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RESEARCH ARTICLE

Isolation and Identify the Antibiotic from the Local Isolates of the *Streptomyces* antibiotics and Test their Antifungal Activity against Plant Pathogens

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Abstract

A bacterial isolates of (80) from (17) soil samples has been collected from the rhizosphere of different plants of many regions Mosul city; the isolates were initially identified as a *Streptomyces* genus according to the chalky appearance and the odor of the moist earth. Antagonistic activity against fungi isolated from the soil rhizosphere of plantshas been tested. Only (49) isolates (i.e. 57.64%) proved their antagonized ability against one or more of the tested fungi. The best isolation that gave antifungal efficacy against all fungi, and was the largest diameter of the inhibition area against *A. flavus* (48 mm) followed by *M. Phaseolina* (27 mm), the least inhibition zonewas (12 mm) of *Sclerotiumcepivorum* (12 mm). The isolation elected to the level of the species was identified by the morphological, physiological and hematological tests and was found to belong to the species of *Streptomyces lydicus*. The identification of the antibiotic extracted by ethyl acetate was done using three solutions that are butanol: pyridine: water (6:3:4), methanol: dichloromethyne: water (1:1:1) and butanol: acetic acid: water (6:4:3). A single spot appeared on a thin-layer chromatography plate (TLC) indicating the production of one type of antibiotic. It was noticed that R_f for the spot in the 3 solvents was 0.82, 0.47, and 0.80 respectively. The solvent with R_f 0.86 was the best hence it gave the highest inhibition for the growth of at least 2 fungi. Ultra violet absorption for the antibiotic was (286) and appeared that this compound was a Macrolides. Also Infra red (IR) was measured suggesting that the expected structure of the antibiotic is Oleandomyein.

Key words: *Streptomyces antibiotics, Bacteria, Antifungal, TCL plate.*

Introduction

The continued success of biotechnical scientists in the process of researching or detecting metabolic compounds of macrobiotics such as beneficial antibiotics, which have the potential to eliminate diseases that affect humans, animals and plants, which are used in pharmaceutical and physiological. Medical and agricultural applications [1]. As it have the ability to produce antibiotics, antibiotics and enzyme production [3, 2], The nematode bacteria, especially the species of *Streptomyces* genus, are widely prevalent in soils, especially in the rhizosphere of plants, as soils are considered as important source for different groups of microbes that affect the safety and health of the plant. It is known that *Streptomyces* genus species have a great importance in the production of antibiotics that are used to combat many plant pathogenic bacteria and fungi and their use in the field of control plant pathogenic fungi depends on their ability to colonize rhizosphere of plants and their inhibitory impact of harmful microbes [4].The species belongs to *Streptomyces* have the potential to produce effective metabolic substances, Bull and Stach [5] indicate that approximately (80%) of natural products produced by the species of this genus as indicated to its producing of (6000) compounds, as well as (75-80) of antibiotics, commercial, medical compounds and plant growth factors processed by this genus. The *Streptomyces*genus is the most important species of the group of nematodes, which is then isolated over the course of history.

The economic importance of the fact that it represents the hardenability of many of the effective biological substances of antibiotics, vitamins and enzymes [6]. The study aimed to obtain the isolations of *Streptomyces* genus from rhizosphere of the plant and test its effect on the growth of some fungi and then extract the antibiotic separation, identification and study of the of anti-fungal efficacy.

Materials and Methods

Isolating Actinomycetes from the Soils:

Twenty samples of soil were obtained from the rhizosphere of sunflower plants, these isolates were purified by the method of casting dishes [7]. The soil samples were previously treated with calcium carbonate CaCO3 by (10:1 g soil/CaCO3) and incubated in the incubator at temperature(37 ^{o})for (4) days, after the end of the incubation period, an extract soil is made using the sterile ringer solution and that by adding (1) g of soil to (9) ml of the solution in sterile test tube for dilution (10^{-1}), the procedure was repeated to the extent of dilution (10^{-5}) and then took (0.5) ml of the last two dilutions (10^{-4} and 10^{-5}), placed in sterilized dishes and then added for each dish nearly (15) ml of the dissolved medium at the temperature of (50^{o}), the dishes trolled quietly to blend the ingredients and left to harden and then incubated in an incubator at the temperature degree (28 ± 1^{o}) for (7-14) days until the growth of colonies bacteria, The chalky-looking elected dishes to the center of the glycerol-asparagine agar [8] and then incubate at a temperature (28 ± 1) Silesian for 7 days, and after obtaining pure colonies placed at temperature(4^{o}) until the other tests were performed.

Testing of Antibiotic Production by Streptomyces Isolates

For the purpose of studying isolates ability to produce antibiotics, Agar disc diffusion method was used by Egrov method [9]. The dishes of the glycerol asparagine agar dishes medium were vaccinated with isolates of bacteria to be tested and incubated at temperature (28 ± 1^{o}) for (7-14) days, after the growth of isolates, a discs in diameter (8mm) mediated by the piercing of the sterile withalcohol flaming are taken from the colonies of growing bacteria, and transported to Agar of potatoand sucrose medium(PSA) with rate (5-3discs per dish) and vaccinated with one of the following species of fungi: *Alternaria alternaria, Aspergillus flavus, Curvularia inaequlis, Fusarium Equiseti, Fusarium heterosporum, Fusarium oxysporum, Fusarium Solani, Helminthsporum sp., Macrophomina Phasolina and Sclerotium cepivorum.*

Morphological Features

Isolations are initially identified depending on chalky appearance of the colonies and production of of moist earthodor. The identification was confirmed using the implant technique on the glass slide to observe the ground and antenna yarn, arrange spores strings and observe the colors of the air spinning on the starch mineral salts agar medium[10]and the production of melanin pigment on the of tyrosine agar medium [11].

Chemobiotic and Enzymatic Characteristics:

The biochemical and enzymatic tests were performed based on the tests mentioned by both from [11, 12, 13, 14, 15].

Antibiotic Separation

The process of antibiotic extraction was carried out using Ilic method and others [16], after the expiry of the specific incubation period, the *Streptomyces* bacteria culture filtered by a glass funnel and using Whatman No.1 filtration papers and after the exclusion of bacterial cells from the culture medium added to the ethyl acetate by rate (1:1 size/size) per jug, the mixture put in the rocking incubator for one hour for the purpose of full extraction, then put in separating funnel (250ml), separation funnel was shaken well then leave for a period of time until the formation of two layers.

The upper layer represents the dissolvent (ethyl acetate) containing the antibiotic was taken, the lower layer representing the aqueous phase which was neglected. The solvent was vaporized using Rotary Vacuum Evaporator model (Buchi, Swiss) at a temperature of (50^{o}), and after evaporation a fatty substance brown color melted by methanol was obtained.

The Biological Efficacy Test of the Extracted Antibiotic

For the purpose of studying the biological efficacy of this substance, the disc diffusion

method was used, as Whatman No.1 filter paper discs in diameter (6mm) and flooded with the special extracted material produced from the isolation elected, then dried by exposing it to air well and placed using sterile forceps in the dishes of the container On the Center (PSA) and pollinator *F. Solani* and *M.phaseolina*, incubated at temperatures (28 ± 1) Silesian for (48 hours) and the results were recorded by measuring the growth-free zone.

Spectral Analysis

The spectral analysis of the extracted antibiotic was done using an UV-Visible-ir device, Spectrophotometer-(Camspec M330 UK) at the Faculty of Education/Chemistry department. BRUKER, TENSOR, 27, Germany.

Results and Discussion

Isolation of Nematodes Bacteria:

EightIsolates from (17) Soil samples from rhizosphere of different plants and from different areas of the city of Mosul. It was found that all the isolates belonged to the genus *Sterptomyces* depending on the chalky appearance of the slips and the production of the Wetlands [17].The number of colonies (1-30) was a colony/gr of soil and these results were close to what it obtained [18]. As it obtained (29.2) colony/G while the number of colonies obtained in the study of Qasim and Suleiman [19] (1-45) colony/G and was to treat soil with calcium carbonate CaCO3 by (1:10) and drying it to a (40^{o}) for (4) days "important" role in increasing the numbers Bacterial *Streptomyces* in the initial insulation.

Diagnostic:

The isolates of the *Streptomyces* genus have been diagnosed using the implant technique on the glass slide, which is one of the best methods in the species diagnosis, as the different form of air and terrestrial yarn [20] characterizes the species of the nematodes. The arrangement of spore's chains for elected isolation with a spiral shape and highly branched ground yarndoes not carry spores (Fig. 1).

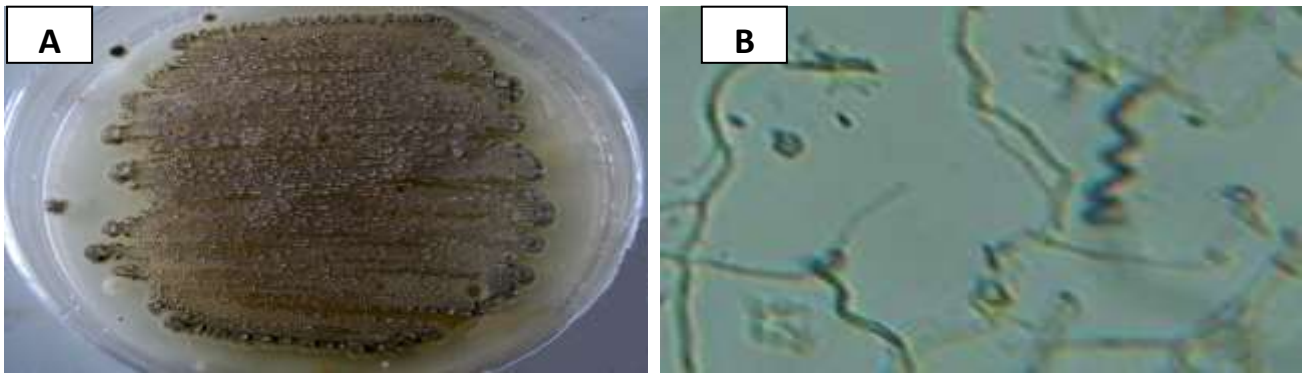


Fig. 1: Air and ground yarn to isolate the *Streptomyces* antibiotics magnification 40XA. Ground yarn. B. Pneumatic yarn

Testing the Antagonistic Activity of *Streptomyces* Isolates Isolated from rhizosphere of the Plant's Soil

In the study of the ability of all isolates of *Streptomyces*genus to inhibit fungi, (49) isolates (57.64) percentage of the *Streptomyces*-tested isolates proved to be of their highly antagonistic activity against one or more of the tested fungus (Table 1).

Table 1: The ability of *Streptomyces* genus isolates to produce antibiotics against a number of pathogenic fungi (diameters of the areas of inhibition in mm)

Tested fungi	Number of antifungal Isolates	Percentage of inhibition	Diameter rate of inhibition areas (mm)
Alt. alternata	30	%(61.22)	13.7
A. flavus	11	%(22.44)	14.0
C. inequlis	31	%(63.26)	12.7
F. equiseti	21	%(42.58)	12.5
F. heterosporum	27	%(55.10)	15.0
F. oxysporum	15	%(30.61)	13.6
F. solani	18	%(36.73)	13.0
Helminthsporum sp.	14	%(28.57)	11.5
M. phaesolina	17	%(34.69)	15.0
S.cepivorum	20	%(40.81)	12.7

This result is an approach to Bharti et al. [20], (59.18)% of which were effective against a number of tested fungi, including *Candida albicans, Aspergillus, Flavus and A. Niger*

and A.fumigatus. Atta [21] referred to antagonistic ability of *Streptomyces* genus isolates, obtained from soil samples, to inhibit a number of pathogenic fungi are *A. flavus, Fusarium, Oxysporum, Alternaria, Alternata, Botrytis, Fabae, A. Fumigatus, A. Niger, C. albicans, Rhizoctonia, Solani, Penicillium, Chrysogenum* and *Saccharomyces cerevisiae* as well as a number of positive and negative bacteria for the formula *Pseudomonas aeruginosa, Esherichia coli and Bacillus subtilis* (Fig. 2).

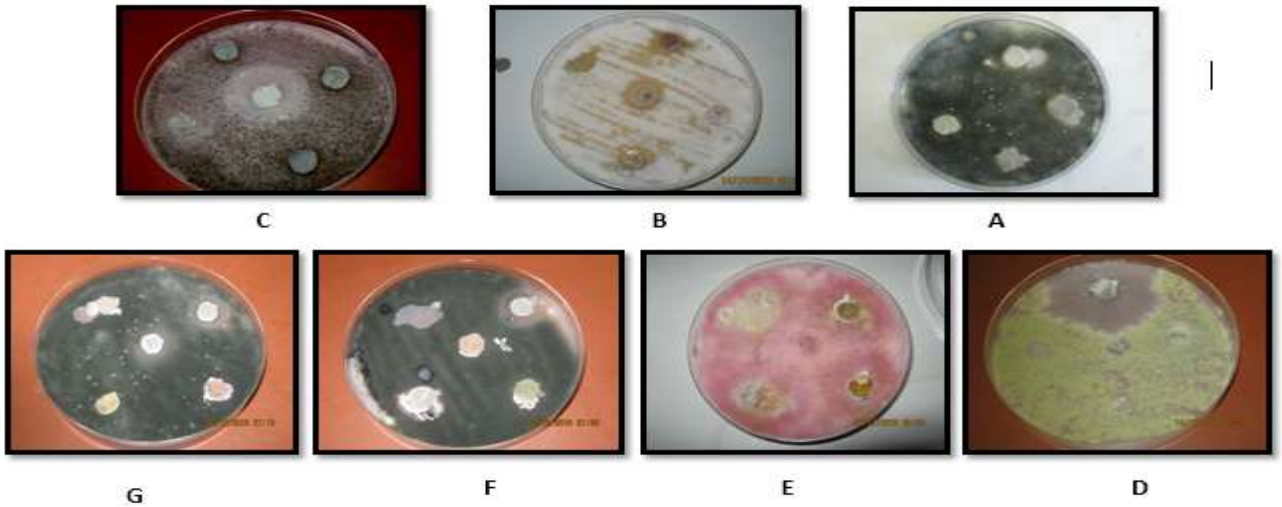


Fig. 2: Test the antagonistic activity of the local isolation *Streptomyces antibiotics* against pathogenic fungi
A. *M. Phaseolinak* B .*F. solani*،C. *Alt. Alternata* D. *A. flavus*
E. *F. oxysporum*، F. *C. Inaequlis*،G. *Helminsporium*

Table 2: Diagnostic tests for local isolation *Streptomyces* antibiotics *

Tests		**Sensitivity of *Streptomyces* of antibodies**	
Gram stain	+	(ml/µg 10) Streptomycin	R
Acid-resistant stain	-	(ml/µg 15) Erythromycin	R
Color of pneumatic yarn	Gray	(ml/µg 30) Gentamycin	R
Spore chain shape	Spindle	(ml/µg 30) Tetracycline	R
Spores formation on ground yarn	-	(ml/µg 2) Lineomycin	R
Melanin stain production on the center of tyrosine agar	+	(ml/µg 10) Vancomycin	S
Production of other stains	+	(ml/µg 30) Neomycim	R
(0.01)% sodium azide	-	(ml/µg 5) Rifampin	R
(0.1))% phenol	+	(ml/µg 30) Chloromephenicol	S
Growth shape in the liquid medium Nutrient broth	Small balls shape		
Nitrate reduction	+		
H2S Production	-		

Enzyme Tests		**Consumption of Carbonic resources**		**The ability of inhibition of growth of pathogenic fungi of plant**	
The catalase	-	Glucose	++++	*Alternaria alternata*	++++
Oxidase	-	Galactose	+++	*Aspergillus flavus*	+++
Hydrolysis of starch	+	Fructose	++	*Curvularia inaequlis*	++++
Hydrolysis of Gelatin	+	Lactose	++	*Fusarium equiseti*	++++
Glycolysis	-	Arabinose	+	*Fusarium heterosporum*	++++
Hydrolysis of gasoline	-	Xylose	±	*Fusarium solani*	+++
Hydrolysis of Urea	+	Cellulose	+	*Fusarium oxysporum*	+++
Hydrolysis Blood	+	Starch	++++	*Helminthsporium* sp.	+++
Hydrolysis of isocline	-	Glycerol	++	*Macrophomina phaseolina*	++++
				Sclerotium cepivorum.	+++

Ability to grow at different temperatures		**The ability to grow different concentration of Nacl**	
Nacl % (1.5)	+	25 Silesian	±
Nacl % (3)	+	30 Silesian	+
Nacl % (5)	+	37 Silesian	+
Nacl % (7)	±	45 Silesian	+
Nacl % (15)	-		
Nacl % (20)	-		

* + = Positive result-= Negative result + + + + + = excellent + + = very good
+ + = good ± = Few S = Sensitive R = Resistance

Antibiotic Diagnosis

The process of antibiotic separation was mediated by the thin layer chromatography

(TLC) and by using three solutions that drink the first solution: n-Butanol: Pyridine: Water with rate (4:3:6) second solution methanol: Dichloromethane: Water with rate (1:1:1) and the third solution Biotanol: Acetic acid: Water and by descent (3:4:6) all solutions showed a single yellow spot (Fig. 3). This indicates that these bacteria produce a "one" type of antibiotic and may produce more than one type of antibiotic such as *Cacaoi Streptomyces*, which produces Polyoxin D &B (28).Nematodes bacteria produces many of antibiotics that have different chemical structures such as Polketides, B-lactams and peptides, as well as various types of secondary metabolic substances such as fungal and bacterial antibiotics and antioncotic (25).

Fig. 3: Antibiotic separation with the use of the thin-layer Chromatography

The antibiotic (R_f) value was different by the use of the three solutions by giving the first solution (R_f) 0.08 second solution (R_f) 0.47 and the third solution (R_f) 0.82, after exposure to UV rays as in Figure (3) and this is similar to that obtained by Sahin and Ugur (26) the value (R_f) (0.8) to the antibiotic Bio-product by two types S.antibiotics and S.rimosus. The activity of the extracted antibiotic was tested using the three solutions against the *F. Solani* and *M. Phaseolina* as the first solution was the best in the antibiotic separation and gave a high capacity to inhibit *F. Solani*, the inhibition area was 46 mm, while the inhibition area using the second solution was reached (27) mm The third solution was (9) mm as in Table (3).

Table 3: Shows the diameters of inhibition areas and antibiotic (Rf) values extracted from *S.antibiotics*

Solvent	Diameter of inhibition areas (mm)		Value (R_f)
	M. Phasolina	*F. solani*	
Butanol: pyridine: Water	46	30	0.80
Methanol: dichloromethane: Water	25	21	0.47
Butanol: Acetic acid: water	9	0.0	0.82

While the diameters of the inhibition area of *M. Phasolina* were (30mm) for the first solution, (21mm) for the second solution, the third solution did not give any activity against *m. Phaseolina* compared to the standard antibiotic *nystatin* as in (Fig. 4).

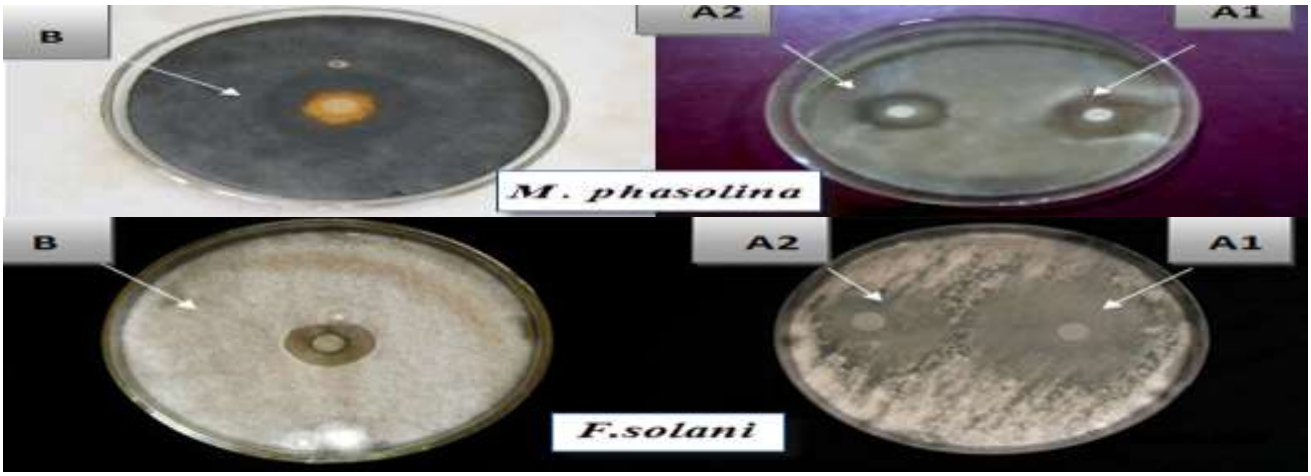


Fig. 4: The effect of the antibiotic product of isolation *s.lydicus* on the growth of *F.solani* and *M. Phasolina* using various drinking solutions: A1. Butanol: pyridine: Water A2. Methanol: dichloromethane: Water B. Nystatin standard antifungal effect

The inhibition area of *M. Phaseolina* was (15 mm) for and (20mm) of *f. Solani* and this is consistent with [22] antibiotics produced by pathogenic anti-fungal bacteria are more efficient than the bacteria themselves and vary with different bacteria. The UV spectrum of the antibiotic extracted methanol was measured using the optical spectrophotometer for visual and ultraviolet radiation as it reached the peak of absorption (289). Depend on Berdy [23]. The antibiotic returns to the Macrolides group. (Fig. 5), [24] mentioned the importance of antibiotics that belong to the Macrolides group as anti-fungal growth that pathogenic for humans, animals and plants.

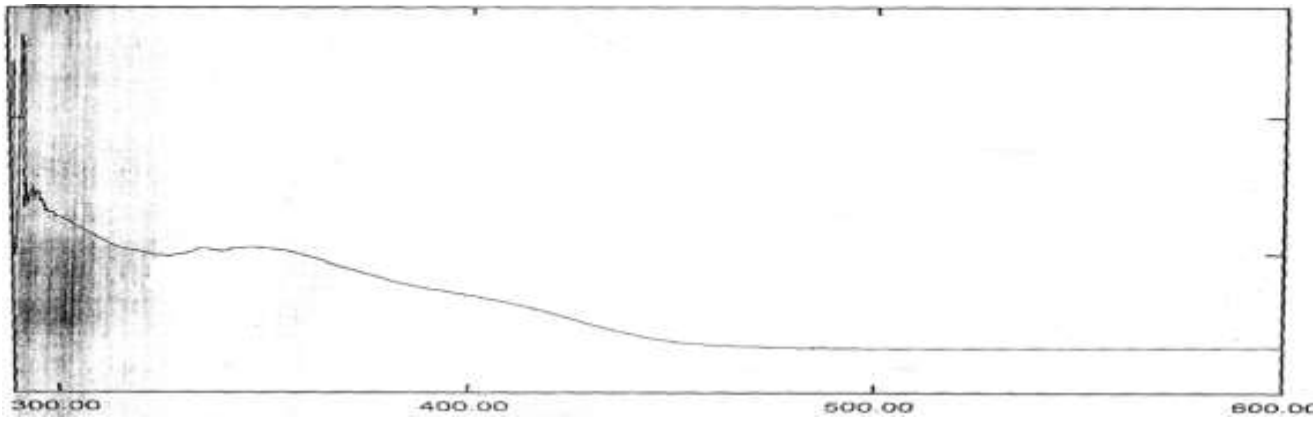
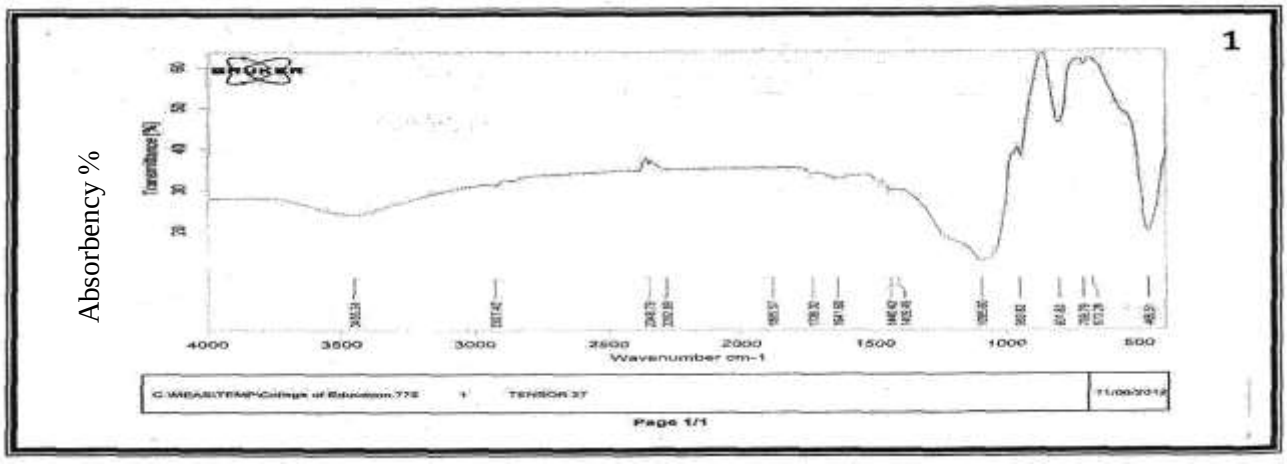


Fig.5: UV spectrum measurement for antibiotics

The antibiotic was diagnosed using infrared (IR), Absorption package has been shown 1736 cm^{-1}(C = O), 1095 cm^{-1} (C-o), 1641 cm^{-1}(C = C), 3455 cm^{-1}(OH) (Fig. 6). Through these tests it is clear that the compound is belong to the group macrolides and specifically Oleandomyein. These results are comparable to the compound obtained by [25] at bioleaching mediated by methanol and ethyl acetate and the flux rate (R_f) for this compound was 0.86 and the same (R_f) 0.86 That we got in this study [26-32].



Wave length cm^{-1}

Fig.6: Antibiotic infrared spectrum measurement

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