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Cytotoxic Activity of Secondary Metabioltic Agent Produce by Actinomycetes

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Abstract

In this work, total of 50 samples of different soil types (sandy, loomy, clayey, and river deposits) were collected randomly. The physico-chemical properties such as pH, soil temperature and electrical conductivity of the soil samples were studied and analysed Soil characteristics all were within the limits of growth and reproduction of Actinomycetes. Of the 50 soil samples, only 35 were sourced to obtain 18 colonies, were suspected to be Actinomycetes. These isolates were purified by sub culturing more than one time on International Streptomyces project-2 agar (ISP-2),the pigmentation of the selected colonies exhibited a spectrum of colors ,including shades of white, cream, gray, green ,black to yellow. These isolates were identified by culture properties which showed that they were Gram positive varieties of aerial mycelia and substrate mycelia and spore chain formation. By the third day of incubation, the isolate had attained the stationary phase of its growth cycle.at maximum OD 0.619nm. Extraction of Extracellular secondary metabolites from Each of the Actinomycetes by ethyl acetate. According to the quantity of the product and the speed of its production, 3 isolates (NI- RS 15, AM - AG 18 and MA-CL2) were selected to complete the study. All extracts with high solubility with water, had melting point range 287°C- 300°C and were powder in natural. Chemical Characterization of secondary metabolites were as follow λ max ranged from 274 nm to 320 nm FTR show several bands of absorption as functional chemical group and HPLC clearly sharp peaks that appeared at 2.1 - 9.9 minutes which indicated a very well separated. The cytotoxicity of extracts were assessed by MTT on normal cell line was which inhibited cells range from 9.9-13.9% in 200 µg / mL.

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1. Introduction

Actinomycetes is a varied collection of prokaryotic heterotrophs that produce hyphae at certain stages of their growth [1 2]. For this reason, it is called filamentous prokaryotes. For more than a century, the existence of Actinomycetes has been acknowledged.,They were identified as an unusual class of creatures that have affinities for both fungus as well as bacteria. Nonetheless, their prokaryotic nature was validated by the analysis of their fine structure and chemical makeup. The categorization and identification of Actinomycete genera and species have improved as a result of the use of modern taxonomic techniques. Actinomycetes continue to be studied as a distinct group of bacteria due to their widespread presence, unique structural features, Ability to produce a wide array of secondary metabolites and enzymes, and their overall significance to human applications [3]. They represent a prolific group of microorganisms that thrive in diverse natural and artificial environments [4-7]. Aerobic actinomycetes are predominantly isolated from soil environments, with a viable counts reaching over four million per gram in fertile soil [1]. These microorganisms are widely acknowledged for their ability to produce antibiotics and other valuable secondary metabolites, as elucidated by [8]. A significant proportion -ranging between 70 and 80% - of antibiotics and antimicrobial secondary metabolites used commercially, have been derived from diverse species of Actinomycetes [9] This current study aims to investigate and identify the secondary metabolites produced by actinomycetes in soil.

2. Materials and methods

2.1. Sample collection:

Soil samples were randomly collected from various locations across the Hilla city. The sampling sites include clay, loomy (agricultural) sandy and river sediment soils, as summarized in Table 1. The collection was conducted during the study period to ensure a representative sample diversity of soil types.

Table 1: The site location, sample numbers, and soil types were documented for purposes of isolating Actinomycetes from various areas within Babylon province

Site	Sample No.	Soil Type
Al-Mahaweel	1,2	Clay
	3,4,5	Sandy
	6,7,8	Agricultural
Al-Nile	9,10,11	Agricultural
	12,13	Clay
	14.15.16	River sediment
	17	Sandy
Al-Muhandseen	18,19,20	Agricultural
Al-Karama	21,22,23	Clay
Al-Athar	25,26,27	River sediment
Al-Amam city	28,29,30,50	Agricultural
	31.32,33	Clay
	34,35,49	River sediment
	36	Sandy
Al-Kasim	37,38,39	Agricultural
	40,41,42,43	Clay
	44,45,46	River sediment
	47,48	Sandy

2.2. Testing Soil properties :

i. pH measurement of the soil:

Containers are usually filled with roughly 10 grams of soil, weighing it down to the nearest half gram, then adding fifty milliliters of water that has been distilled into the soil. In order to ensure that a 1:5 dilution is achieved, any approximate measurement will be sufficient. The container should be agitated for roughly two to three minutes, and then the soil sample allowed to stabilize for two minutes. Check the pH level of the water that is above the soil that is contained within the compartment.[10]

ii. Measurement of soil temperature:

- Utilizing a soil thermometer at a depth of ten centimeters through:[10] Mark the location that is 12 cm up from the soil thermometer's tip.
- Calculating the distance between the 12 cm mark and the soil thermometer's dial base.
- The process of making a spacer by cutting a piece of wood or plastic tubing to the length that has been set. Create a hole in the middle of the wooden block by drilling with a drill.
- The spacer through the soil thermometer. The thermometer should protrude 12 cm from the spacer's bottom.
- Labeling this spacer 10 cm Measurement

iii. Measurement of soil electric conduction (EC)

Electric conduction of soil samples were measured by using EC meter (HANA/214).

2.3. Treatment of the Collected Samples:

Samples of soil weighing one hundred grams taken from the locations that were indicated at a depth of ten to fifteen centimeters were then placed in plastic bags that measured twenty centimeters by forty centimeters. The soil samples were produced with $CaCO_3$ at a ratio

of 10:1 of soil to $CaCO_3$, which were kept at an ambient temperature of 28 degrees Celsius for a period of one week. This was done in order to increase the number of actinomycetes, which are normally found in alkaline settings, and to reduce the amount of contamination caused by molds and yeast, as described in [11;12].

2.4. Isolation of actinomycetes from soil samples :

Once each sample has been homogenized to get rid of waste, big stones, and gravel, the sample was then sieved through a 2mm metal sieve to remove the gravel . Then, 9 ml of D.W. was mixed with 1 g of each soil sample, and Serial dilution of the soil suspension were prepared up to 10^{-1} to 10^{-5} Aliquots from each dilution were aseptically spread onto the surface of international Streptomyces projected type 2(ISP-2) agar medium. In order to inhibit fungal contamination and promote the selective isolation of Actinomycetes, Nystatin was added into the culture medium, and the inoculated plates were subsequently incubated at 28 °C for 5 to 7 days. Actinomycetes isolates purified bysubculturing on on ISP-2 agar medium. Next, the light microscope was used to examine the purified colonies. Subsequently, the standard colonies of Actinomycetes were grown on International Streptomyces Project type-2 agar slants and preserved at 4°C for further uses [13].

2.5. Morphological Identification of Actinomycetes spp:

Actinomycetes isolates were identified through the analysis of morphological criteria, including colony morphology, aerial mycelia, substrate mycelia, and cell shape [14]. In order to assess the properties of Actinomycetes isolates, these bacteria were cultured on a variety of agar medium obtained from the International Streptomyces Project (ISP), including (ISP-2) [15]. in line with the International Streptomyces Project (ISP) and documented their features [16].

2.6. Fermentation Condition

One loopful of spores with their mycelial development was used to screen for the generation of secondary metabolites. After that, the spores were moved to a conical flask that contained 250 ml of sterile ISP2 and were cultured for seven days in a shaking incubator at a temperature of 29±1 degrees Celsius and a rotational speed of 150 revolutions per minute. The technique was specifically carried out in the Biology department, [17;18], relatively depending on the quantity of the product and the speed of its production, 3 isolates were selected to complete the study

2.7. Growth (Kinetic) Curve Study for Maximum secondary metabolites Production.

To attain growth patterns of locally isolated Actinomycetes, The stationary phase of microbial growth curve is known to provide the highest yields of secondary metabolites. Additionally, to calculate the isolate's growth curve in order to attain the stationary phase, 1 cc of stock culture solution was added to 250 ml of ISP2 prepared broth for inoculation. Twice daily, broth media were gently shaken while incubated at 28°C. After incubation for twenty-four hours, the absorbance of the inoculated broths was measured at a wavelength of 600 nm. Up until the point where the optical density (OD) reached its highest point, the procedure continued. The phase was considered to be one of stagnation [17].

2.8. Extraction of Extracellular secondary metabolites.

A 500 ml flask filled with 250 ml of pH 7.2 ISP-2 broth was used to inoculate each of the Actinomycetes isolates, which were then cultured for 10 days at 28°C. Following the period of incubation, the fermented broth is filtered using a Whatman No.1 filter paper to separating the cellular components from the culture filtrate, then broths were centrifuged for 15 minutes at 6000 rpm to extract the cell-free supernatant. To complete the extraction process the culture supernatants were mixed an equal volume of (1:1 v/v) of ethyl acetate and vigorously shaken for one hour to facilitate phase separation into two distinct layers: an organic layer and an aqueous layer. The ethyl acetate phase, which contained the extracted antibiotics, was carefully separated from the aqueous phase. Subsequently, the organic solvent was evaporated using a rotary evaporator to obtain the crude extract. Using a rotary evaporator at temperatures ranging from 80 to 90 degrees Celsius in order to achieve dryness, and the residue that was left over was weighed [19]. A secondary screening test is performed by weighing the residue that is collected from the flask that has been evaporated in the water bath. This residue is then used for antibacterial analysis. Typical pathogenic microorganisms were used in this experiment, which was carried out utilizing the agar well diffusion method [9].

2.9. Physical characteristics Produced secondary metabolites.

After secondary metabolites have been extracted from Actinomycetes fermentation broth culture after seven days from incubation period by treated with ethyl acetate. Physical characteristics of the secondary metabolites agents included the color and solvent solubility (water, methanol, ethanol, and ethyl acetate) that were investigated. Additionally, the Organic Chemistry Lab's Stuart SMP30 equipment was used to determine the melting point. Department, University of Babylon, College of Science.

2.10. Chemical Characterization of secondary metabolites

Secondary metabolites' physical properties were described using FTIR, HPLC, and UV visible

2.11. UV-Visible Spectroscopy

The University of Babylon's College of Science, Department of Chemistry's service chemistry laboratory used Shimadzu UV-visible 1800 UV-visible spectroscopy to confirm the PVD production and size estimation within the composite solution.

2.12. Fourier Transform Infrared Spectroscopy

FT-IR spectrophotometer, PVD for FTIR in the service chemistry laboratory of the University of Babylon, College of Science, Department of Chemistry, was used to measure the transmittance of the produced formulations using Fourier Transform Infrared.

2.13. High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) was used to examine and partially purify the extract in order to identify molecular weight and determine purity. To make the HPLC solution, 100 mg of well-shacked ethyl acetate were passed through a filter. Utilizing a disposable syringe to pass through a millipore micro filter (0.2 µm pores). The separation was performed using an HPLC (Waters 2545-USA) quaternary gradient module, integrated with a system fluidics organizer (Waters-SFO) and coupled to a SQ detector, operating in positive ionization mode within the m/z range of 200-900 at a scan rate of 2 scans per minute. The analysis was performed using a photodiode array detector (Waters 2998) operating at a sampling rate of 2 points per second over a wavelength range of 190–800 nm. Chromatographic separation was achieved using a reversed-phase C18 column (X Bridge, 4.5 × 155 mm). The mobile phase consisted of solvent A (de-ionized water with 0.1% formic acid) and solvent B (methanol with 0.1% formic acid). The gradient program applied was as follows: 30% A and 70% B from 0 to 2 minutes; followed by a linear increase of A to 95% and a decrease of B to 5% from 2 to 10 minutes. A 10 µL aliquot of the extract was injected using an autosampler (Waters 2767). All analytical procedures were carried out at the Advanced Research Center, College of Health and Sciences, Asyut University [20]. The concentration of the extract was set on by following the equation which depended on the area under the peak.

$$Conc.\,of\ sample\left(\mu\frac{g}{ml}\right) = \frac{Conc.\,of\ standard\ \times\ A\ of\ sample}{A\ of\ Standard} \times DF$$

Where Conc = Concentration, DF= Delusion factor, A = Area.

2.14. MTT Cytotoxicity Assay:

A non-radioactive colorimetric method for assessing how cells react to different cytotoxic stimuli. The MTT dye transitioned from yellow to purple formazan in living cells due to the action of the NADH enzyme (Mosumann; 1983), the cytotoxic effect of the extract on two normal cell lines was determined. Non tumorigenic fetal hepatic cell line (WRL 68)) and (Dermal Fibroblast Normal Human Neonatal (HdFn) [22].

3. Results and Discussion

Every sample was gathered at random from several sites inside Hilla City based on the soil types—clay, agricultural, sandy, and river sediment. In order to isolate Actinomycetes from fifty different soil samples, the serial dilution approach was utilized. This procedure involved inoculating Plates containing ISP-2 agar were inculcated with soil suspension and subsequently incubated under appropriate conditions to promote microbial growth while they were incubating for seven to fourteen days. The dilutions ranged from 10-1 to 10-5. Table 2 summarizes all probable Actinomycetes isolated from various soil sources, based on their ability to form colonies with inhibitory or clear zones surrounding them, characterized as tiny, white, rough, and chalky, as advised by [23].Out of 50 soil sample, only 35 soil from this soil sources were obtained 18 colonies (table 1) were suspected to contain Actinomycetes with different morphological types were gathered from every source of soil. The chosen suspect colonies were white, creamy, and gray in color, green, black to yellow.

Table 2: Site , Number &Soil types using for the isolation of locally Actinomycetes

No.	Site	Sample no.	Abbreviated	Soil condition
1	Al-Mahaweel	2	MA-CL2	Clay
2	Al-Mahaweel	7	MA-AG7	Agricultural
3	Al-Nile	11	NI -AG11	Agricultural
4	Al-Nile	10	NI-AG10	Agricultural
5	Al-Nile	17	NI -SA17	Sandy
6	Al-Nile	15	NI- RS 15	River sediment
7	Al- Muhandseen	18	AM - AG 18	Agricultural
8	Al- Muhandseen	19	AM - AG 19	Agricultural
9	Al- Muhandseen	20	AM-AG20	Agricultural
10	Al-Kasim	40	KA - CL 40	Clay
11	Al-Kasim	37	KA - AG 37	Agricultural
12	Al-Kasim	41	KA-SA41	Sandy
13	Al-Athar	25	AA- RS 25	River sediment
14	Al-Athar	27	AA - RS 27	River sediment
15	Al-Amam	30	AM –AG30	Agricultural
16	Al-Amam	49	AM- RS 49	River sediment
17	Al-Amam	28	AM-AG 28	Agricultural
18	Al-Karama	23	KA- CL 23	Clay

3.1. Soil properties Determination

There is a substantial relationship between the number and variety of actinomycetes that are present in a particular soil and the factors that are associated with the geographical location of the soil. These factors include the soil temperature, humidity, soil type, soil pH, organic matter concentration, cultivation practices, aeration, and moisture content all play a role as shown in Table 3. As an example, streptomyces spp. are predominantly found in lower pH soils whereas arid soils of alkaline pH contain fewer streptomyces spp [24]. There is a substantial relationship between the number and variety of actinomycetes that are present in a particular soil and the factors that are associated with the geographical location of the soil. These factors include the soil temperature, soil type, humility, soil pH, organic matter concentration, cultivation practices, aeration, and moisture content all play a role. The features of the soil sources from which Actinomycetes isolates were obtained varied, and conforms in agreement with the discovery made in [25], Actinomycetes , particularly those belonging to the genus Streptomyces, are widely distributed across various soil types. These are especially prevalent in surface soil layers and tend to thrive in alkaline soils, compost, river sediment, and riverbeds, owing to their highly active aerobic metabolism [26]. Key environmental factors influencing the abundance and distribution of *Streptomyces* include physical soil properties, organic matter content, pH, moisture levels, soil chemistry and texture Table 4.

Table 3: Number of Actinomycetes isolates according to soil type

Soil Type	No. of Actinomycetes isolates
Agricultural	9
Clay	4
River sediment	3
Sandy	2

Table 4: Properties of the soil sample that containing Actinomycetse isolate.

Soil type	pH mean	Temperature mean	EC mean
Clay	7.0	22	22.1
Agricultural	7.3	18.9	22.49
Sandy	7.2	27.8	66.5
River sediment	7.2	15.8	41.9

3.2. Morphological and cultural characteristics of *Actinomycetes* isolates

The features of Actinomycetes isolates show they have different mycelium shapes , different substrates color and differed in present or no soluble pigments as show described in table(5) and figure (1).

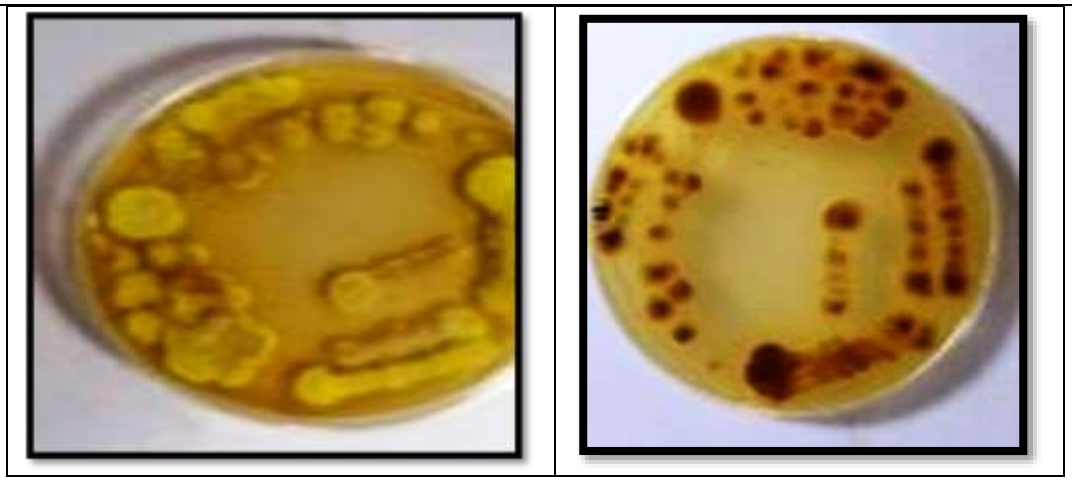
Table 5: Features of isolated Actinomycetres' morphology on ISP2

Isolate no.	Growth state mycelium	Colony texture	Aerial mycelium color	Substrate Color	Soluble pigments	
					Presence	Color
1	Very good		White	Brown		
2	Moderate	Powder	Green	Brown	-	-
2	Very good	Filamentous	White	Yellow	-	-
3	Good	Filamentous	White	Yellow	-	
4	Very good	Powder	Brown	Black	+	Brown
5	Very good	Filamentous	White	Yellow	+	Yellow
6	Very good	Filamentous	Yellow	Brown	+	Yellow
7	Good	Powder	Black	Dark black		-

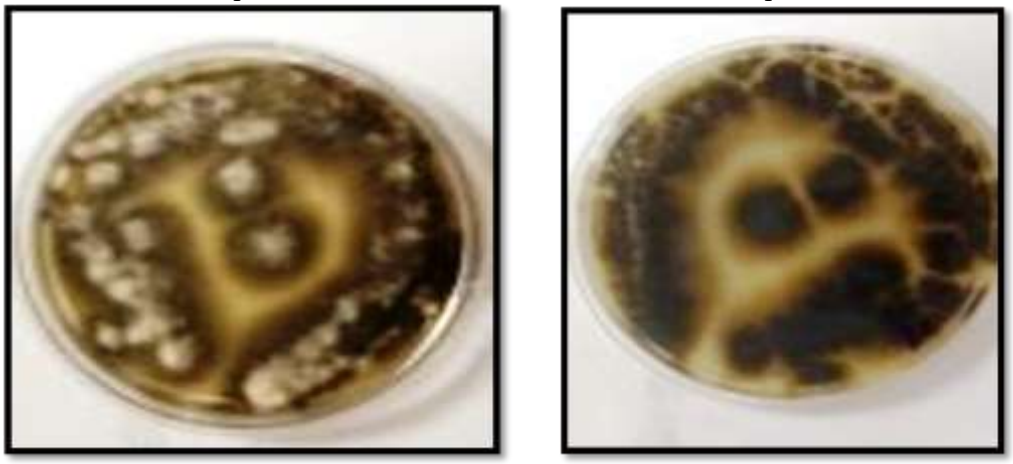
The ISP-2 medium, a commercially prepared agar medium for the development and characterisation of actinomycetes, includes several essential components like yeast and malt extract that are high in nutrients including vitamins, amino acids, and nitrogen converted to dextrose, which provides the carbon these bacteria need to develop. Furthermore, more cultural characteristics for Actinomycetes were exhibited on ISP-2 medium such as the colors of variety of aerial mycelia and substrate mycelia, while those appearing homogenous on other media, this supports ISP-2 is suitable medium for enhances the cultural characteristics of *Streptomyces* spp [27;1]. The results of [27;1] which isolated the Actinomycetes spp. from the soil of 53 colonies, were in agreement with the current results in table 4 and figure 1 of colony characteristics for 18 colonies chosen from 53 colonies. The morphological characteristics of colonies, including the forms and pigmentation of aerial and substrate mycelia with examined on ISP-2 medium at peripheral locations of the culture plate at the University of Putra in Malaysia [27, 1] Also the presented results were agreed with the results of [28; 29] who isolated the Streptomyces spp. from Indian soils and investigate these bacteria's morphological traits [28].

The shape of the actinomycete bacteria was also examined using gram stain and its spore shape by an electron microscope, as shown in figure 2.

The present findings on mycelial morphology align with the results of [30; 31], who isolated Streptomyces from soil and investigated its antibacterial properties against various human pathogenic bacteria, as well as examined the morphological characteristics of these bacteria's mycelia using a light microscope while submerged in oil [30]. These qualities can be confirmed through the application of the Gram stain to ascertain the positive attributes of these microorganisms [22]. The bacteria were inspected and identified using a light microscope. The shapes of substrate and aerial mycelium magnification 1000 X [32]. The use of a light microscope allows for the observation of a number of characteristics, such as the presence or absence of aerial mycelium, substrate fragmentation, spore chain morphology, and spore mass colors. Furthermore, the properties of the colony, such as its size, shape, and color, contribute to the ability to differentiate between these bacteria [9].



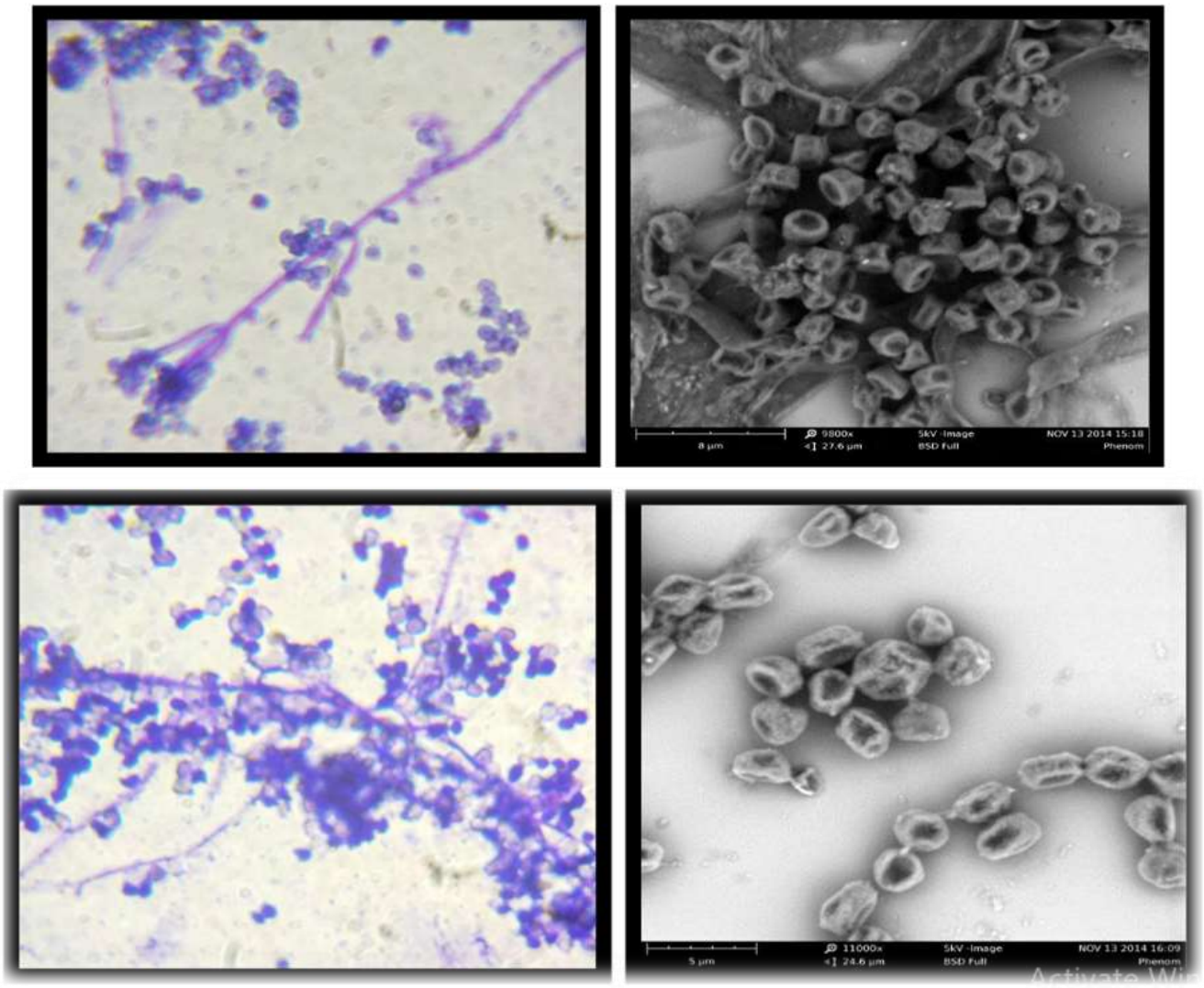
Front shape\ NI- RS 15 | Rear shape NI- RS 15



Front shape\ AM - AG 18 | Rear shape\ AM - AG 18

Front shape\ MA-CL2 | Rear shape\ MA-CL2

Figure 1: Morphological and cultural of Actinomycetes isolates.



Shape of mycelium under light microscope at 1000 X after staining by Gram stain

Shape of spores under scanning electron microscope at 9800 X

Figure 2: Shape of mycelium and Shape of spores

3.3. Extraction of Extracellular secondary

Extraction of extracellular secondary metabolites from each of the Actinomycetes by ethyl acetate. According to the quantity of the product and the speed of its production, 3 isolates (NI- RS 15, AM - AG 18 and MA-CL2) were selected to complete the study for the following steps.

3.4. Evaluating Growth Curve (Growth Kinetics)

The stationary phase of the bacterial growth curve is identified as the period that yields the largest quantity of secondary metabolites, as noted by [17]. A malt extract yeast extract broth ISP2 was used to cultivate the isolated KH14 in order to monitor growth through the measurement of absorbance and to detect the stationary phase at an optical density (OD) of 600 nm. After 24 hours of incubation with the initial inoculum, the growth activity of the isolate KH14 is depicted in Figure (3). The isolate completed its lag and exponential (log) phases within the first two days of growth curve, with the subsequent progression into the stationary phase optical density reaching a maximum of 0.619 on the third day of incubation. On the first day, the OD was approximately 0.067, and on the second day, it reached 0.315. After three days of incubation, the isolate would be able to begin producing secondary metabolites since it had entered the stationary

phase, whereas Figure () shows that the curve would start to decline after three days of incubation. The findings concurred with those reported by [20] about maximal growth after 3 days of incubation, [33] indicating a maximum at 72 hours maintained until 129 hours; [34] recorded a maximum after 3 days. Conversely, the research in [17] indicated that isolate MJA 1105 entered the stationary phase by the fourth day. Upon reaching the stationary phase in four days and concluding the incubation period in six days, [34] likewise achieved the same outcomes in their investigation with GZO24. At log phase, when the isolate began generating antimicrobial metabolites, the concentrated duration in the stationary period activity was discovered. The focused activity was observed in the stationary period. Kojiri et al. (1992) documented peak activity at 6 days [36] commenced at 7 days and extended to 9 days, whereas [36] initiated at 7 days and persisted until 10 days of incubation.

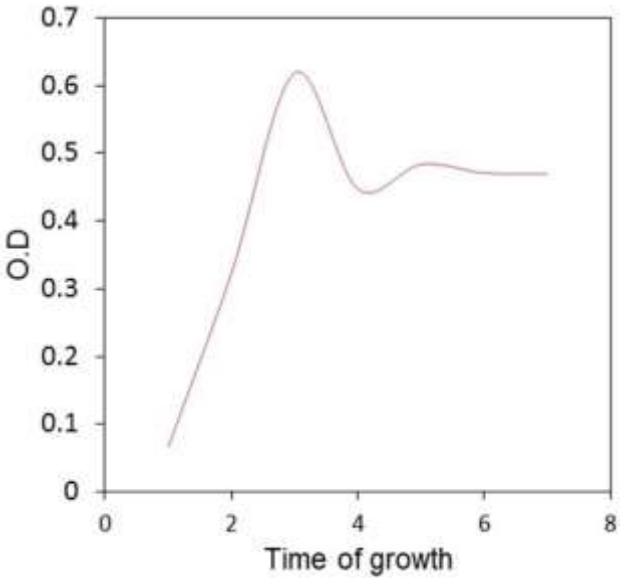


Figure 3: Growth kinetic curve of actinomycetes isolated locally, cultivating ISP2 for six days

3.5. Physical characteristics of the secondary metabolites substance

The **secondary metabolites** extracts had different physical characteristics as show in table (5). The results in table (5) appeared that secondary metabolites extract appeared in NI- RS 15 isolates had white color high solubility with water methanol, ethanol and ethyl acetate and powder texture. The melting point of antibacterial substance was 287ºC and black material remained after this test methanol, ethanol and ethyl acetate and powder texture. The melting point of secondary metabolites substance was 287ºC and black material remained after this test .The secondary metabolites extract of AM - AG 18 isolates had white color high solubility with water, methanol and ethyl acetate but not in ethanol the melting point of secondary metabolites substance was 300°C. The extra cellular extract of MA-CL2 had white to yellowish color high solubility with water, methanol and ethyl acetate but not in ethanol the melting point of antibacterial substance was 295°C.

Table (6). Physical characteristics of Actinomycetes extracts

Actinomycetes isolates	Physical characteristics of extracts						
	Color	Solubility				melting point	Natural
		water	methanol	ethanol	ethyl acetate		
NI- RS 15	white	+	+	+	+	287ºC	Powder
AM - AG 18	white	+	+	-	+	300°C.	Powder
MA-CL2	White to yellowish	+		-		295°C	Powder

Numerous researchers employed various solvents to extract antibiotics, and these secondary metabolites' capacity to dissolve in solvents varied according to their compositions, structures, and presence of hydrophilic or hydrophobic groups; still, the antibiotic associated with Glycopeptide groups are more soluble in highly polar solvents like methanol, ethanol, and acetone than in solvents with low or intermediate polarity like ethyl acetate or chloroform (Oskay, 2011). Some antibiotics that are generated from Actinomycetes and include an aminoglycoside group are soluble in methanol or any other solvent with a high polarity. On the other hand, other antibiotics, such as ethyl acetate, are more soluble in solvents with a medium polarity [37, 38]. Secondary metabolites exhibited varying colors and distinct physical properties based on their composition, including the presence of carbohydrate and peptide moieties, with the antibacterial agents potentially associated with aminoglycoside or glycopeptide groups [39].

3.6. Chemical Characterization of secondary metabolites

To measure the λ max for secondary metabolites, which ranged from 274 nm to 320 nm, ultraviolet (UV) spectroscopy was employed. This provides insight into the kinds of these substances. (Figures 4-6).

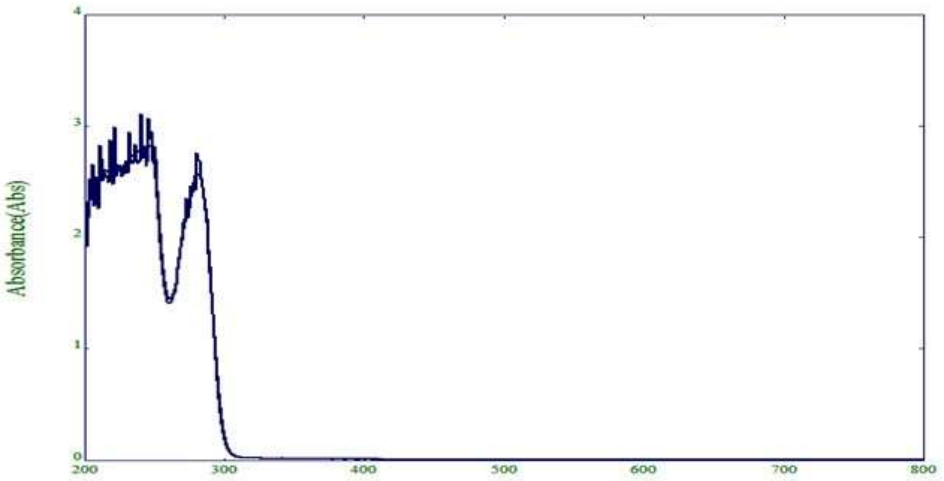


Figure 4: show the λ max was 281.00nm. For NI- RS 15 isolate

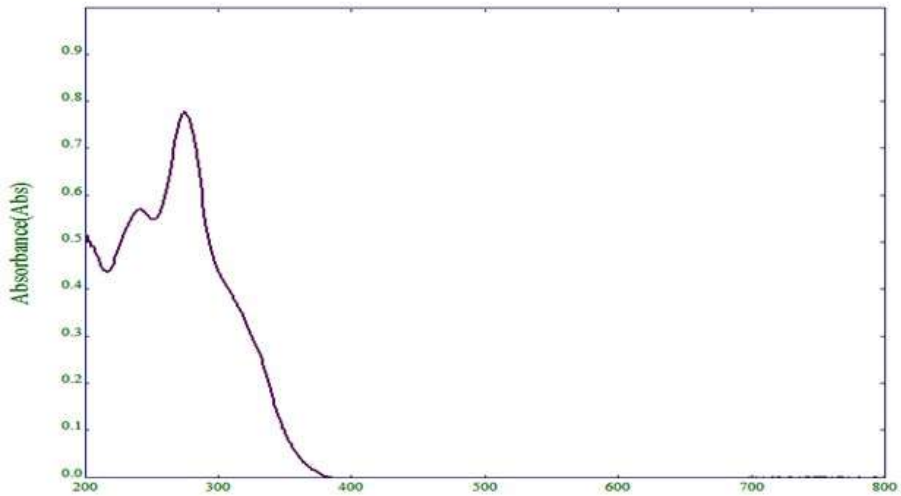


Figure 5: show the λ max was275. 00.nm. For MA-CL2 isolate

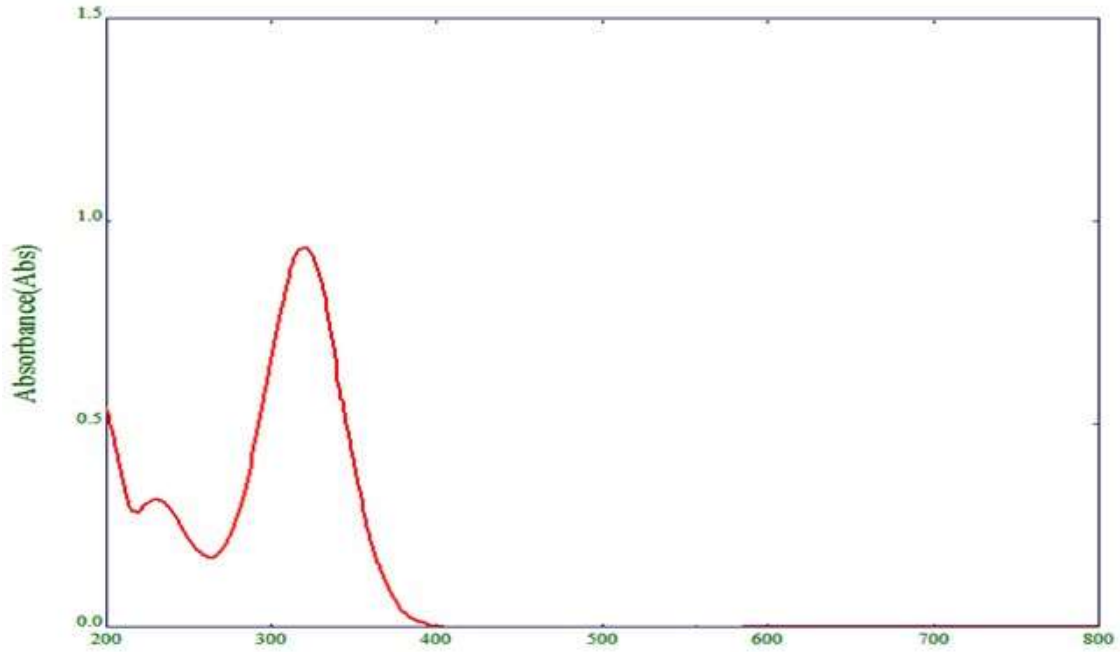


Figure 6: show the λ max was320. 00.nm. For AM - AG 18 isolate

Actinomycetes spp. isolates' secondary metabolite characterisation using FT-IR spectroscopy For the purpose of identifying the significant chemical functional groups, Infrared Red (FT-IR) spectroscopy was employed found in secondary metabolisms that are created (Figures 7-9).

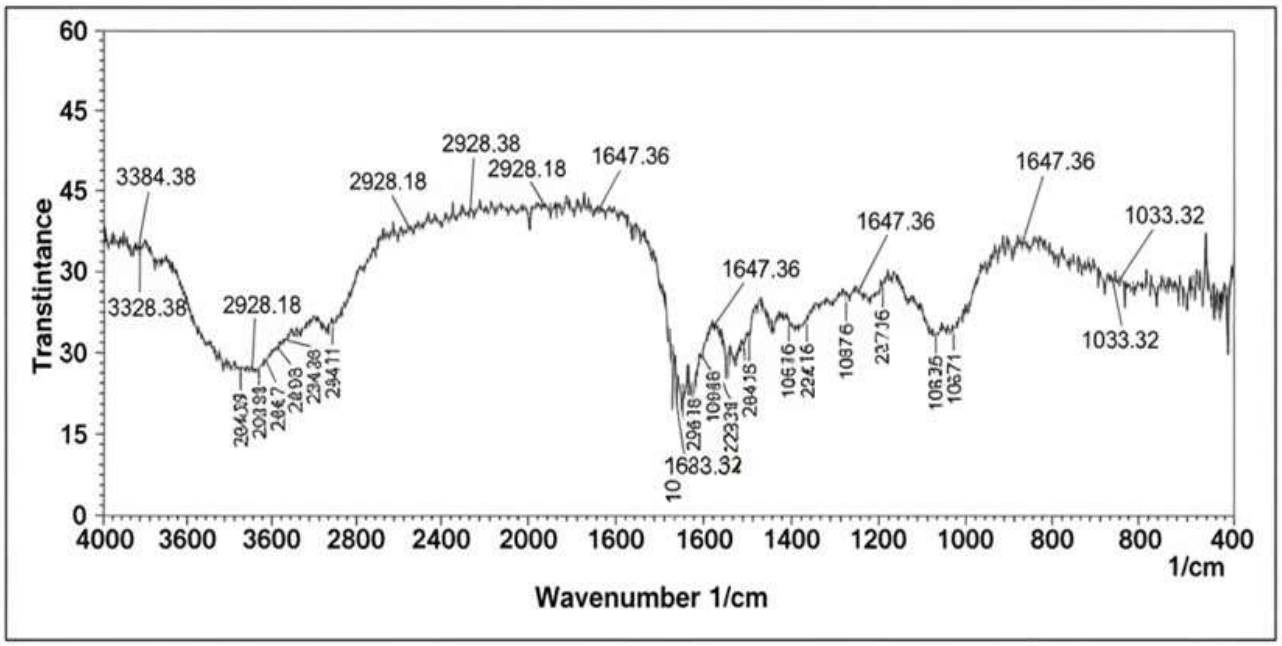


Figure 7: Infrared Red spectrum for Actinomycetes isolate NI- RS 15 extracellular.

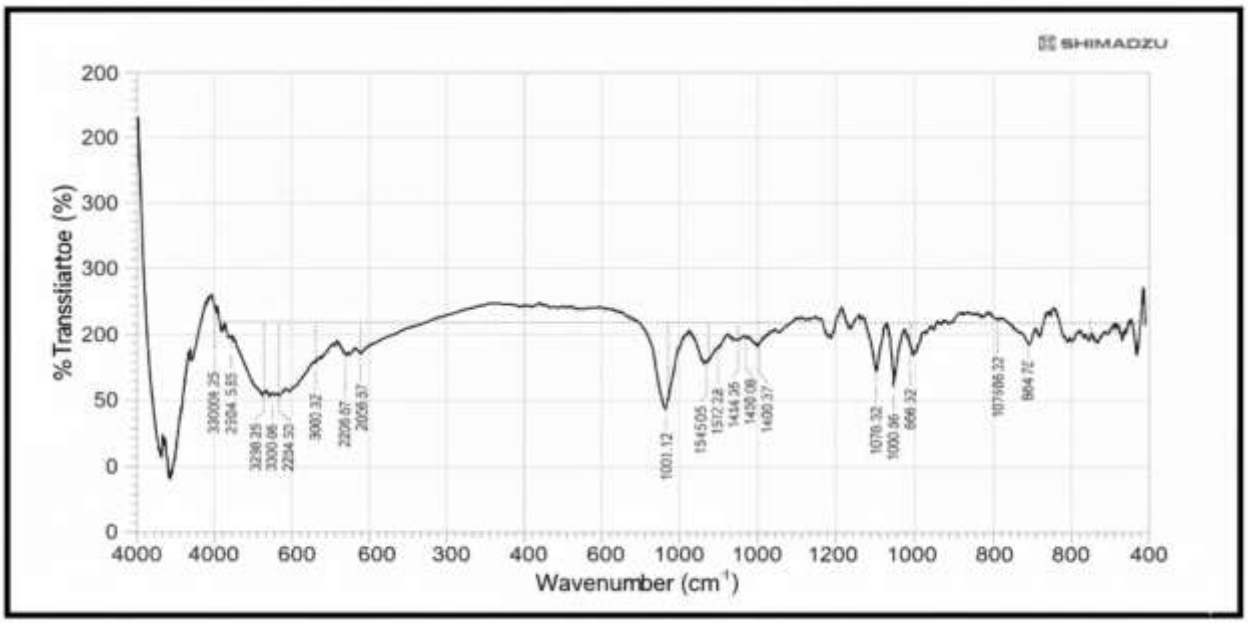


Figure 8: Infrared Red spectrum for MA-CL2 isolate

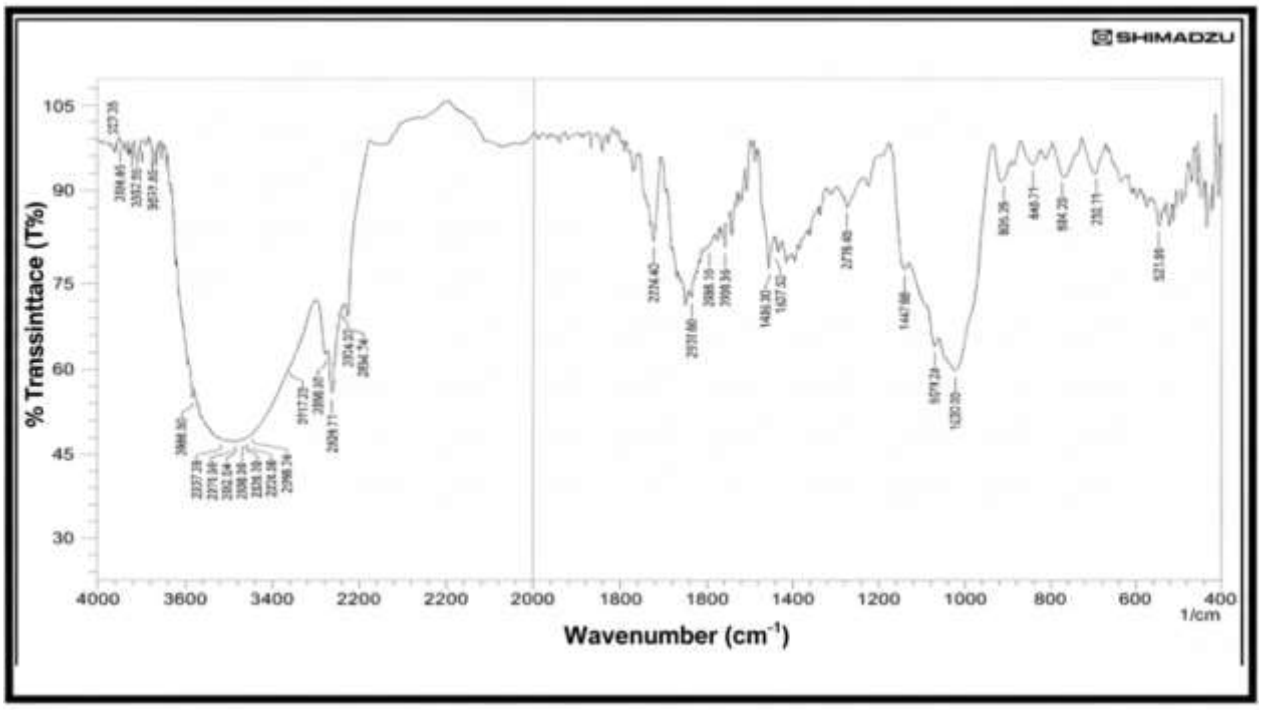


Figure 9: Infrared Red spectrum for AM - AG 18 isolate

Using its array of photodiodes, High Performance Liquid Chromatography was utilized to identify and detect chemicals in the samples for the ethyl acetate extract as show in figures (10,11,12) and tables(6,7,8). whereby the samples were analyzed using light from an HPLC-UV detector by detecting the sample's absorption of UV light at various retention times and creating a Figure (relation) between X- axis that represented the retention duration in relation to the Y-axis, which represented the absorption units (AU).

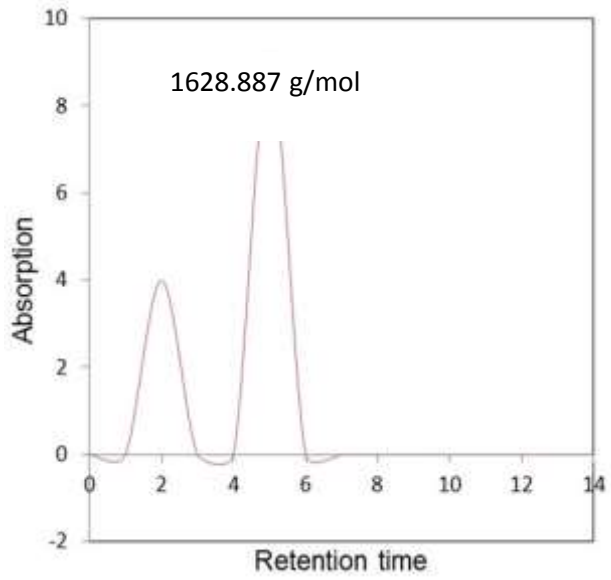


Figure.10 : HPLC spectrum of a secondary metabolisms of ethyl acetate extract from isolate NI- RS 15

Table 7: Profile of using HPLC to diagnose secondary metabolisms extracted from NI- RS 15 isolate

Type of extract	No.of Peak	Reten. Time [min]	Area [mAU.s]	Height [mAu]	Area%	Height %	W05 min
ethyl acetate extract	1	2.1	52360.89	590.24	85.00	85.00	0.25
	2	5.0	22147.63	360.44	15.00	15.00	0.15
		Total	74508.52	950.44	100.00	100.00	

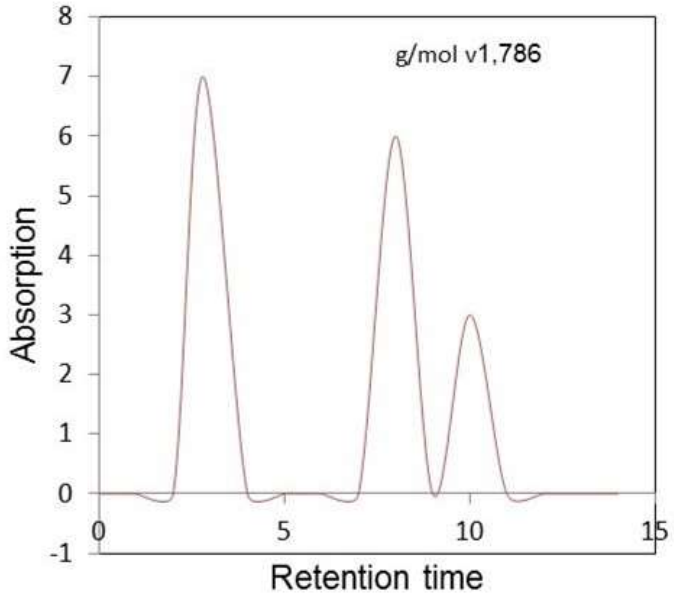


Figure.11 : HPLC spectrum of a secondary metabolisms extracted for ethyl acetate extract from MA-CL2 isolate

Table 8: Profile of using HPLC to diagnose secondary metabolisms extracted from MA-CL2 isolate

Type of extract	No. of peak	Reten. Time [min]	Area [mAU.s]	Height [mAu]	Area%	Height%	W05 min
ethyl acetate extract	1	2.8	50360.85	590.24	85.00	85.00	0.25
	2	8	21147.60	320.44	10.00	10.00	0.15
	3	9.9	10021.01	224.01	5.00	5.00	0.15
		Total	71629.56	1134.69	100.00	100.00	

Following the extraction of secondary metabolisms from Actinomycetes spp fermentation cultures. Measured the λ max for these **secondary metabolisms** using ultraviolet light and discovered that the λ max ranged from 274 nm to 320 nm. It is then possible to establish a correlation between the types of bonds present in a particular molecule and the peaks that are produced as a consequence of analyzing the structure of materials. These peaks are extremely useful in establishing the functional groups that are contained inside a molecule [40]. These findings were corroborated by [41], who determined that the λ max of two antibacterial extracts for Actinomycetes isolated from soils in Babylon city was 210 nm and 247 nm for these strains [41]. Additionally, the present findings were in agreement with those of [42], The individuals who conducted the measurement of the λ max for antibiotic extracts in Actinomycetes spp isolates came to the conclusion that the range of λ max was between 215 and 320 nm.

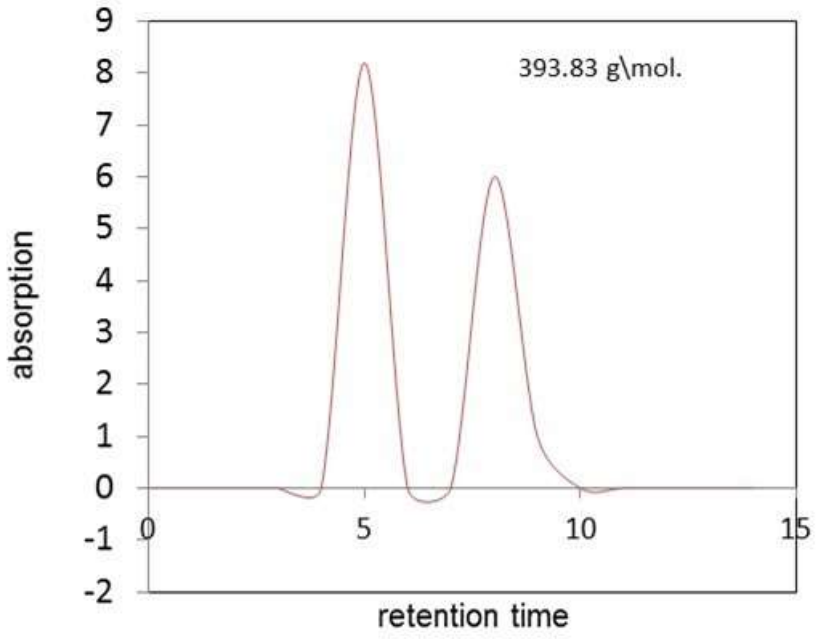


Figure.12 : HPLC spectrum of a secondary metabolisms extracted for ethyl acetate extract from AM - AG 18 isolate

Table 9: Profile of using HPLC to diagnose secondary metabolisms extracted from

Type of extract	No.of peak	Reten. Time [min]	Area [mAU.s]	Height [mAu]	Area%	Height%	W05 min
ethyl acetate extract	1	5.2	52360.89	590.24	85.00	75.00	0.25
	2	8.6	22147.63	360.44	15.00	25.00	0.15
		Total	74508.52	950.44	100.00	100.00	

The FTIR spectra revealed multiple absorption bands, each corresponding to specific functional chemical groups. These are interpreted as follows: the absorption bands observed in the range of 3352.39 cm-1 to 3148.88 cm-1 are attributed to the stretching vibrations of free O—H groups, typically found in alcohol and phenols. Absorption band 2926.11 cm −1 refer to C-H (Alkanes stretch) or O-H (carboxylic acid. Absorption band 1647.26 cm−1 cm−1 refer to N-H (primary and secondary amines and amides stretch)). Absorption band 1381.08 cm−1 refer to C-X (fluoride Absorption band 1234.48 refer to C-O (alcohols, ethers, carboxylic acids. anhydrides). Absorption band 1076.32 cm −1 - 1037.74 cm −1 refer to N- H (Amines) or C-O (alcohols, ethers, carboxylic acids, anhydrides [46]. The results presented in Figures (10-12) The UV chromatogram of the ethyl acetate extract exhibited two distinct sharp peaks at 2.1 and 5.0 minutes. The MA-CL2 isolate displayed three prominent sharp peaks at 2.8, 8.0, and 9.9 minutes. Additionally, two sharp peaks were observed at 5.2 and 8.5 minutes, indicating a well-separated mixture at the specified times, with clear absorption units detected by the UV detector [47]. The cytotoxic effect of the extract on two normal cell lines was determined .Non tumorigenic fetal hepatic cell line (WRL 68)) and (Dermal Fibroblast Normal Human Neonatal (HdFn)... This was followed by the method [22]. As we notice in the figures (12), the IC50 is very high and higher than the concentrations used for all the extracts obtained, whereas the lowest IC50 (395.64) against HdFn cells was for the NI-RS 15 extracts.

Table 10: Cytotoxic effect of AM - AG 18 extract on normal cell(HdFn) and WRL 68

Cell viability				
AM - AG 18 extract	HdFn		WRL 68	
Conc.(µg/ml)	mean	SD	Mean	SD
200	86.07267	3.076421	88.69133	2.552461
100	90.08467	1.049913	89.19733	2.411951
50	93.86533	1.104054	93.98167	0.530051
25	94.63733	0.481986	94.67567	0.601599
Control	Dimethyl Sulfoxide (DMSO) 100% viability			

Table 11: Cytotoxic effect of MA-CL2 extract on normal cell(HdFn) and WRL 68

Cell viability				
MA-CL2	HdFn		WRL 68	
Conc.(µg/ml)	mean	SD	mean	SD
200	90.07447	1.02221	90.19833	2.234571
100	92.08345	1.00813	95.13463	2.52871
50	95.44876	1.43298	96.32912	0.410051
25	99.60903	0.481900	98.39712	0.501229
Control	Dimethyl Sulfoxide (DMSO) 100% viability			

Table 12: Cytotoxic effect of NI- RS 15 extract on normal cell(HdFn) and WRL 68

Cell viability				
NI- RS 15 extract	HdFn		WRL 68	
Conc.(µg/ml)	mean	SD	mean	SD
200	87.07864	2.07876	88.76543	2.52912
100	94.08467	1.87654	92.15567	1.27543
50	96.34786	1.02378	97.90217	0.39712
25	98.64221	0.30123	99.60098	0.45682
Control	Dimethyl Sulfoxide (DMSO) 100% viability			

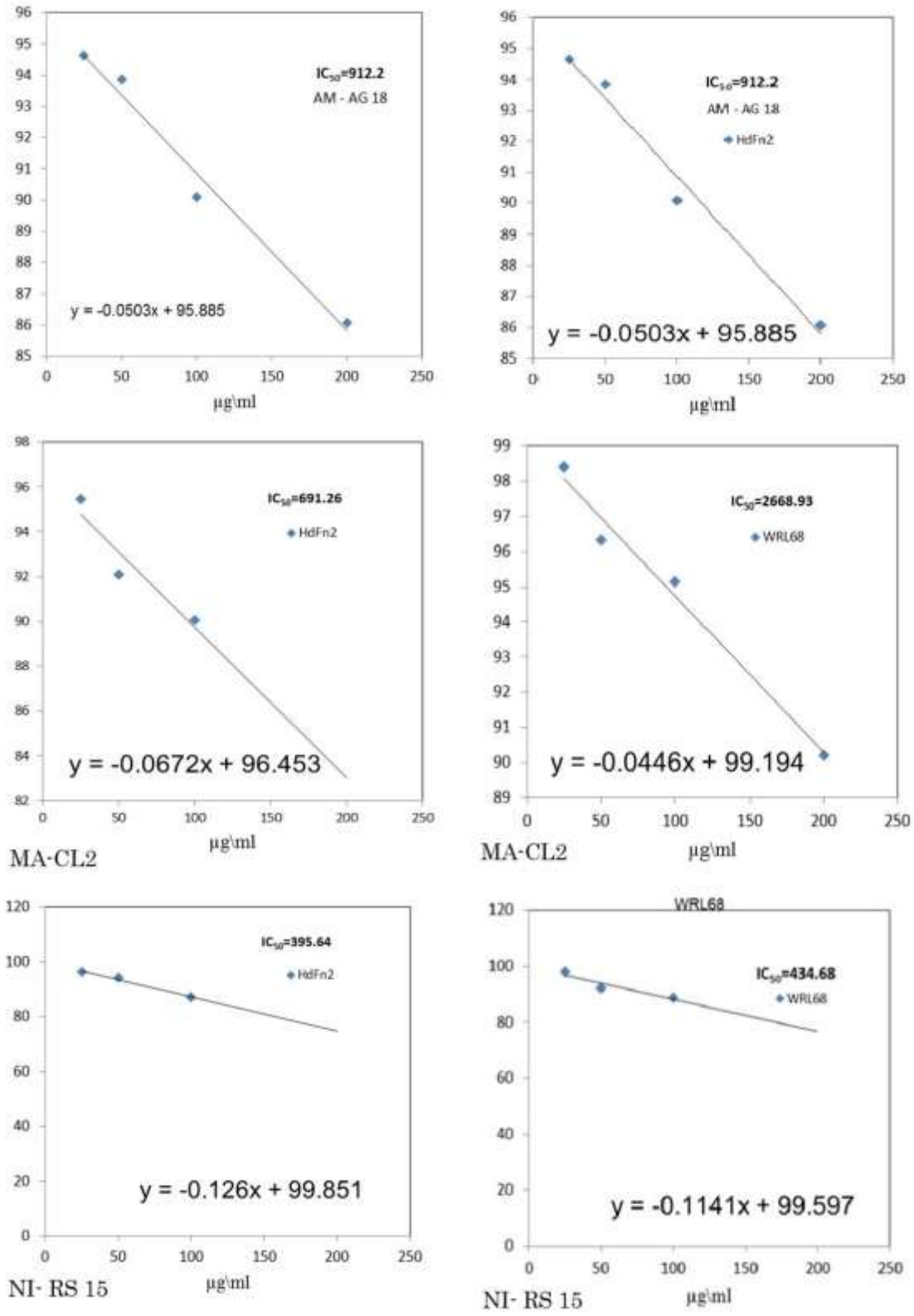


Figure 12: IC50 and Cell viability

Then, statistical analysis of the optical density readings was performed to calculate the IC_{50}. The effect of the extract was very low or almost non-existent against cell lines, as shown in Tables 9, 10, and 11. Secondary metabolisms has been shown to have little effect on the normal cell line (even at greater concentrations, which makes it an excellent to use in other biological activity. According to study of [48] the categorization of chemicals based on their observed cellular damage at the individual cell level was crucial in identifying toxicity, utilizing flow cytometry and cellular imaging techniques.These findings concurred with study of [49]. The human breast cancer cell line 7cell line and the non-tumorigenic fetal MCF hepatic cell line were utilized to illustrate the cytotoxicity of bioactive chemicals derived from rare Actinobacteria. WRL- The effect of our product on natural cellsWRL-68) at a concentration of 100 was (7.5%) less than the effect of study done by Omran et al. which was 7.6% [49].

4. Conclusions

After the systematic screening of various soil types across Hilla city, multiple isolates of Actinomycetes spp., with diverse morphological and bio-active properties were successfully obtained. Many of these isolates demonstrated a significant capacity to produce secondary metabolites, achieving optimal production during the stationary phase, which commenced on the third day of incubation Importantly. Cytotoxicity essays conducted on normal human cell lines (WRL 68 and HdFn) demonstrated that the extracts exhibited very low to negligible toxicity, with IC50 values exceeding the tested concentrations (up to 200 µg/mL). The high cell viability observed across both cell lines indicates that the secondary metabolites possess a favorable safety profile. This suggests their promising potential for future development in pharmaceutical and therapeutic applications, with minimal cytotoxic risk to normal cells

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