamycin antibiotic has a peaks at υ>3000 cm1 indicated that there is a typical carboxyl-structure; the υ=1716 cm-1 peak revealed a conjugated ester; the υ=1570 cm-1 peak corresponded to a primary amine; and the υ=1294-1116 cm-1 peaks showed the existence of different C-O [26].

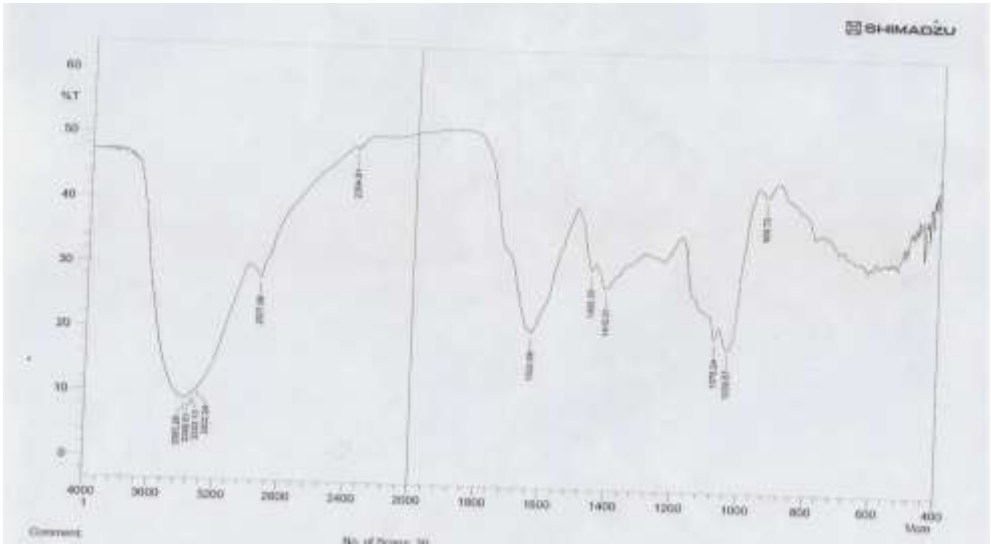


Figure (2): Infrared spectra of antibacterial agent produced by *Streptomyces* spp.4

Nuclear magnetic resonance (NMR):

The proton magnetic resonance spectrum of antibacterial agent (Figure 3) indicated to a sharp singlet at δ4 may attributed to OH group. Another singlet at δ1.2 indicated to presence of peripheral CH3 group. Also Singlet at δ2.4 may be assigned to presence of CH2 group surrounded by carbonyl bond (C=O).

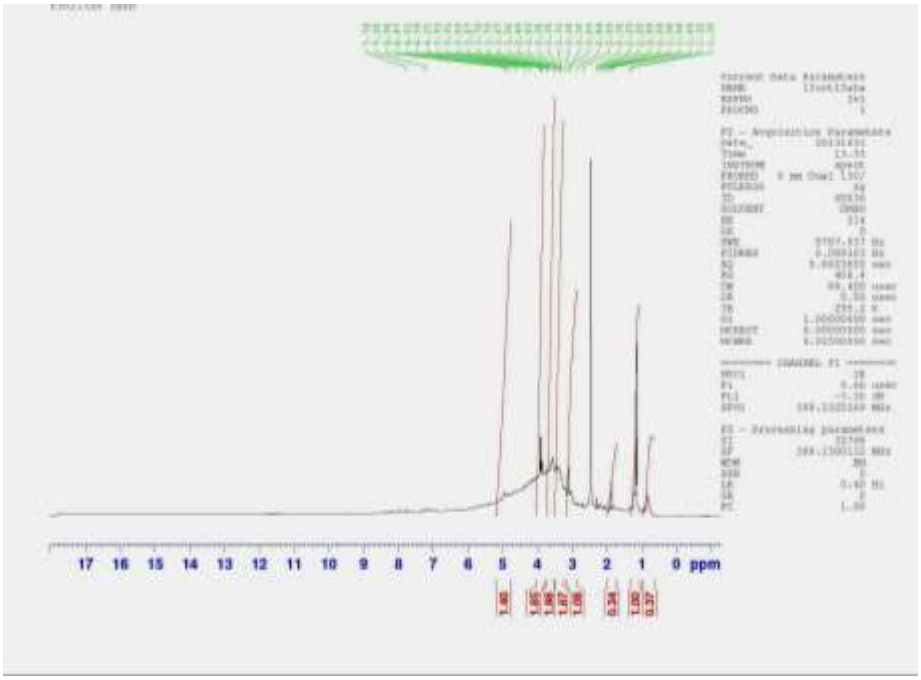


Figure (3): Proton magnetic resonance spectrum of antibacterial agent

Spectral analysis including infrared (IR), ultraviolet (UV), NMR (nuclear magnetic resonance) spectroscopy and mass spectroscopy are used in the identification of antibiotics [27].

The data obtained from spectral analysis can be interpreted to give the most probable structure of the antibiotic [28].

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