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ايمان محمد جار الله

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Antibacterial Activity of Actinomycetes spp. Extract on *Pseudomonas aeruginosa* Isolated from Clinical Samples

A Thesis

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By

Zainab Hasan Abed Alwan

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Msc. / Microbiology / 2019

Supervised by

Prof. Dr. Eman Mohammad Jarallah

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الخلاصة

تم جمع 40عينة من الترب الزراعية بشكل عشوائي من مناطق مختلفة من محافظة بابل.وقد درست بعض خصائص التربة الفيزيوكيميائية مثل دالة الحموضة ، حرارة التربة. وكانت خصائص التربة مختلفة بين عينات الترب ولكن جميعها ضمن حدود نمو وتكاثر البكتيريا الشعاعية . Actinomycetesمن مجموع 40عينة تربة تم عزل 68عزلة من البكتيريا الشعاعية Actinomycetes.نقيت هذه العزلات بالزرع المتكرر لعدة مرات على وسط استخلاص الستربتومايسيس *Streptomyces* الدولي النوع الثاني(ISP2) . وقد درست خصائص هذة العزلات بالاعتماد على الفحوصات البايوكيميائية وقد اظهرت النتائج انها موجبة لصبغة جرام ولها القابلية على افراز بعض الانزيمات مثل (الكاتليز) شخصت العزلات جزيئيا باستخدام تقنية تفاعل انزيم البلمرة المتسلسل واكدت النتائج ان 23 عزلة تابعة لجنس .*Streptomyces spp* .

درست الفعالية المضادة للبكتيريا لهذه العزلات ضد 3 انواع من البكتيريا السالبة لصبغة غرام وهي *Klebsilla pneumonie, Escherichiag coli, Pseudomonas aeruginosa* بواسطة طريقة التخطيط العمودي على وسط مولر- هنتون الصلب وقد اظهرت 7 عزلات من بكتريا الستربتومايسس فعالية مضادة للبكتيريا الممرضة .

ومن ناحية اخرى تم جمع 150عينة من كلا الجنسين باختلاف الأعمار من مستشفيات بابل تنوعت بين مسحات من الجروح والحروق ومن الادرار ومسحات من الاذن ونتائج العزل اظهرت نسبة عالية من بكتريا الزائفة الزنجارية (50 عزلة) توزعت كالاتي 50/14 (28%) مرضى التهاب المجاري البولية والجروح 50/6 (12%) والحروق 50/26 (52%) ومرضى التهاب الاذن الوسطى 50/4 (8%) وشخصت اعتمادا على الصفات المضهرية، الزرعية، المجهرية، الاختبارات الكيموحيوية و compact 2 Vitek . اكد تشخيص *P. aeruginosa* باستخدام تقنية تفاعل البلمرة المتسلسل (PCR) للجين النوعي *P. aeruginosa* ، وكانت مطابقة بنسبة 100 % مع النتائج التي تم الحصول عليها من فحوصات الصفات المظهرية، الزرعية، المجهرية، الاختبارات الكيموحيوية .

تم فحص حساسية المضادات الحيوية ل 17 نوعًا من المضادات الحيوية ، باستخدام طريقة نشر القرص . تم اختيار العزلات التي ابدت مقاومة لاغلب انواع المضادات وتم اختبار بكتريا الستربتومايسس ومستخلصها على هذه العزلات المقاومة وابدت تاثير متباين . فقد اظهرت اربع

عزلات من العزلات السبعة فعالية عالية ضد بكتريا الزائفة الزنجارية متعددة المقاومة للمضادات . وكما تبين ان اقل تركيز مثبط للبكتيرياMIC واقل تركيز قاتل للبكترياMBC كان يتراوح بين 0,625 - 0.312 مايكرو غرام /مل لكل عزلات الزائفة الزنجارية المتعددة المقاومة للمضادات و قد تم توصيف العوامل المضادة للبكتيريا المنتجة من العزلات قيد الدراسة بواسطة تحديد المجاميع الفعالة الموجوده فيها بطريقتين هي مطياف الاشعة فوق البنفسجية لقياس اعلى امتصاص maxللمضادات الحيوية المنتجة حيث تراوح معدل الامتصاص ما بين (210) الى nm(250) و مطياف الاشعة تحت الحمراء وقد استخدم بمدى من 400 الى (4000) cm-1 وقد اعطت نتائج هذا الفحص انواع مختلفة من القمم وان كل مسافة بين قمتين تعبر عن مجموعة كيميائية معينة . ثالثا التحليل بواسطة جهاز GC-MS للكشف عن نوع المركبات الكيميائية الموجودة بالمستخلص .

اضافة الى ذلك فقد درست الفعالية المضادة ضد خلايا السرطان عن طريق الفحص MTTوقد اظهرت النتائج ان العوامل المستخلصة من العزلة st12 تمتلك تأثير ضد الخط السرطاني المستخدم A375 (خلايا سرطان الجلد) وذلك بتثبيط 71.61 % عند تركيز (400مايكروغرام/مل) من الخلايا السرطانية بعد 72ساعة من الحضن . وتثبيط 69.95 % (400مايكرو غرام / مل) من الخلايا السرطانية .وان هذا التأثير تم مقارنته مع عوامل السيطرة التي اظهرت فرق كبير. وكما تم تقدير الفعالية السمية للعوامل الحيوية المستخلصة من العزلتين st6 و st12 عن طريق فحص MTT وتم دراسة تأثيرها على الخلايا الليفية البشرية الطبيعية (HDFn) حيث تبين ان مستخلص العزلة st12 ثبط بنسبة 4.79٪ فقط (25ميكروغرام / مل) من الخلاية الطبيعية اما كمية الخلايا التي تم تثبيطها بواسطة مستخلص العزلة st6 كانت 6.18٪ (25 ميكروغرام / مل) .

من ثم تم ايداع تتابعات منطقة 16S rRNA من *Streptomyces spp.* لعزلتين محلية في قاعدة بنك الجينات العالمي تحت ارقام النظام [OQ119140.1 و OQ119141.1] .

Summary

A total of 40 samples of agricultural soil were collected randomly from different areas of Babylon Governorate. The physico-chemical properties such as pH and soil temperature of the soil samples were studied . Soil characteristics were different among soil samples, but all were within the limits of growth and reproduction of Actinomycetes. Out of the 40 soil samples, 68 isolates of Actinomycetes were obtained . these isolstes were purified by sub-culturing many time on International *Streptomyces* project-2 agar medium (ISP-2). These isolates were identified by culture properties and biochemical tests which showed that they were Gram positive and had the ability to release some enzymes such as (catalase, oxidase). 23 of these isolates were identified as *Streptomyces* and this identification was confirmed by *16S rRNA* PCR amplification. All of *Actinomycetes* isolates were tested for their antibacterial activity against 3 Gram negative pathogenic bacterial (*Klebsilla pneumonie, Escherichia coli, Pseudomonas aeruginosa,*) by perpendicular streak method on Muller - Hinton agar. Results were showed that 7 *streptomyces spp.* isolates were posess antibacterial activity against pathogenic bacteria.

On the other hand , 150 clinical specimens of both genders and different ages were collected from Babylon hospitals , The results of isolation revealed high percentage of *P. aeruginosa* 50 isolates distributed as UTIs patients 14/50 (28%) , burn infection patients 26/50 (52%), wounds infection 6/50 (12%) while 4/50 (8%) for otitis media , *Pseudomonas aeruginosa* using morphological, cultureal and microscopical properties, biochemical tests, and Vitek 2 compact . The identification of *P. aeruginosa*

was confirmed using polymerase chain reaction (PCR) 16S rRNA . The antibiotic susceptibility test was performed against 17 types of antibiotics, using the disc diffusion method . The isolates that showed resistance to most types of antibiotics were selected, and *Streptomyces spp.* and its extract were tested on these resistant isolates, and they showed a variable effect. 4 isolates show high anibacterial activity against MDR *P. aeruginosa* The results showed that the minimum concentration inhibitors (MICs) and the minimum bactericidal concentration (MBCs) ranged from 0.312-0.625µg/ml for selected isolates of MDR *P. aeruginosa* .

The antibacterial agents that produced by these isolates were characterized by three different methods; First: was the ultraviolet spectroscopy(UV) for measuring the λmax for producing antibacterial, the results showed the ranged of this assay was from (210) to (250) nm. Second: infrared spectroscopy (FT-IR) was used in the range from (400) to (4000) cm-1. The results were showed different types of peaks and any region between two peaks refer to specific chemical group that mean the chemical functional groups were present in produced antibiotics . third by GC-MS analysis which revealed the presence of a variety of chemical substances.

The antitumor activities of these extract were determined by MTT assay, which indicated that extract of st12 isolate possesses a powerful effect against used cancerous cell line (A375) by inhibiting 71.61% of them at (400 µg concentration) of cancer cells after 72 hours of incubation and inhibiting 69.95 % of cancer cells by extract of st6 isolate . This effect was compared with control factors that showed significant difference. The

cytotoxicity of st6 and st12 extracts were assessed by MTT on human normal fibroblast (HDFn)was investigated. The extract of st12 isolate was only inhibited 4.79% (25 µg / mL) of normal cell and the amount of cells that were inhibited by extract of st6 isolate were 6.18 % (25 µg / mL)

The two isolates were sequences for the 16S rRNA region from *Streptomyces* spp obtained in this study were deposited in the GenBank sequence database under the accession numbers [OQ119140.1 and OQ119141.1] .

بِسْمِ اللَّـهِ الرَّحْمَـٰنِ الرَّحِيمِ

" وَعَلَّمَكَ مَا لَمْ تَكُنْ تَعْلَمُ ۚ وَكَانَ فَضْلُ اللَّهِ عَلَيْكَ عَظِيمًا "

صدق الله العلي العظيم

النساء (١١٣)

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Four: List of Abbreviations

Symbol	Mean
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide	MTT
4-(2-Hydroxyethyl)-1piperazine-Ethane Sulfonic Acid	HEPES
ATP	Adenosine Triphosphate
CAP	Community-Aquired Pneumonia

D.W	Distal water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribosenucleic Acid
EC	Electric conduction
EDTA	Ethylene-Diamine-Tetra Acetic Acid
ESBL	Extended-spectrum β- Lactamase
FT-IR	Fourier Transform-Infrared
FT-IR	Fourier- Transform Infrared Spectroscopy
G+C	Guanine + Cytosine
GC-MS	Gas-Chromotography- Mass Spectroscopy
HdFn	Dermal Fibroblast Normal Human Neonatal
ISP	International Streptomyces project
IU	International Unit
MBC	Minimum Bactericidal concentration
MBC	Minimum Bactericidal Concentrations
MDR	Multi-Drug Resistant
MIC	Minimum inhibition concentration
MIC	Minimum Inhibitory Concentrations
MRSA	Methicillin -resistant *Staphylococcus aureus*
No	Number
PBS	Phosphate Buffer Saline
pH	Power of Hydrogen Ion
PTA	Peptone tween agar
RPMI	Roswell Park Memorial Institute
rRNA	Ribosomal Ribosenucleic Acid
SDS	Sodium dodecyl sulfate
TE	Tris-EDTAbuffer
UV	Ultraviolet

v/v	Volume/Volume
VRSA	Vancomycin - resistant *Staphylococcus aureus*
w/v	Weight/ Volume

Five : List of Appendices

Introduction

Actinomycetes are a diverse genus of bacteria that form filaments in the soil that resemble threads .They are widely distributed in the natural environment , They are members of the phylum Actinobateria, one of the 18 primary lineages that has been identified in the bacterial domain with the most variations within the soil category , and involved in a variety of biological and metabolic activities, such as the production of extracellular enzymes (Sapkota *et al*., 2020) . The contribution is significant under unfavourable conditions , such as saline and alkaline habitats, drought stress, and high temperatures. The cellular characteristics (i.e., Gram-positive, elongated cells forming filamentous or hyphal structures, and spores' formation) and metabolic versatility allow these bacteria to be present and survive in a wide range of soil environments (Farda *et al*., 2022)

Moreover, almost 90% of the Actinomycetes genera have been isolated from soil and are safe for use in a variety of industries, including the pharmaceutical and industrial sectors . Futhermore , actinomycetes secrete red, green, yellow, brown, and black instinctive pigments on the media (Bawazir, & Shantaram, 2018) . The common characteristics of actinomycetes exist as free , spore forming , saprophytic microorganisms found broadly scattered in the soil , colonizing the plants and present in water . At the beginning the actinomycetes were recognized primarily on their morphological criteria , furthermore , actinomycetes taxonomy was in the past thought to be associated with their morphology that is insufficient for differentiating between different related species and among a lot of genera. In addition, the phylogenetic and molecular approaches when applied have been given a huge impact to facilitie their classification methods (Hozzein and Goodfellow, 2011)

Collectively, both culture and microscopic characteristics help researchers to organize the members of actinomycetes till reaching the genus such as *Streptomyces* genus. Numerous studies have been accepted out where the actinomycetes isolates were recognized as a species of *Streptomyces* based on these properties and /or

characteristics (Kekuda *et al.,* 2012) . Molecular studies of *Streptomyces spp*. are more specific for identification of these bacteria , and identification of the *Streptomyces spp*. is commonly derived from 16S rDNA by use of polymerase chain reaction (Isik *et al*., 2014).

Nocardia and *Micromonospora*, which are inferior in nature to Streptomyces in their ability to create antibiotics, are thought to follow Streptomyces as possible species that encompass antibiotic synthesis (Carey *et al.*, 2018) . The isolation and screening of actinomycetes that produce antimicrobials has been the subject of numerous investigations. According to a theory put out by researchers, screening soil isolates led to the discovery of the majority of new antibiotics (Baniya *et al.,* 2018). the increase resistance rates for antibiotic from pathogenic bacteria especially G-ve bacteria such as *Pseudomonas aeruginosa*, which is harmful to the health of the vast community because of the introduction of diseases that are multidrug resistant . Furthermore, it has been a significant obstacle to the treatment of infectious diseases, requiring further research to find novel antibiotics that can assist manage the issue (Pathalam *et al.,* 2017) . Furthermore, the study of Suthindhiran and Kannabiran (2010) and Raja and Prabakarana (2011) explain that the actinomycetes which produced bioactive compounds, and their produced compounds are of diverse components including anthracyclins, glycopeptides, aminoglycosides, macrolides, polyenes, β-lactums, peptides, nucleosides, terpenes, polyethers and tetracyclines, which have a broad variety of biological activities .

Numerous techniques and analytical systems, such as gas chromatography-mass spectrometry (GC-MS) analyses, Fourier transform-infrared spectrum (FT-IR) , nuclear magnetic resonance (H-NMR) , and mass spectrometry (MS), have been developed for the analysis and characterization of active compounds from microorganisms.

The ultraviolet (UV) assay is important in determines of antibiotics produced by *Streptomyces* spp.. Whereas the infrared spectroscopy (FT-IR) used for purification of

antibiotics that produced by *Streptomyces* spp This assay gives idea about functional groups that present in the antibiotics molecules and (GC-MS) It reveals the type of chemical compounds present in the extract of bacteria . (Sanghvi *et al*., 2014).

Identifying the toxicity of new antibiotic is considered to be an important step before starting marketing and consuming the new antibiotic. Many procedures are currently used to detect the level of cytotoxicity of compounds or drugs. Multi-parametric analysis is one of the valued approaches using flow cytometry and cellular imaging–based techniques such as high-content screening (HCS) to detect the level of compound toxicity and classification of compounds based on observed patterns of reversible and irreversible cellular injury (Abraham *et al*., 2008).

Aim of the Study:

The current study aimed to evaluate antimicrobial activity of bioactive agents – producing Actinomycetes extracts on *Pseudomonas aeruginosa* isolated from clinical samples .

Objectives of study:

1- Isolation and identification of the Actinomycetes isolates .

2- Extraction and partial purification of crude bioactive agents .

3- Isolation and identification of *Pseudomonas aeruginosa* from clinical samples.

4- Study antibiotics susceptibility profile of *Pseudomonas aeruginosa* isolates .

5- Evaluation of the antibacterial activities of extracellular crude extracts against MDR *Pseudomonas aeruginosa* resistant isolates .

7- Exploration the safety of secondary metabolite produced by evaluating its cytotoxicity property.

8- Determining the antitumor activity of the extracellular crude extract via MTT assay against cancer cell lines

2. Chapter Two: Literatures Review

2.1: General Characteristics of Actinomycetes

Actinomycetes are aerobic gram-positive bacteria that form spore ; have a high guanine cytosine (57-75%) in their genomes , they are members of the order Actinomycetales, which is distinguished by substrate and aerial mycelium growth . They have true aerial hyphae and are filamentous like fungi. Actinomycetes are widespread and constitute a steady population in a variety of environments, particularly in the soil , where they predominate in arid, alkaline soil. They demonstrate a wide range of life cycles that are distinct from those of prokaryotes and appear to be crucial for the cycling of organic materials in the soil ecosystem (Bhatti *et al*., 2017). The majority of actinomycetes in the soil are neutrophils, growing well between pH (5.0 and 9.0) (Goodfellow *et al*., 1983) .The word "Actinomycetes" originally came from the Greek words "attacks," which means "a ray," and "mykes," which means "fungus," therefore it shares traits with both fungi and bacteria (Das *et al*., 2008). Actinomycetes have been viewed as a group well apart from typical bacteria because of their highly developed cultural and morphological traits (Das *et al*.,2006).

These intricate bacteria are prokaryotic creatures. Although they lack a nuclear membrane, they have a cell wall. They can divide by fissions or conidia when they form spores. Actinomycetes are known as filamentous bacteria because, when they grow, they produce branching filaments or hyphae that resemble the mycelia of fungi. Despite this, their filaments are smaller than those of fungi, with a diameter of only one micron (μ). This set the Actinomycetes apart from the fungi (Buller, 2014).

These bacteria are found in all types of natural sources, , but soil is where they are most commonly found . The primary natural habitat is the

soil, which is complex and changeable in terms of nutrition, biology, and physical properties. As a result, they can perform a variety of metabolic activities, such as producing a wide variety of bioactive secondary metabolites, such as antibiotics (Anderson and Wellington., 2001). The actinomycetes have the potential to develop antibiotics and other substances with medicinal use. Actinomycetes produce a variety of bioactive secondary metabolites, such as enzymes, antibiotics, anticancer, and immunosuppressive compounds. There is knowledge that these metabolites have neuritogenic, anti-cancer, antibacterial, antifungal, antioxidant anti-helminthic , anti-malarial, anti-inflammatory, and anti-algal (Kekuda *et al.*, 2010; Ravikumar *et al.*, 2011).

They exhibit a variety of life cycles that are distinct from those of other prokaryotes, and they seem to be crucial to the cycling of organic materials in the soil ecosystem (Veiga *et al.*, 1983) . The most prevalent organisms that form filaments in the soil are actinomycetes. They grow as hyphae like fungi responsible for the characteristically "earthy" odor of freshly turned healthy soil (Sprusansky *et al.*, 2005) The actinomycetes are a pervasive group of bacteria found in naturally occurring ecosystems all over the world (George *et al*., 2012) . They have been found in harsh environments specially at cryophilic region for example soil taken from Antarctica and even from desert soil (Diraviyam *et al*., 2010).

The development of typically branching threads or rods distinguishes actinomycetes from other organisms. Although the hyphae are typically nonseptate, septa may occasionally be seen in some forms under specific conditions . The mycelium that is producing spores might be straight or spiral in shape, branching or nonbranching. The spores might be round, cylinder-shaped, or oval. Actinomycetes create initial microclusters made of branching filaments that disintegrate after 24–48 hours. into short chain, coccobacillary, and diptheroid types.

The actinomycetes cell wall is a hard structure that preserves the form of the cell and resists cell bursting as a result of excessive osmotic pressure. (Manuselis and Mahon, 2007).

The wall is made up of a wide range of intricate substances, including as polysaccharides, teichoic and teichuronic acid, and peptidoglycan.

The glycan makes up the peptidoglycan (polysaccharides) chains of alternate N-acetyl-d-glucosamine (NAG) and N-acetyl-d-muramic acid (NAM) and diaminopimelic acid (DAP), It is specific to the walls of prokaryotic cells.

Chemical bonds between teichoic and teichuronic acids and peptidoglycan exist (Davenport *et al.*, 2000) . Although the chemistry of their cell walls is similar to that of Gram positive bacteria, actinomycetes have been thought of as a distinct category from other common bacteria because of their well-developed morphological (hyphae) and cultural traits . Actinomycetes have been considered as a group, well separated from other common bacteria (Das *et al.*, 2008).

At the microscopic level of categorization, the life cycle of actinomycetes generally consisted of three main distinct appearances, namely vegetative mycelium (growth), aerial mycelium bearing cuffs of spores, the characteristic agreement of spores, and finally the spore arrangement. The last two characteristics provided the most investigative information for identification (Taddei *et al.*, 2006). Together, culture and microscopic traits enable scientists to categorize actinomycetes until they reach the genus level, such as the *Streptomyces* genus. Numerous research that used these traits and/or characteristics to identify the actinomycetes isolates as a species of Streptomyces have been carried out (Kekuda *et al*., 2012). Until recently, a large number of actinomycetes species have provided 70–80% of the antibiotics and antimicrobial secondary metabolites that have been acquired commercially. These released secondary metabolites are known to be a rich source of biologically active substances such enzymes, antibiotics,

antiparasitics, immunological suppressants, and anticancer agents. However, due to the declining success of discovering novel strains over the past ten years, the search for antibiotics and other secondary bioactive metabolites in actinomycetes species and other microorganisms has decreased in popularity. As a result, screening for new potential chemical compounds increased, but it didn't result in much progress, which is not remarkable given that actinomycetes have developed their capabilities over a long period of time. (Khanna *et al*., 2011)

2.1.1 Life Cycle of Actinomycetes

Actinomycetes serves as an important model of how bacteria develop ; because of their exceptional complexity, diversity of cell types (spores, vegetative and reproductive mycelia), and morphological alterations that are closely linked to physiological segregation. Due to the availability of the complete genome sequence, studies of the life cycle of Streptomyces coelicolor have been used to inform the biology of actinomycetes (Bentley *et al*., 2002). The multicellular prokaryotic model organism Streptomyces coelicolor, which has sporulation and programmed cell death (apoptosis) ،
The consideration of actinomycetes biology has depended on general studies in the life cycle of *Streptomyces coelicolor* because of the availability of the whole genome sequence (Bentley *et al.*, 2002). The Streptomyces *coelicolor* considered as a model of multicellular prokaryotic and it includes a sporulation and programmed cell death (apoptosis). When the spore germinated, the development of its vegetative components resulted in the formation of a mycelium, which is made up of ramifying arrangements of hyphae that enter a wet substrate by the extension of hyphal tips and sub-apical branches. Reproductive growth was then initiated, frequently accompanied by the formation of filamentous aerial hyphae, which underwent differentiation into chains of uni-genomic spores. This

bacterium's multicellular prokaryotic model is created by the apoptotic processes that are a component of its complex developing life cycle (Rioseras *et al.,* 2014)

Streptomycetes have a complex life cycle that is neither multicellular nor unicellular in nature (Maguelez *et al.*, 2000). They typically define Streptomyces' life cycle as seen in (Figure1.1), which begins with spore germination and ends with filaments dispersing into the solid medium to provide an external appearance of vegetative mycelium. After that, aerial mycelium is generated from the vegetative mycelium as sporophores, which spread vertically on their way to the surface. Additionally, aerial mycelium divides into spirals and ultimately into filaments to generate the polynucleated aerial. The final sheaths turn into spores, and the cycle then starts over. Antibiotics, poisons, pigments, and anticancer agents are among the secondary metabolites of microbes, which are essentially organic substances in nature (Martin *et al.,* 2005).

On the other hand, throughout the stationary phase of their developed life cycle, microorganisms produce their secondary metabolites. According to Waksman (1940), the filamentous actinomycetes' life cycle consistently produces either spores or tiny pieces of mycelium. When growth circumstances are favorable , germination takes place through the development of branching threads or rods into unicellular mycelia. The produced hyphae are often non-septated, and the vegetative mycelia develop within the solid media substrate, while the aerial mycelia protrude from the vegetative growth. The majority of actinomycetes reproduce from particular sporulation structures, such as those seen *Streptomycetes,* or from the hyphal tips part of the formed mycelium in the case of none - sporulating genus such as, *Mycobacterium, Nocardia* and *Rhodococcus* .

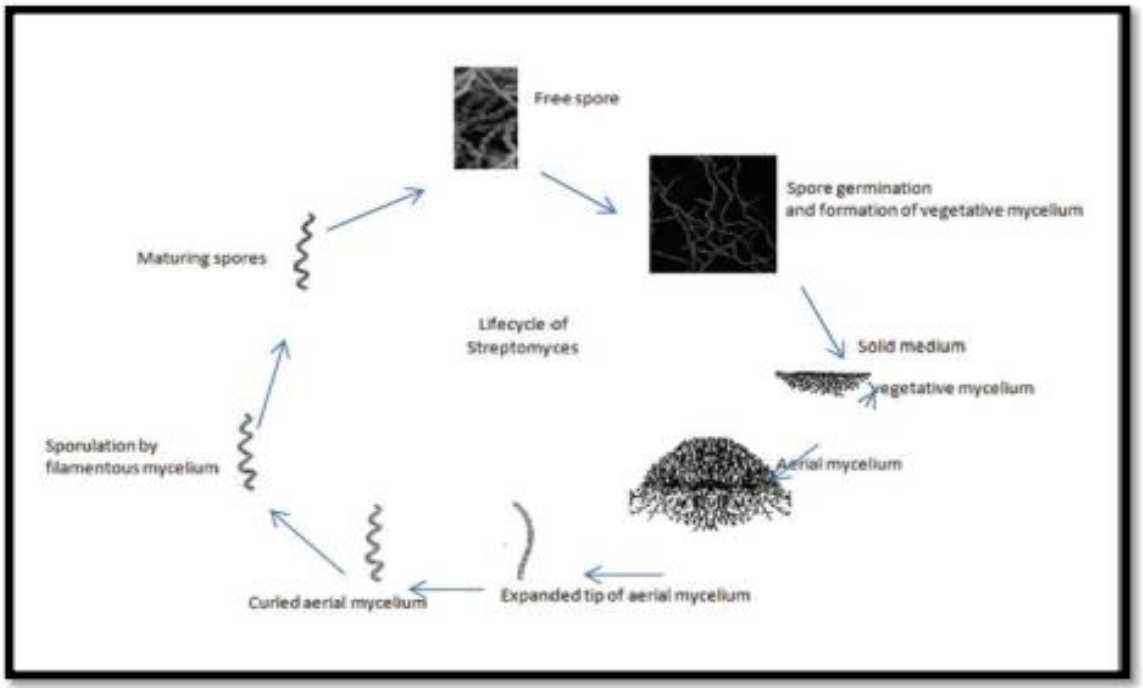


Figure 2.1: Life cycle of actinomycetes in general, especially Streptomycetes (Maguelez *et al.*, 2000).

2.1.2: Taxonomy of *Actinomycetes*

Actinomycetes are typically categorized as belonging to the bacterial kingdom. Volume 4 of the Bergey's Manual of Determinative Bacteriology contains extensive sections on actinomycetes. The order Actinomycetales includes all Actinomycetes Streptomycetaceae, Actinomycetaceae, Actinoplanaceae, and Mycobacteriaceae comprise the four families that make up the order Actinomycetales (Williams *et al.*, 1989) .

Actinomycetes are categorized as actinobacteria, which have a high guanine-plus-cytosine DNA content of around (69–73%), are known for their extensive branching of substrates, and the presence of aerial mycelia. Additionally, a summary of the taxonomy of actinomycetes can be found in (Figure 2.2).

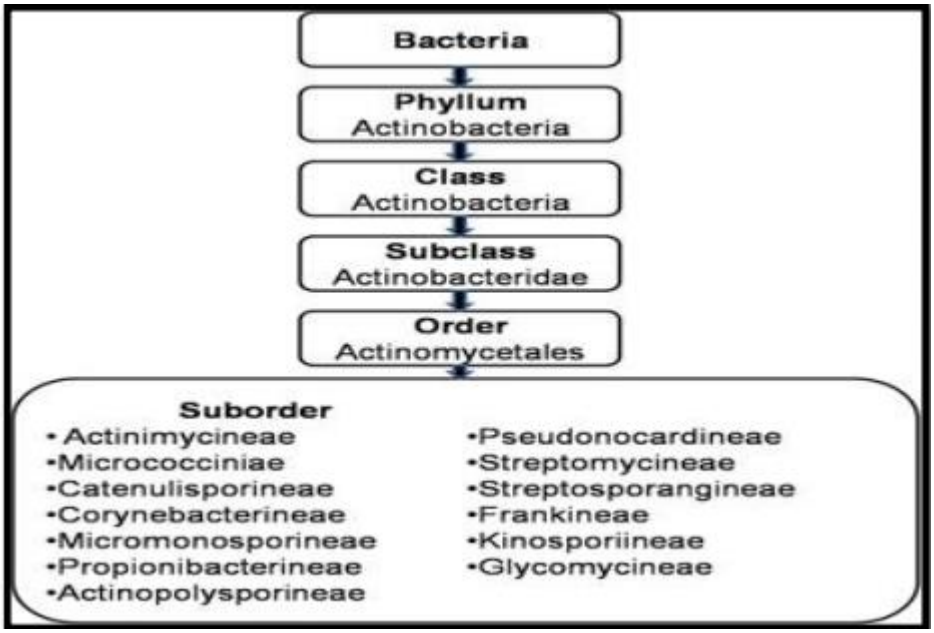


Figure 2.2: Taxonomy of the actinomycetes with all 13 suborders, (Mahajan and Balachandran, 2015)

2.2 Bioactive Compounds from Actinomycetes

Alternative natural product sources must continue to be bioprospected, (Hamaki *et al*., 2005) . According to Demain, secondary metabolites produced by microorganisms and plants are the finest candidates for creating a library of distinctive chemical variety (Demain, 2002). Despite being in great number, less than 1% of all the currently known microorganisms have been named and described, according to (Amann *et al*., 1995) .

In natural habitats, there is still a sizable reservoir of uncultured microorganisms. It is possible to use soil and water, which are abundant resources and provide a plentiful supply of possibly unique microorganisms, in natural product screening processes . It seems that 99% of the various bacterial species have not been studied. In his "crystal ball," Zinder predicted that numerous significant free-living, uncultured microorganisms that can be found in our environment will be cultivated. (Zinder, 2002)

The secondary metabolic pathways of microorganisms evolve to produce chemicals with a remarkable and varied range of biological activity. They have demonstrated ability to introduce functionality, such as chirality, and secondary metabolites through biotransformation. But not all the production of secondary metabolites is not always uniform among microorganisms, though. The prokaryotic filamentous actinomycetes, myxobacteria, pseudomonads, and cyanobacteria, as well as the eukaryotic filamentous fungus, have the capacity to create a variety of chemically varied metabolites (Peric-Concha and Long , 2003).

Over the past 50 years, a number of chemicals generated from these two families have achieved significant success as therapeutic agents. Some well-known examples include penicillin, streptomycin, bacitracin, terramycin, aureomycin, chloromycetin, erythromycin, and vancomycin. In addition to being essential for reasons of public health, antimicrobial agents also hold a significant deal of interest for organic chemists. But these issues of novelty, variation, and structural complexity have never been present in any other discipline of natural product research. Microorganisms that have been extensively screened are more likely to produce known metabolites than those that haven't been used as much.

It is beyond dispute that microbial diversity enables ongoing discovery of neo-bioactive substances. Additionally, these compounds selectively block their molecular targets and have novel, unexpected architectures . (Tiwari and Gupta , 2012)

The fact that different strains of the same species of microbes can produce various secondary metabolites has long caused confusion in the search for novel secondary metabolites. Taxonomically distinct strains, however, are capable of producing the same metabolites. According to a study, widely dispersed bacterial populations can exhibit the species-specific, phenotypic feature of producing secondary metabolites. According to Jensen's research, the correlation between actinomycete phylotype and

chemotype is a successful, diversity-based method for discovering new natural compounds. Unlike popular belief, the generation of secondary metabolites is strain-specific (Larsen *et al.*, 2005) .

Actinomycetes are widely spread in soil and make up a significant component of the microflora there. Actinomycetes have already been isolated in large numbers from soils all over the world, and they have made important contributions to the discovery of numerous useful bioactive substances like antibiotics, anticancer medicines, and immunosuppressive agents. They can produce chemicals with exceptional structure and biological activity due to their new biosynthetic genes. However, conventional bioprospecting of soil actinomycetes, which has been the main source of novel antibiotics in the twentieth century, has mostly led to the rediscovery of already recognized substances (Fischbach & Walsh, 2009).

Evidently, numerous terrestrial streptomycetes, despite isolated from various environments, may yet synthesize the same recognized compounds. This is likely because the species frequently interchange genetic material, as documented in 420 Studies in Natural Products Chemistry (Bredholdt *et al.*, 2007). Therefore, it is unlikely that isolation and screening of huge libraries of actinomycetes will yield any truly novel bioactive compounds. The fact that the common Streptomyces species are the easiest to isolate and cultivate since they continue to dominate culture collections around the world makes the issue much worse. On the other hand, uncommon actinomycetes that are more challenging to isolate and cultivate could be an unequaled source of novel bioactive compounds. (Busti *et al.*, 2006)

More than 10,000 antibacterial agents are produced by the actinomycetales family, which is also responsible for the generation of bioactive chemicals used in pharmaceuticals. (Sharma *et al.*, 2014)

2.2.1Antibiotics

Bacteria, fungi, and actinomycetes produce antibiotics, a special type of chemotherapeutic agent that, in small doses, can prevent the growth of germs or even kill them. Low molecular weight organic substances produced by microorganisms are referred to as antibiotics (Adegboye et al., 2013). Actinomycetes produce around 75% of antibiotics, primarily antibacterials. Numerous antibacterials exhibit a wide spectrum of actions and functional systems. They showed strong resistance to a large variety of Gram +ve and Gram -ve bacteria. (Hasani *et al.*, 2014)

When compared to bacteria and fungi, historically, streptomycetes was the source of the majority of new antibiotic medications (Hong et al., 2017). Roughly 45% of the known bioactive metabolites were produced by this order alone; more than 10,000 compounds were extracted from various actinomycetales species, with about 34% originating from Streptomyces and 11% from the other actinomycetes (*Baltz ,* 2009).

2.2.2Biopesticide agents

Insects can be naturally controlled by using microorganisms, such as those that are antagonistic to them. Actinomycetes produce insecticidal substances that are active against the domestic house fly, Musca domestica (Sundarapandian *et al*., 2002). After employing the insecticide actinomycetes, a large percentage of larval and pupal stages perished—up to 90%. Actinomycetes functioned well to control the Culex quinquefasciatus mosquito (Sharma *et al.*, 2014).

2.2.3 Plant growth hormone

Actinomycetes are utilized to produce plant growth hormones like auxins and gibberellin-like substances, which are used to improve plant development

(Persello et al., 2003). Indole-3-acetic acid (IAA), the primary type of auxin produced by actinomycetes, is what causes cell division, elongation, and differentiation. anti-tumor substances Streptomycetes are distinct groups that have the ability to produce unique cytotoxic substances with anticancer action. (Sahu *et al.*, 2008).

2.2.4 Antiviral agents

Peptides called complestatins were synthesized by Streptomyces lavendulae. These peptides function by interacting with the cell surface molecules of the target cells and inhibiting the adsorption of human immunodeficiency virus type 1 (HIV-1) to the cells, but they did not exhibit inhibitory activity against HIV enzymes (Chiu et al., 2001). From its culture supernatants, Streptomyces chromofuscus developed the protease inhibitor (PISC-2002). As an antiviral drug against influenza virus A/Rostock/34, PISC-2002 is crucial (H7N7). Streptomyces Pimprinine, an extracellular alkaloid, was created by sp. Pimprinine exhibits noteworthy physical and chemical characteristics, antibacterial and anticonvulsant effects, and antiviral efficacy against Enterovirus 71. (EV71) (Angelova *et al*.,2006)

2.2.5 Pharmacological compounds

One of the most crucial vitamins in the B complex—which also includes B1, B2, B3, B6, and folic acid—is vitamin B12. It originated from Streptomyces griseus and was first noted in 1948. This vitamin can only be produced by microorganisms (Bhawsar *et al.*, 2011)

2.2.6 Pigments

Actinomycetes are characteristic of the different production of pigments on artificial or natural media. These pigments are typically red, rose, yellow,

green, blue, violet, purple, and sometimes even black. According to Thompson *et al.* (2002), the pigments may diffuse into the media or they may stay in the mycelium. Actinomycetes were found to create various types of antibiotics, and these antibiotics also contain a variety of pigments (Wawrik *et al.,* 2007). Pharmacology and cosmetics frequently employ melanin. In a liquid medium containing peptone, yeast extract, and iron, Streptomyces virginiae produced the most pigment, followed by tyrosine. The actinomycete that produces pigment Streptomyces hygroscopicus shown antibacterial efficacy against numerous drug-resistant organisms, including strains of extended-spectrum -lactamases (ESBL), methicillin-resistant Staphylococcus aureus (MRSA), and vancomycin-resistant Staphylococcus aureus (VRSA). Yellowish 4 hydroxynitrobenzene is an antibiotic pigment that was generated by Streptomyces species. both Shigella shiga and Bacillus subtilis were active against the yellow pigment after their extraction in chloroform. Microbiological pigments are safe for use by humans and have antibiotic or anticancer properties. A select few of them have also been shown to be pigments of food grade. They are inexpensive and simple to manufacture (Amal *et al.*, 2011).

Streptomycetes, a significant class with industrial significance, have the capacity to manufacture a wide range of antibiotics and pigments. The diverse nutrition and cultivation conditions affect these organisms' ability to produce pigments, which can either be significantly enhanced or completely lost. Therefore, it is crucial to enhance the ideal fusion of many cultural factors in order to promote development and pigment production. Streptomyces coelicolor, Streptomyces violaceusruber, and Streptomyces lividans generate the biological pigment actinorhodin. It is blue and depends on the pH. (Moore *et al*., 1999). Actinorhodin is a pigment that can be used in a variety of ways, including as an antibiotic against Gram +ve bacteria, an indicator in scientific conditions due to its capacity to exhibit distinct colors in acid and alkaline environments, and

possibly even in the cosmetics industry. The whole range of pigment applications has not yet been investigated. (Morens *et al.*, 2004)

2.2.7 Commercial enzymes

The value of commercial enzymes has increased significantly as a result of their various applications in the food, pharmaceutical, and detergent industries. A unique active enzyme found in actinomycetes can catalyze several biochemical reactions with novel enzymes. (Kumar *et al.,* 2014). Important enzymes like amylase, protease, and cellulose were synthesized by streptomyces species and have industrial uses (Kundu et al., 2006). In natural water and sediments, L-glutaminase, Lasparaginase, and -galactosidase play an important part in the biocycling of carbon and nitrogen. L-glutaminase and L-asparaginase were generated by marine streptomycetes and had anticancer properties. Due to several mergers and acquisitions, the global market for industrial enzymes has been growing steadily. Enzymes used in food and beverages have received a significant amount of attention recently. (Prakash *et al.,* 2017)

2.2.8 Enzyme inhibitors

Enzyme inhibitors are becoming recognized more and more as useful tools for the pharmacological industry as well as for the study of enzyme structures and reaction processes. It has been observed that numerous forms of enzyme inhibitors, such as N-acetyl—D-glucosaminidase, pyroglutamyl peptidase, and -amylase inhibitors, are produced from marine actinomycetes, making them viable sources for the synthesis of enzyme inhibitors. Since they contain ingredients that prevent the body from consuming dietary starches, amylase inhibitors are also known as starch blockers. Weight loss may benefit from the inhibitors. According to Sun *et al.* (2015), Streptomyces corchorushii and *Streptomyces sp.* CC5 respectively generate alpha-amylase inhibitors.

Lipstatin, a very special inhibitor of pancreatic lipase, was generated by Streptomyces toxytricini (Weibel *et al.*,1987).

2.3 *Streptomyces* the Major Antibiotics Producers

Antibiotics can be found in an endless supply from actinomycetes. The majority of currently used antimicrobials were first discovered in actinomycetes, particularly in the genus Streptomyces. All significant medication classes now employed in clinics, such as -lactams, tetracyclines, macrolides, aminoglycosides, or glycopeptides, are included in the generated compounds. However, in recent years, the development of bacterial pathogens that pose a threat to human life has put these amazing weapons' efficacy in jeopardy. Additionally, it was believed that the antibiotic pipeline would run dry because the golden age of antibiotic discovery has long since passed. The subject of antibiotic research was changed by next-generation sequencing methods combined with genome mining strategies, and the pipeline may soon be restarted. First Streptomyces genome sequenced in 2002 (Bentley *et al.*, 2002) .

Streptomyces coelicolor, a representative actinomycete, with the following genomic sequence. The analysis of this sequence showed that *Streptomyces* Despite having 22 secondary metabolite gene clusters, *Streptomyces* coelicolor can only synthesize four of the encoded compounds in a lab environment. Only the genus Streptomyces' more than 625 genome sequences are now accessible (Blin *et al.*, 2019).

Less than 10% of the genetic potential of antibiotic manufacturers, according to genome mining assessments, is currently being employed, indicating that there is a vast untapped genetic reservoir ready to be used for drug discovery. Additionally, according to metagenomic data, there are a lot more potential antibiotic manufacturers in nature that are still waiting to be

isolated and studied . Actinomycin was the first antibiotic to be isolated from an actinomycete, thus over 80 years after Selman Waksman introduced the Streptomyces genus, these bacteria continue to be a gold mine for the discovery of new antibiotics (Nett & Moore, 2009).

2.4 Mechanisms of Antibiotic Action

Traditional definitions of antibiotics include natural substances made by microbes that have selective antibacterial action but no significant impact on human cells. Their method of action involves either bactericidal effect (killing the bacteria) or bacterial growth inhibition (bacteriostatic effect). When synthetic antibacterial drugs first became available, both synthetic and natural compounds were included in the term "antimicrobial agents," but since the idea of antibiotics had already acquired widespread acceptance, this term eventually took over and is now commonly used to refer to all antibacterial agents (Livermore, 2003).

Penicillin's discovery paved the way for the investigation of diverse natural substances with various targets inside the bacterial cell. Penicillin kills bacteria by preventing the manufacture of the cell wall, rendering the cell wall vulnerable, and inducing cell lysis. Other compounds have distinct effects on the bacteria and target diverse locations, such as inhibiting protein synthesis, RNA synthesis, and DNA replication (Andersson, 2005).

There are five major modes of bacterial antibiotic mechanisms of activity :-

2.4.1 Interference with cell wall synthesis

Penicillins and cephalosporins are examples of -lactam antibiotics that interfere with the enzymes required to produce the peptidoglycan layer. By attaching to the D-alanyl-D-alanine termini of the peptidoglycan chain, glycopeptides (Vancomycin, Teicoplanin, and Oritavancin) specifically target the bacterial cell wall and inhibit the cross-linking processes. (Benton *et al*., 2007).

2.4.2 Inhibition of protein synthesis

Macrolides bind to the (50S) ribosomal subunits and interfere with the elongation of nascent polypeptide chains. Aminoglycosides inhibit initiation of protein synthesis and bind to the (30S) ribosomal subunits . Chloramphenicol binds to the (50S) ribosomal subunit blocking peptidyl-transferase reaction . Tetracyclines inhibit protein synthesis by binding to (30S) subunit of ribosome , thereby decreasing the ribosome-tRNA interaction. (Leach *et al*., 2007).

2.4.3 Interference with nucleic acid synthesis

Rifampicin interferes with DNA-directed RNA polymerase Quinolones interfere with type II topoisomerases DNA gyrase and topoisomerase IV during replication and cause double strand breaks, which prevent DNA synthesis from completing (Leach *et al*., 2007).

2.4.4 Inhibition of a metabolic pathway

The sulfonamides (e.g. trimethoprim and sulfamethoxazole) each block the key steps in folate synthesis, which is a cofactor in the biosynthesis of nucleotides, the building blocks of DNA and RNA stands (Dzidic *et al*., 2008).

2.4.5 Disorganizing of the cell membrane

The primary site of action is the cytoplasmic membrane of G+ve bacteria , or the inner membrane of G-ve bacteria . It is believed that polymyxins cause leakage of bacterial content by increasing the permeability of bacterial membranes, which is how they exercise their inhibitory effects. (Tenover, 2006). By binding to the cytoplasmic membrane in a calcium-dependent manner and oligomerizing in the membrane, the cyclic lipopeptide daptomycin exhibits fast bactericidal activity, causing an efflux of potassium from the bacterial cell and cell death (Straus and Hancock, 2006).

2.5 Some Partial Purification Methods For CrudeExtract

2.5.1 Gas Chromatography–Mass Spectrometry (GC-MS) Technique

Gas Chromatography–Mass Spectrometry (GC-MS) is a hyphenated analytical technique that combines the separation properties of gas-liquid chromatography with the detection feature of mass spectrometry to identify different substances within a test sample . GC is used to separate the volatile and thermally stable substitutes in a sample whereas GC-MS fragments the analyte to be identified on the basis of its mass. The further addition of mass spectrometer in it leads to GC-MS/MS. Superior performance is achieved by single and triple quadrupole modes (Sahil *et al.*, 2011).

GC requires the analyte to have significant vapor pressure between 30 and 300°C. GC presents an insufficient proof of the nature of the detected compounds. The identification is based on retention time matching that may be inaccurate or misleading. GC-MS represents the mass of a given particle (Da) to the number (z) of electrostatic charges (e) that the particle carries. The term m/z is measured in DA/e. GCMS commonly uses electron impact (EI) and chemical ionization (CI) techniques. The main features of enhanced molecular ion, improved confidence in sample identification, significantly increased range of thermally labile and low volatility samples amenable for analysis, much faster analysis, improved sensitivity particularly for compounds that are hard to analyze and the many other features and options provide compelling reasons to use the GC-MS in broad range of areas (Sahil *et al.*, 2011) . **Applications of GC-MS** :

1-Environmental monitoring. 2-Food, beverage, flavor and fragrance analysis. 3-Forensic and criminal cases. 4-Biological and pesticides detections. 5-Security and chemical warfare agent detection. 6-Astro chemistry and Geo chemical Research . 7-Petrochemical and hydrocarbons

analysis. 8-Clinical toxicology. 9-Medicine and Pharmaceutical Applications.

2.5.2 Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) is a largely used technique to identify the functional groups in the materials (gas, liquid, and solid) by using the beam of infrared radiations. An infrared spectroscopy measured the absorption of IR radiation made by each bond in the molecule and as a result gives spectrum which is commonly designated as % transmittance versus wavenumber (cm−1). A diverse range of materials containing the covalent bond absorbed electromagnetic radiation in the IR region. The IR region is at lower energy and higher wavelength than the UV-visible light and has higher energy or shorter wavelength than the microwave radiations. For the determination of functional groups in a molecule, it must be IR active. An IR active molecule is the one which has dipole moment. When the IR radiation interacts with the covalent bond of the materials having an electric dipole, the molecule absorbed energy, and the bond starts back and forth oscillation. Therefore, the oscillation which caused the change in the net dipole moment of the molecule should absorbed IR radiations. (Khan *et al.,* 2018). **Applications of FTIR** :

1- FTIR in Biomedical Imaging . 2- FTIR in Proteins Study.

3-Miscellaneous Applications of FTIR Spectroscopy.

2.5.3 Ultraviolet (UV) spectroscopy

Ultraviolet spectroscopy is one important and advanced analytical instrument in Pharmaceutical industry and used since last 35 years and. The method of analysis is based on measuring the absorption of a monochromatic light by colorless compounds in the near ultraviolet path of spectrum (200-

400nm). The pharmaceutical analysis comprises the procedures necessary to determine the "identity, strength, quality and purity" of such compounds. It also includes the analysis of raw material and intermediates during manufacturing process of drugs. The fundamental principle of operation of spectrophotometer covering UV region consists in that light of definite interval of wavelength passes through a cell with solvent and falls on to the photoelectric cell that transforms the radiant energy into electrical energy measured by a galvanometer. Ultraviolet-visible spectroscopy is used to obtain the absorbance spectra of a compound in solution or as a solid. (Shinde *et al*., 2020).

2.6 History and description of *Pseudomonas aeruginosa*

Throughout its history, the opportunistic bacterium pathogen *P. aeruginosa* has known by a variety of names based on the distinctive blue-green coloration it produces when cultured. The first person to observe that a transferring agent was associated to the darkening of surgical wound dressings was Sédillot in 1850. Fordos isolated the pigment that gives the color its blue color in 1860, and Lucke was the first to associate rod-shaped microorganisms and this pigment in 1862 (Pitt, 1998) .

Carle Gessard described the growth of the organism from cutaneous wounds of two patients with bluish-green pus in a work titled "On the Blue and Green Coloration of Bandages" in 1882. At that time, *P. aeruginosa* had not yet been effectively isolated in pure culture (Gessard, 1984). Additional reports between 1889 and 1894 identified P. aeruginosa (Bacillus pyocyaneus) as the reason behind patients' wounds turning blue-green (Villavicencio, 1998). In a 1916 publication, Freeman presented the methods of *P. aeruginosa* invasion and spread that result in acute or chronic infection in greater detail. (Philips *et al.*, 2009).

Pseudomonas aeruginosa is an aerobic, non-fermentative rod with dimensions of 0.5 to 0.8 m by 1.5 to 3.0 m. A single polar flagellum is used by nearly all strains to move about. It has an amazing capacity to colonize ecological niches where nutrients are rare, from water and soil to plant and animal tissues, as it often survives in wet climates and requires a wide spectrum of organic molecules for growth. Positive oxidase test results, growth at 42°C, hydrolysis of arginine and gelatine, and nitrate reduction are typical biochemical characteristics of *P. aeruginosa* isolates. Pyoverdin and pyocyanin are two different forms of soluble pigments that *P. aeruginosa* strains produce. The later blue pigment, which is produced in large quantities in low-iron environments, enhances the bacterium's metabolism of iron. Pyocyanin, which comes from the Greek word "pyocyaneus," refers to the "blue pus" that *P. aeruginosa* suppurative infections are known for producing (Murray *et al*., 2003).

Pyocin, a chemical similar to an antibiotic that Pseudomonas aeruginosa also makes, is active against many strains of the same species as well as other species. These ingredients are used to diagnose species and type P. aeroginsa (Aboud, 2001).

Rarely does *Pseudomonas aeruginosa* appear in the human body's typical microbial flora. Skin, nasal mucosa, throat, and fecal samples all have representative colonization rates of 0 to 2%, 0 to 3.3%, 0 to 6.6%, and 2.6 to 24%, respectively, in people (Morrison and Wenzel, 1984). However, colonization rates during hospitalization may surpass 50% (Pollack, 1995), particularly in patients who have had surgery, mechanical ventilation, tracheostomy, catheters, or serious burns that have damaged or breached their cutaneous or mucosal barriers. (Valles, *et al*., 2004).

According to Morrison and Wenzel (1984), patients with compromised immunity are more likely to become colonized by this bacterium, and antibiotic medication has also been demonstrated to enhance *P. aeruginosa*

colonization (Takesue *et al*., 2002). Despite *P. aeruginosa*'s widespread distribution in nature and the possibility of community-acquired infections, major infections with this bacterium are typically obtained in hospitals. *P. aeruginosa* was found to be the fifth most frequently isolated nosocomial pathogen, accounting for 9% of all hospital-acquired infections in the United States, according to a review of surveillance data gathered by the Centers for Disease Control and Prevention (CDC), National Nosocomial Infections surveillance system from 1986 to 1998. (Emori, and Gaynes, 1993).

Pseudomonas aeruginosa was the fourth most frequently isolated pathogen in surgical site infections (8%), the third most frequent cause of urinary tract infections (7 to 11%), the second most common cause of nosocomial pneumonia (14 to 16%), and the seventh most common cause of bloodstream infections (2 to 6%). P. aeruginosa is still the primary cause of pneumonia in pediatric patients in the critical care unit and the second most frequent cause of nosocomial pneumonia, health care-associated pneumonia, and ventilator-associated pneumonia, according to data from previous research (ICU) (Kolleff *et al*., 2005)

2.6.1 Pathogenicity of *Pseudomonas aeruginosa*

Almost every organ or external location can become infected by Pseudomonas aeruginosa. Infections are often mild and superficial in the general population, but they are more frequent, more severe, and more varied in hospitalized patients. Only when *P. aeruginosa* is introduced into regions without natural defenses, such as when mucous membranes and skin are damaged directly, when intravenous or urinary catheters are used, or when neutropenia is present, as in cancer chemotherapy, does it become harmful. The bacterium colonizes the skin or mucous membranes by attaching to

them, spreads locally, and causes systemic disease (Sambrook and Rusell, 2001).

The Pili, enzymes, and toxins all help to stimulate these activities. Lipopolysaccharide directly contributes to the development of adult respiratory distress syndrome, disseminated intravascular coagulation, oliguria, leukocytosis, and leucopenia. (Pollack, 2000).

Pseudomonas aeruginosa is a opportunistic pathogen related to infectious eye illnesses that can cause blindness, including keratitis (Green et al., 2008). Pseudomonas keratitis is a serious ocular infection that, if aggressive and adequate treatment is not initiated immediately can cause corneal scarring and severe visual impairment (Stapleton *et al*., 2007).

Community-acquired pneumonia (CAP) is caused by *Pseudomonas aeruginosa* , and the majority of nosocomial infections areassociated to this organism since it is commonly found in hospital environments (Hatchette *et al*., 2000)

The primary problems for hospitalized patients with underlying malignancies, cardiovascular disease, renal failure, or diabetes remain to be bacteremia and septic shock caused by *P. aeruginosa* . It caused bacteremia in intravenous drug users is typically accompanied by bacterial endocarditis and may also result in osteomyelitis of various bones. (Boffi *et al*., 2001).

The infection process for an opportunistic pathogen as *P. aeruginosa* begins with some alterations or evasion of the normal host defenses. The multitude and variety of virulence determinants that *Pseudomonas* infections exhibit suggest that the pathogenesis of these infections involves multiple factors. The majority of Pseudomonas infections are toxic and invasive. There are three main stages that compose of the full Pseudomonas infection: (1) bacterial colonization and adhesion; (2) local invasion; and (3) widespread systemic illness (Brooks *et al*., 2010).

2.6.2 Antibiotic resistance

Antibiotic resistance has remained a significant problem over time, and research has revealed that resistance can appear abruptly after the introduction of an antibiotic. Patients were first given penicillin in 1941, and by 1942, it had developed a resistance. Another case included the 1960 introduction of methicillin, which led to the discovery of methicillin-resistant bacteria in 1961. (Landecker, 2015). Multi-resistant bacteria are some types of bacteria that have developed resistance to multiple antibiotics. Such situations are considerably more challenging to manage. Bacteria can quickly adapt to new conditions, especially if those environments present a threat to their ability to survive. They can improve/activate their ability to adapt to such environmental circumstances, Their capacity for adaptation allows them to enhance/activate survival mechanisms that will help them develop better, and one potential method of doing this is by changing the way they regulate the expression of their genes (Guo & Gross, 2014). According to Hottes *et al.* (2013), the loss of function mutations in a few bacterial genes, particularly those that the environment has chosen to target, may be a mechanism for the bacterium to adapt to difficult environmental conditions like high temperatures and nutritional shortages. Curiously, it is believed that these changes that cause resistance in some bacteria originated long before the bacterium was ever exposed to an antibiotic. This demonstrates that bacterial resistance existed before the use of antibiotics (Davies & Davies, 2010). Another traditional manner in which bacteria become resistant is through horizontal gene transfer (HGT) . HGT is a process in which one organism obtains new genetic material from another organism rather from parent to offspring (Wiedenbeck & Cohan, 2011). The most frequent of the three forms of HGT via which bacteria develop antibiotic resistance is conjugation. One bacterial cell will directly transfer its plasmid DNA to another bacterial cell during conjugation (Greenwood, 2012). While transformation occurs when one bacterial cell acquires foreign DNA from another bacterial cell, transduction occurs when a

bacteriophage transfers DNA material from one bacterium to another (Tenover, 2006). The same or distinct genera can experience horizontal gene transfer.

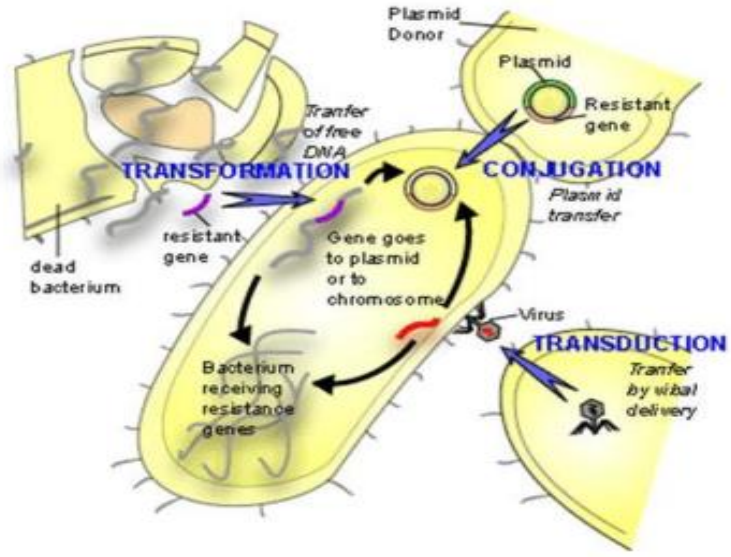


Figure 2.3 : Bacteria use different methods of horizontal gene transfer as a mechanism of preventing the activity of antibiotics. These methods include transformation, conjugation and transduction (Tadakamalla & Evans, 2014).

Resistance acquired due to plasmid transfer occurs more rapidly than resistance that involves genes on a chromosome , this is as chromosomal mutations are exceedingly rare and if they do occur , they occur at a very moderate rate (Giedraitienė *et al.*, 2011). For chromosomal mutations that results in resistance to occur , many genes are required to change before a desirable effect is achieved. As chromosomal mutations are extremely rare and, when they do occur, happen at a relatively low rate, resistance acquired through plasmid transfer occurs more quickly than resistance involving genes on a chromosome (Giedraitien *et al.*, 2011). Numerous genes must alter

before a desired effect is realized for chromosomal changes that result in resistance to develop.

Biofilm formation is another widely investigated idea that contributes to the emergence of resistance. In biofilms, which are communities of bacteria and connected cells at the surface, many bacteria are likely to proliferate (Vu *et al.*, 2009).

These bacterial cell communities, which can arise from the same species or others, "work together." The bacteria produce polysaccharides, proteins, and amino acids that give them stability and defense against stressors or invaders (Hughes & Anderson, 2017). Most bacteria, including Staphylococcus epidermis and *Pseudomonas aeruginosa*, are known to be affected by biofilms, and it has been demonstrated that growing a susceptible strain in a biofilm causes it to develop antibiotic resistance more quickly than growing the strain alone (Wu *et al.*, 2015). However, as soon as the bacterium was removed from the bacterial biofilms, it returned to being susceptible to the antibiotic (Stewart, 2002). Once these biofilms have developed, it can be difficult to remove them, which is why antibiotics and immune responses do not always effectively eradicate them. In cystic fibrosis, *P. aeruginosa* (and other infection-causing organisms) has been observed to develop biofilms during infection which contributes to the problems of treating the infection with the prescribed drugs (Wu *et al.*, 2015).

2.7 Cytotoxicity Assay

When a novel medicine, whether from a natural source or a synthetic one, is being researched, it's important to look at its host cell safety or the cytotoxic effect on cancer cells. It is commonly referred to as the cell

viability test. This viability cell test can range from the most basic to the most extensive. (Stepanenko *et al*., 2016).

Many techniques are employed in toxicological screening to evaluate the viability or proliferation of cells following direct or indirect exposure to substances. Most frequently utilized among the tetrazolium substances is 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) . (Pintor *et al.* 2020)

The MTT reducing , a colorimetric assay is regarded as the "gold standard" for cytotoxicity testing . However, this test has attracted criticism for potentially overestimating or underestimating the vitality of cells for certain drugs , and in anti-cancer medication screening, investigations on tumor cells have been conducted. (van Tonder *et al*. 2015)

The biochemical mechanism MTT assay based on the reduction of MTT (yellow colored) and other tetrazolium dyes depends upon cellular metabolic activities due to NAD(P)H-dependent cellular oxidoreductase enzymes (Figure 2.4). The healthy and rapidly growing cells exhibit high rates of MTT reduction to formazan while the dead or inactive cells fail to do so. The final product of MTT reduction is a purple color formazan that can be easily dissolved in dimethyl sulfoxide (DMSO) . Viability in the MTT assay is connected with the quantification of formazan at (540 nm) which is linearly associated with the enzyme activity and indirectly the number of viable cells. High purple color intensity denotes higher cell viability while the decrease in purple color intensity signifies the reduced cell number and thus cytotoxicity of the given substance. (Bahuguna *et al.* 2017)

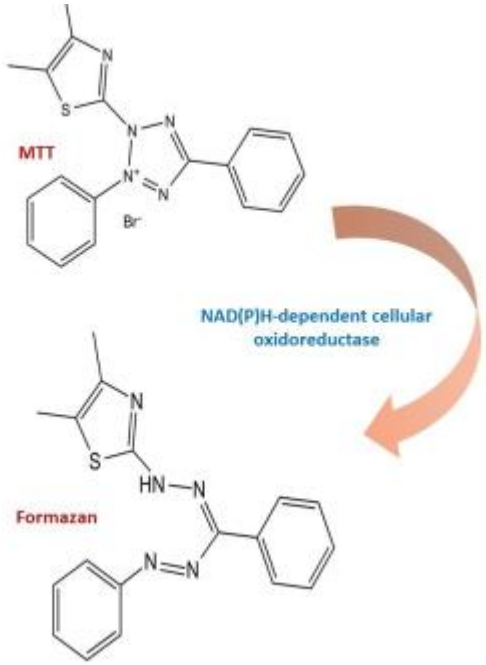


Figure 2.4 : Flow diagram representing the systematic conversion of MTT to formazan

2.8 *Streptomyces* genome

Among bacteria, streptomycetes have some of the largest genomes, typically within the range .The genome size of *Streptomyces* between (10-6) Mbp is estimated ratio of G-C (62-71)% in DNA , which reflects their complex life cycle and their metabolic, physiological and metabolic capability. The process of understanding the functions and composition of genetic material in Streptomyces can be interpreted the role of these bacteria in pharmaceutical, applied, environmental and biological terms. *(*Mariita *et al.,* 2015) . *Streptomyces coelicolor* was considered one of the first species to detect total genome sequencing. Studies showed that the Streptomyces have a linear chromosomes after it has prevailed that the chromosomes was circular with Centrally located origin of repliction (oriC) . (Undabarrena *et al*., 2017) figure (2-5)

Studies of a wide range of species indicate that the genome contains a central region region that contains responsible essential genes as DNA replication, cell division, and genes responsible for metabolism and a terminal regions are responsible for secondary metabolites and the production of antibiotics and exogenous enzymes , This explains the difference in their ability to secrete antibiotics and the ability of one type of them to secrete more than a second metabolite. The genus Streptomyces also contains several types of plasmids that have a role in the production of antibiotics and resistance to it . (Sevillano *et al*.,2013)

The future of ***streptomyces*** **genomes** : The availability of cheap sequencing has led to the generation of numerous genome sequences for *Streptomyces* and related species with the objective of discovering novel metabolic products (Liu *et al*., 2013). However, sequencing the genome and discovering novel gene clusters is just the beginning; many of the metabolic products of these gene clusters are 'cryptic', not being expressed under normal laboratory conditions. Productive 'genome mining' requires either genetic modification of the cluster to force expression or cloning and expression of the cluster in a heterologous host (Gomez-Escribano and Bibb, 2014). The value of this approach, even starting from rather poor-quality draft genome sequences, has been demonstrated by the discovery of the gene cluster encoding cypemycin in *Streptomyces* sp. strain OH-4156, revealing an unusual class of post-translationally modified ribosomally synthesized peptides (Claesen and Bibb, 2010). There will inevitably be a lag between the initial frenzy of genome sequencing and the characterization of novel useful products as the biochemical investigations are more laborious than the sequencing. Another interesting emerging theme is the role of endophytic

streptomycetes and the emerging picture that secondary metabolites contribute to the medicinal properties of their host plants . (Akshatha *et al*., 2014). The most recently published *Streptomyces* genome comes from strain PRh5, an endophyte of wild rice that produces nigericin, an antibiotic effective against mycobacteria (Yang *et al*., 2014).

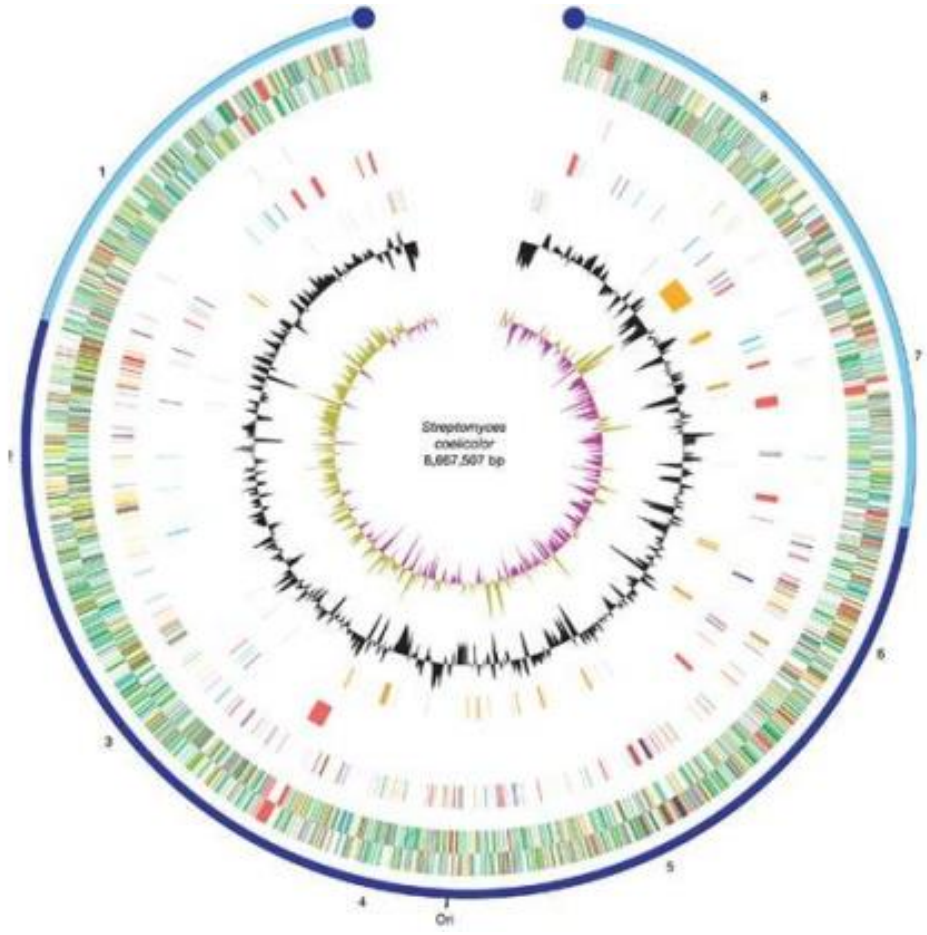


Figure 2-5 *: Streptomyces coelicolor* Genome

(Undabarrena *et al*., 2017)

3. Chapter Three: Materials and Methods

3.1: Materials and Apparatuses

Different Apparatuses were used through the study from different manufactured company as shown in table (3.1) .

Table: 3-1. Apparatuses and Equipment used in the study

Apparatuses	Manufactured Company (Origin)
Autoclave	Webeco-GmBH (Germany)
Automated DNA Extraction	Bioneer (Korea)
Centrifuge	Heidolph MR-Heat Standard (China)
Cold centrifuge	Hermle (Germany)
Distiller	Ogawa Seiki (Japan)
Electric Balance	Meter PJ600 (Japan)
Electric Oven	Gallenkamp (England)
Electrophoresis	Consort (England)
Spectrophotometer	Lab. Tech / Korean
Flask (250, 500, 1000) ml	Afco Dispo (China)
FT-IR Analyzer	Shimadzu (Germany)
Heat Plate	Heidolph MR-Heat Standard (China)
Incubator	Gallenkamp (England)
Laminar flow	Bioneer (Korea)
Light Microscope	Olympus (Japan)
Loop	Heidolph MR-Heat Standard (China)
pH Meter	Hoeleze and Cheluis K.G.(Germany)

Polymerase Chain Reaction	Cleaver Scientific (Japan)
Refrigerators	Vestal (Turkey)
Sensitive Balance	Sartorious (USA)
Shaker incubator	Scientific Industries (USA)
UV-Analyzer	Shimadzu (Germany)
Vacuum Evaporator	Stuart (Netherlands)
Vortex	Scientific Industries (USA)
Water Path	Gallenkamp (England)
Microwave	Sanyo electric Japan
Nanodrop	Optizen Korea
Oven	Memmert (Germany)
Vitek 2 system	Biomerieux France
Vortex mixer	Eppendorf (Germany)
Centrifuge Cooling	Eppendorf (Germany)
Incubator	Memaret (Germany)
Micro-titer Plate Reader	Bio-Rad (Germany)
Haemocytometer	Sigma (USA)
Para film	BDH (England)
Petri Dish	Afco Dispo (China)
Plain Tube	Afco Dispo (China)
Slander (250, 500, 1000) ml	Afco Dispo (China)
Syringes	Afco Dispo (China)
Whatman Filter Paper	Shimadzu (Germany)
Finn tips with different sizes (20µl, 100 µl, 500 µl, 1000 µl)	Eppendorf (Germany)
Eppendorf tubes	Eppendorf (Germany)
Burner	Turkey Amal
Micropipette (0.5-10 µl , 20-200 µl , 100-1000 µl)	Dragonlab China

3.2: Chemical Materials

Table: 3-2. Chemical Material and Manufactured Companies

Biological and Chemical Materials	Company (Origin)
Acetone	BDH (England)
Agar-agar	BDH (England)
Catalase reagent	Merk (England)
Crystal violet	BDH (England)
Ethanol 99%	Merck (India)
Glycerol (C3H8O3)	Himedia (Switzerland)
Gram stain solution	Fluka (USA)
Glucose	Sigma (England)
RPMI / medium	HIMEDIA (India)
trypsin/EDTA solution	Sigma (England)
Kovac's reagent	HIMEDIA (India)
Hydrogen peroxide (H2O2) 3%	Merck (England)
Naldixic acid suplement	Mast (U.K)
Normal Saline solution	S.D.I (Iraq)
Oxidase reagent indicator	BDH (England)
Peptone water	HIMEDIA (India)
Urea Solution	SD-Fine (India)
Calcium carbonate	Sigma (England)
Ethanol	BDH (England)
Ethyl acetate	BDH (England)

Glycerol	BDH (England)
Malt extract	Difco (USA)
Master Mix Kit primers	Bioneer (Korea)
Methyl red	BDH (England)
Sodium chloride	BDH (England)
Sucrose	Himedia (India)
Sufranine	BDH (England)
Yeast extract	Difco (USA)
EDTA, Dimethylsulfoxide(DMSO), Trypsin, Fetal bovine Serum, RPMI-1640 Media, Phosphate Buffer Saline, Sodium bicarbonate	Sigma (USA)
Absolute Ethanol	Hemadia (India)

Table: 3-3 Specific PCR Material

Material	**Company (origin)**
DNA extraction Kit	Bioneer (Korea)
DNA ladder (1kbp)	Bioneer (Korea)
DNA loading buffer	Fermentas (Germany)
Primers	Bioneer (Korea)
Safe Red	Fisher (USA)
Tris-Borate EDTA buffer (TBE)	Promega (USA)
DNA ladder marker (100-1500) bp	Bioneer (Korea)
Agarose	Bio Basic (Canada)

3.3: Culture Media

3.3.1: Ready-To-Use Powder Media

Culture media used in the present study were prepared according to the manufacturer's instruction. The media were sterilized by the autoclave at 121°C for 15 min, and kept at 4°C until use, All the media in this study were perchased from HIMEDIA company/ India. Table 3-3 shows the media used in this study**.**

Table: 3-4 Ready-to-use powdered media

Medium	**manufactory companies**
Luria broth	Himedia (India)
Brain heart infusion broth	Himedia (India)
Cetrimide agar	Himedia (India)
Kliglers iron agar	Himedia (India)
MacConkey Agar	Himedia (India)
Muller Hinton agar	Difco (USA)
Muller Hinton broth	Difco (USA)
Nutrient agar	Himedia (India)
Nutrient broth	Himedia (India)
Simmons citrate agar	Himedia (India)

3.3.2: Laboratory-Made Culture Media

Table 3-5 : Composition of International *Streptomyces* Propjet System (ISP2 Media)

N0.	Media name	Abbreviation	Composition	Amount of Composition
1-	Yeast extract- ISP2 Yeast Extract 4gm malt extract agar (Shirling and Göttlieb، 1966)	ISP2	Yeast Extract	4 gm
			Malt Extract	10 gm
			Dextrose	4 gm
			Distilled water	1000 ml
			Agar agar	20 gm
	Preparation condition	PH 7.3	121°C, 15 lb and 15minutes	

3.4 Commercial Kits and primers

3.4.1 Commercial Kits

The commercial kits used in the study are illustrated in Table 3-6.

Table 3-6 Commercial kits used in this study

No.	Kit	Company	Origin
1.	DNA extraction Kit	Favorgen	Taiwan
2.	DNA ladder	IntronBio	Korea
3.	Primers	Macrogen	Korea
4.	*P. aeruginosa* Specific Primer	Macrogen	Korea

5.	*Streptomyces spp.* Specific Primer	Macrogen	Korea
6.	PCR master mix	IntronBio	Korea
7.	VITEK® 2 Compact	Biomerieux	France
8.	MTT Kit	Intron Biotech	Korea

3.4.2 Polymer Chain Reaction (PCR) Mixture

The PCR reaction mixture used in the study are listed in Table 3-7.

Table 3-7 PCR Reaction Mixture

No.	Contents of reaction mixture	Volume
1.	Forward primer (10 pmol/µl)	2 µl
2.	Master Mix	12µl
3.	Nuclease free water	6 µl
4.	Reverse primer (10 pmol/µl)	2 µl
5.	Template DNA	3 µl
6.	Total volume	25 µl

3.4.3 Green Master Mix Materials

The Master Mix Materials used in the study are listed in Table 3-8.

Table 3-8 contents of master mix

No.	Materials
1.	DNA polymerase enzyme (Taq)
2.	dNTPs (400µm dATP, 400µm d GTP, 400µm dCTP, 400µm
3.	$MgCl_2$ (3mM)
4.	Reaction buffer (pH 8.3)

3.4.4 Commercial Primers

The commercial Primers used in the present study are illustrated in Table 3-9.

Table 3-9 Commercial Primers used in this study

Gene	**Sequence (5----------›3)**	**Amplicon size (bp)**	**Conditions**	Reference
Streptomyces specific primer *16S rRNA*	**F** TCACGGAGAGTTTGATCCTG	500	97˚C/30 sec 50˚C/1 min 72˚C/1 min 35 cycle	(Al-Rubaye, 2016)
	R GCGGCTGCTGGCACGTAGTT			
Universal primer	**27F** AGAGTTTGATCCTGGCTCA	1500	95˚C/30 sec 50˚C/1 min 72˚C/1 min 40 cycle	(Chen, 2015)
	1492R GGTTACCTTGTTACGACTT			
P. aeruginosa specific gene 16S rRNA	**F** GGGGGATCTTCGGACCTCA	956	95˚C/30 sec 61˚C/1 min 72˚C/1 min 35 cycle	(Spilker *et al*., 2004)
	R TCCTTAGAGTGCCCACCCG			

3.5 Antimicrobial susceptibility test

This test performed according to clinical laboratory guidelines (CLSI-2021) (0.5 McFarland tube was used to obtain 1.5×10^8 CFU/mL bacterial culture). Table 3-8 shows the antimicrobial disks used in this Study.

Table 3-10 Antimicrobial Disks used in this Study

Antibiotics	**Antibiotics Classes**	**Symbol**	**µg / disk**	**Company/ origin**
Aztreonam	Monobactams	ATM	30	India/Himedia
Ceftazidime	Cephems	CAZ	30	Roseto /Italy
Cefepime		CEP	30	Roseto /Italy
Cefriaxone		CIP	30	
Piperacillin tazobactam	β-Lactams combinations	PTZ	100/10	MAST/U.K
Piperacillin	Penicillins	PRL	100	Roseto /Italy
Gentamicin	Aminoglycosides	CN	10	Condalab/Spain
Tobramycin		TOB	5	Tur/ Bioanalyse
Netilmicin		NET	30	Roseto /Italy
Amikacin		AK	30	Roseto /Italy
Ciprofloxacin		CIP	5	Himedia/ India

Norfloxacin	Fluoroquinolones	NX	10	Roseto /Italy
Gatifloxacin		GAT	5	Roseto /Italy
Levofloxacin		LEV	5	Roseto /Italy
Ofloxacin		OFX	5	Bioanalyse /Turkey
Imipenem	Carbepenem	IPM	10	Roseto /Italy
Doripenem		DOR	10	Bioanalyse /Turkey
Meropenem		MEM	30	Bioanalyse /Turkey

3.6 Methods

3.6.1. Laboratory Preparation of Culture Media

All media were preparation according to the instructions of the manufacturing company Sterilization of culture media and solutions were achieved by autoclaving at 121°C ⁄ 15 minutes. After sterilization urea agar base was supplemented with 20%sterile urea solution and blood agar base was supplemented with 5% fresh human blood, then media poured on petri dish or plane tubes, and incubated at 37 for 24 hours to ensure their sterility. Storage of sterile media in the refrigerator to prevent dehydration . pH was adjusted to 7.0 and the media sterilized by autoclaving (Brown and Smith, 2017) (Table 3.9).

Table 3-11 Culture media used in the diagnosis of bacteria with the purposes. MacFaddin (2000)

No.	Media name	The purpose
1.	InternationalStreptomyces	Selective media used for isolation of

	project ISP2	actinomycetes from different sources
2.	MacConkey agar	is a selective and differential media. It is us in the differentiation of lactose fermenting from lactose non-fermenting gram-negative bacteria
3.	Nutrient broth	this medium was used in a general experiment such as cultivation and activation of bacterial isolates when it necessary
4.	Blood agar	Is an enriched, bacterial growth medium, isolation, identification and determine the type of hemolysis
5.	Cetrimide agar	This medium was used as a selective medium for the isolation of *P. aeruginosa*
6.	Brain heart infusion Broth	This medium used to preserve the bacterial isolated as standard for a long time with 15% glycerol
7.	Müller-Hinton agar	This medium used in the antibiotic sensitivity test
8.	Simmons citrate	It was used to determine the ability of bacteria To utilize sodium citrate as its only carbon source and inorganic ammonium salts as its only nitrogen Source.
9.	Urea agar	It was used to test the ability of bacteria to produce urease enzyme.
10.	TSI agar	It was used to determine the ability of bacteria to utilize carbohydrates supplemented with phenol red as the indicator .
11.	Voges Proskauer	It was used to detection of specific breakdown products of carbohydrate metabolism by bacteria

3.6.2. Preparation of Reagents and Solutions

3.6.2.1. Oxidase reagent

It was prepared by dissolving 1 mg of N, N, N, N-tetramethyl-ρ-phenylenediamine dihydrochloride in 100 ml of D.W. Then stored in a darkbottle and used immediately (Forbes *et al*., 2007).

3.6.2.2. Catalase reagent

It was prepared in a dark bottle by using a 3% concentration from hydrogen peroxide (Forbes *et al*., 2007).

3.6.2.3. Vogas-Proskauer reagent

This substance consisted of two solutions: α-naphthol solution made by dissolving 5 gm of -naphthol in 100 ml of (95 %) ethanol, storing the solution in a dark bottle, and mixing it prior to use. 40 percent Potassium hydroxide solution made by dissolving 40 grams of KOH in 100 milliliters of deionized water and mixing the solution prior to use (MacFaddin, 2000).

3.6.2.4. Methyl red indicator

This solution was prepared by dissolving 0.2 gm of methyl red in 300 ml of (95%) ethanol, and then the volume was completed to 500 ml by D.W. (MacFadden, 2000).

3.6.2.5. Kovacs reagent

Ten grams of dimethyl-amino benzaldehyde were dissolved in 150 milliliters of isoamyl alcohol by heating in a water bath at 50 degrees Celsius, followed by the addition of 50 milliliters of concentrated HCL. Small quantities of the reagent were made and stored in the refrigerator MacFadden, (2000).

3.6.2.6. Gram stains solutions (Jawetz *et al*., 2019).

1-Primary stain: 2 gm Crystal violet, 20ml 95% ethyl alcohol, 0.8gm ammonium oxalate and100 ml distilled water.

2-Stain fixative agent: 2 gm potassium iodide, 1gm iodine crystals and 100 ml distilled water.

3-Decolorize: 70% ethyl alcohol+30% acetone.

4-Counter stain: 4.0 gm safranin, 200 ml 95% ethanol and 800 ml distilled water.

3.6.2.7. Turbidity standard (McFarland)

The turbidity standard (0.5 McFarland solution) was prepared in accordance with Baron and Feingold (1990). In a graduated cylinder, 0.5 ml of 1.175% (w/v) barium chloride dehydrate (BaCl2.H2O) dissolved by D.W was added to 99.5 ml of 1% sulfuric acid; then, 10 ml of the mixture was transferred to a sterile test tube and stored in a dark place at room temperature. At a wavelength of 600 nm, a spectrophotometer measured the absorbance. 0.08 - 0.13 nm is the allowable absorbance range for the standard. Before performing an antibiotic susceptibility test on 46 isolates, this solution was used to set the number of bacterial cells.

3.6.3. Red Safe

Red Safe Nucleic Acid Staining Solution is a new and safe alternative to ethidium bromide (EtBr) for DNA and RNA identification on agarose gels. Red Safe is as sensitive as EtBr, and the staining procedure is virtually comparable; however, compared to EtBr, which is known to be a powerful mutagen, Red Safe produces much fewer mutations in the Ames test.

Importantly, it is non-hazardous, can be disposed of using standard laboratory procedures, and has a long shelf life (Machida and Knowlton, 2012).

3.6.4. Preparation of 1X TBE buffer

1X TBE buffer was prepared by dilution of concentrated 10X TBE buffer, the solution was used to dissolve agarose. Each 10 ml of 10X TBE added to 90 ml of sterile distal water to give final concentration 0.5µg/ml (Sambrook *et al*., 1989).

3.6.5 Agarose gel

According to Green and Sambrook (2012), the agarose gel was made by dissolving 1 gram of agarose in 100 milliliters of 1X TBE buffer (10ml completed with 90ml distal water). The solution was heated to boiling (using a microwave) until all the gel particles dissolved, the solution was cooled to 50-60oC, and 5ml of melting agarose gel was combined with 5ml of simply safe to achieve a final concentration of 0.5g/ml.

3.7: Samples Collection of :

3.7.1 Actinomycetes

fourty soils samples have been collected from different locations in Hilla city, about 50 g of the soil samples were taken from soils top about 5 to 10 cm in depth. Samples were placed in dry and sterile polyethylene tubes and stored at 4°C until use. Soil samples were pretreated with calcium carbonate to inhibition the number of vegetative bacterial cells and allowing *Actinomycetes* spores to survive, this method was required for inhibiting unwanted bacteria and remain only test bacteria (Pordeli *et al*., 2013).

3.7.1.1: Testing Soil properties :

A/Measurement of Soil pH:

weigh out about 10g (to the nearest half gram), of soil into the contair. Add 50ml of distilled water to the soil. Any rough measurement

ensuring a 1:5 diluted will suffice. Shake the container for about 2-3 minutes then allow the soil to settle for 2 minutes. measure the pH value on the water above the soil in the container.(Van Reeuwijk ,2002)

B/ Measurement of soil temperature:

By using soil thermometer in depth 10 cm through :

1. Measuring 12 cm up from the tip of the soil thermometer and mark this spot.
2. Measuring the distance from the base of the soil thermometer dial to the 12 cm mark.
3. Making a spacer by cutting a piece of plastic tubing or wood to this length. (If using wood, drill a hole through the center of the block).

3.7.1.2: Isolation and Purification of *Actinomycetes* from Soil Samples

After homogenizing each samples. It was passed through the 2mm opening to remove gravel, large stone and debris. the samples were then incubated at 55°C in for 5 min. Then 1 g of each soil samples was added to 9 ml of D.W and successive dilutions was made up to 10^{-5}. Each of the serial dilution was spread and placed on the surface (International *Streptomyces* Project type-2 media (ISP-2) agar medium. Nystatin antibiotic was added to minimize fungal contamination. All plates were incubated at 28 °C for 5-7 days. After incubation, *Actinomycetes* growing colonies were selected and purified by subculturing on ISP-2 agar medium plates according to type and forms of these colonies. Then the purified colonies were examined under the oil immersion objective at the light microscope. After this, the typical growing

colonies of *Streptomyces* were cultured on International *Streptomyces* Project type-2 agar slants and stored at 4°C for further uses (Deepthi *et al*., 2012).

3.7.1.3: Morphological Identification of *Actinomycetes*

Streptomyces spp isolates were Identified by the study of morphological characteristics for colonies, aerial mycelia and substrate mycelia (Mantada *et al*., 2013). Characteristics of *Streptomyces* spp isolates were done by cultured of these bacteria on different agar media of the International *Streptomyces* Project (ISP), such as (ISP-2) (Rathna Kala and Chandrika., 1993) in accordance with the International *Streptomyces* Project (ISP) and noted their characteristics (Shirling and Gottlieb., 1966).

3.7.2 *P. aeruginosa*

In this study, a total of 150 collection of specimens from wound swab, burn swab , Otitis media swab and from UTI of patients were hospitalized at Babylon hospitals for both genders with different ages. The specimens collected during the period from February 2022 to April 2022.

3.7.2.1 Culturing

All specimens were cultured on different media for identification of *Pseudomonas* such as blood agar, MacConkey agar, and cetrimid agar, using sterile loop spread on the surface of agar media and incubated at 37 C° for 24 hr. (Jawetz *et al*., 2019). Purified colonies kept in nutrient broth containing glycerol at -20 C° in (Jawetz *et al*., 2019). After final diagnosis of samples, 50 (33.3%) isolates of *P. aeruginosa* were obtained. Forty-six isolates of *P. aeruginosa*, (named Pa1 to Pa50).

3.7.2.2 Microscopic examination

After the growth of bacteria on MacConkey agar, blood agar, cetrimid agar and nutrient agar, their shape, size, texture, and colony arrangement was observed. A single colony was picked up, stained with Gram stain, and examined under the light microscope (100x) using oil emersion (Jawetz *et al*., 2019).

3.7.3 Biochemical tests

3.7.3.1 Catalase test

Few drops of catalase reagent were added on slide with single colony of *P. aeruginosa* by using sterile loop. A positive result indicated the formation of bubbles. This test was used to detect the ability of bacteria to produce the catalase enzyme, which broke down the H_2O_2 into oxygen and water (Brown and Smith, 2017; Cappuccino and Welsh, 2018).

3.7.3.2 Oxidase test

The oxidase reagent was added in few drops on filter paper and mixed with single colony of *P. aeruginosa* using sterile wooden stick. A positive reaction was indicated by the development of purple color within 10 second. This test was used to detect the ability of bacteria to produce the oxidase enzyme (Brown and smith, 2017; Cappuccino and Welsh, 2018).

3.7.3.3 IMVC test

As mentioned by MacFaddin (2000), this test was done in the following way.

A- Indole test

Peptone water medium was inoculated with overnight tested bacterial culture and incubated at 37 Cº for 24 hr. After that 10 drops of Kovac's reagent were added directly to the culture tube; the appearance of the red ring at the top of the broth after gentle shaking indicates a positive result. This test is used to

detect the *P. aeruginosa* capacity to produce a tryptophanase enzyme which hydrolyzed tryptophan to indole, pyruvic acid, and ammonia.

B- Methyl red test

Methyl red-Voges proskauer medium was inoculated with bacterial culture that was tested and incubated at 37 Cº for 24 hours. Then five drops of the methyl red were added. Appositive test changed of medium color from yellow to red. This test was used to detect the bacterial ability to ferment glucose and produce acid as a final product.

C- Voges-Proskauer test

Methyl red-Voges proskauer medium was inoculated with bacterial culture that was tested and incubated at 37 Cº for 24 hours, then few drops of α-naphthol, and KOH were added. A positive reaction was indicated by development of a pink color with15 minutes**.** This test was used to detect the bacterial ability to ferment glucose and produce acetoin.

D- Citrate utilization test

Simmon's citrate slant agar was inoculated with tested bacterial culture by sterile loop and incubated at 37 Cº for 24 hours, a positive result was indicated by changing the color of the medium from green to blue. This test was used to detect the bacterial ability to utilize sodium citrate as carbon source.

3.7.3.4 Urease test

Urea agar slant was inoculated with tested bacterial culture by sterile loop, and then incubated at 37 Cº for 24 hours; existence of pink color indicates a positive result. This test was used to detect the bacterial capacity to produce urease enzyme which hydrolyzes urea to ammonia and carbon dioxide (Cappuccino and Welsh, 2018).

3.7.3.5 Motility test

Semisolid mannitol media were stabbed in the center with an inoculated needle and incubated at 37 °C for 24 hours. Spread out growth from the line of inoculation indicates the existence of motile bacteria (MacFaddin, 2000)

3.7.3.6 Triple sugar iron (TSI) test

The cultured isolates were streaked on surface of slope and stabbed into butt, and then incubated for 37°C for (24) hours. The positive result of *P. aeruginosa* was alkaline / no change or alkaline / alkaline with no produce H2S and gas (Brown and Smith, 2017).

3.8 Molecular identification of *Streptomyces spp.* And *P. aeruginosa*

This involved extraction of genomic DNA from *Streptomyces* cultures and *P. aeruginosa* to be used in their identification by amplification of 16S rDNA gene of *Streptomyces* and by using a species-specific primer for the *P. aeruginosa* specific gene 16S rRNA

3.8.1: Extraction of Genomic DNA from *Streptomyces* Isolates

Genomic DNA was extracted from *Streptomyces spp* cultures as the following

- *Streptomyces* isolates were cultured in polyethylene tubes contained 10 ml of Luria broth medium and incubated at 28°C for at for 7 days.
- The tubes were centrifuged at 5000 rpm for 5 min.

- *Streptomyces* cells were collected after removed the supernatant.
- The cells were resuspended in 200µl of 1 x TE buffer.
- 25 µl of lysozyme (50mg/ml) was added and the tubes were incubated at 37°C for 1h.
- Tubes were centrifuged at 5000 rpm for 3 min and the supernatants were removed.
- The pellets were resuspended with 200µl of resuspension buffer.
- Elution tubes were placed at the rack, tip rack, and buffer Cartridge on the step tray.
- Holes were punched through the buffer cartage sealing films with the aid of 6-hole punch tool in the locations corresponding to the tubes and tips and turn on the *ExiPrep*™ instrument.
- The holes were punched, before make sure to shake buffer cartridge to suspend the beads, then gently swing outward to settle solution to the bottom of the wells.
- 200 µl of samples were loaded into the loading wells.
- The *ExiPrep*™ instrument was Turn on.
- The Store button was pressed for cooling (*ExiPrep*™ 16 pro only).
- The start button was pressed to access the PREP SETUP menu.
- A protocol number was entered from the protocol list that best suits your samples source and target nucleic acid type.
- The Enter button was Presses to proceed to the next step.
- The desired elution volume was select from the screen.

- The elution volume was recommended: 100 µl.
- The ok button was pressed to move to the next step.
- Opened the instrument door and pull out the base plate.
- All racks were placed and buffer cartridge in their correct positions on the base plate.
- Buffer cartridge was placed into its labeled position on the base plate.
- Buffer cartridge was placed into its labeled position on the base plate.
- The elution tube rack was placed into its labeled position on the base plate.
- The disposable filters tips were placed rack into its labeled position on the base plate.
- The waste tray was placed into its proper position (between buffer cartridge).
- The base plate completely pushed back into the instrument and close the door.
- The protocol name was verified on the screen. The first two letters represent the target nucleic acid type, and the next two letters represent the samples source.
- The Run button was placed to start the extraction run.
- The run process was successfully completed, three options will display.
- The door was opened and pull out the base plate.
- The elution tubes were toke from base plate.

- The buffer cartridge was removed and all racks from base plate and closed the door.
- The purified genomic DNA was quantified.
- This procedure was performed according to (Honore *et al*., 2003).

3.8.2 Agarose Gel Electrophoresis

Agarose gel was created by dissolving 1 gram of agarose powder in 100 milliliters of 1X TBE buffer. A melting agarose gel was created by mixing 10ml of TBE buffer with 90ml of cold water. This gel was then melted in a microwave until the solution turned transparent. The amount of agarose that can be dissolved depends on the intended application of the agarose gel. 0.7 percent of agarose gel is utilized for DNA visualization following extraction, while 1.5 percent to 2 percent agarose sheet is used for PCR product visualization (amplicon). The stock solution concentration of simply safe (replacement for ethidium bromide) was 10 mg/ml. To get a final concentration of 0.5 mg/ml, only 5µl of simply safe stock solution was added to 100ml of agarose gel that was melting (Green and Sambrook, 2012). The agarose was poured into the gel tray with the ends capped, the comb was appropriately positioned, and then it was left to dry. 5 µl of the amplified DNA (the result of the PCR process) is loaded into a second well of the gel, while 5µl of the DNA marker is loaded into the first well. The electrodes were properly attached, and the run was performed in accordance with the gel percentage and gel size. (The time required for agarose gel electrophoresis is 45 minutes for genomic DNA and one hour for PCR product.

3.8.3 DNA sequencing analysis

Most two isolates have higher antimicrobial activity were selected for sequencing. Sequences were then compared with the *Streptomyces spp* sequences present in Geneious program version 2022.2.1 and GenBank using the National Center Biotechnology Information (NCBI) online at (http://www.ncbi.nlm.nih.gov) the NCBI program and aligned with the corresponding sequence fragments of the database using the Basic Local Alignment Search Tool (BLAST) BLASTX program, Morgulis et al., 2008 by using Pairwish in BLASTn. Phylogenetic trees were created by the neighbor-joining method using the MEGA_ X program version 10.1.8.

3.9 Screening Test of *Streptomyces spp*.

3.9.1 Primary Screening Test of *Streptomyces spp.*

This antibacterial activity of *Streptomyces* spp isolates were tested by perpendicular streak plate method (Duraipandiyan *et al*., 2010). This method was prepared by cultured of *Streptomyces* spp isolates separately through made cross lines from these isolates in the middle of Muller Hinton agar media and incubated at 28°C for 3 days. After this, columns from the pathogenic test bacteria were streaked at right angles on each side of the *Streptomyces* spp isolates at straight line colony (Zhomghui and Wei., 2000 ; Suthindhiran and Kannabiran., 2009). The media then incubated at 37°C for 24 h. and the results were recorded as positive through inhibition growth of pathogenic test bacteria and (Nanjwade *et al*., 2010).

3.9.2 Extraction of secondary metabolites from Streptomyces *spp.*

Extraction from Muller Hinton Agar Plates Primarily, the antibacterial secondary metabolite was extracted in small quantities using the agar extraction method, which was adopted from Manikindi *et al*. 2016 bacterial

culture was prepared by inoculating a single colony into a 2 mL M-H broth, then incubated in a shaking water bath for 18 hours. The seed culture was grown at 27 °C. To produce the compound, Fifteen large (150 mm × 15 mm) M-H agar Petri dishes were used. The seed inoculum of the Streptomyces spp. was streaked on M-H agar plates by using a sterile cotton swab. Then the plates were kept in an incubator at 19 °C for two weeks. After growth, the agar plate was sliced into small square (1 cm × 1 cm) pieces Figure (3-1) The agar square pieces were collected in a 250 mL beaker and ethyl acetate was added to soak the agar pieces. The beaker was wrapped with parafilm and let stand for one day. The ethyl acetate extract was transferred into a 100 mL beaker and was left under fume hood to evaporate the organic solvent. The left over agar slices were again soaked in a small amount of ethyl acetate solution to remove all the active compound from the agar. Once the collective ethyl acetate extract was evaporated to dryness, the dried extract was dissolved in 1 mL of methanol (MeOH) and transferred to an Eppendorf tube and stored at 4 °C for later use. (**Appendix 1)**

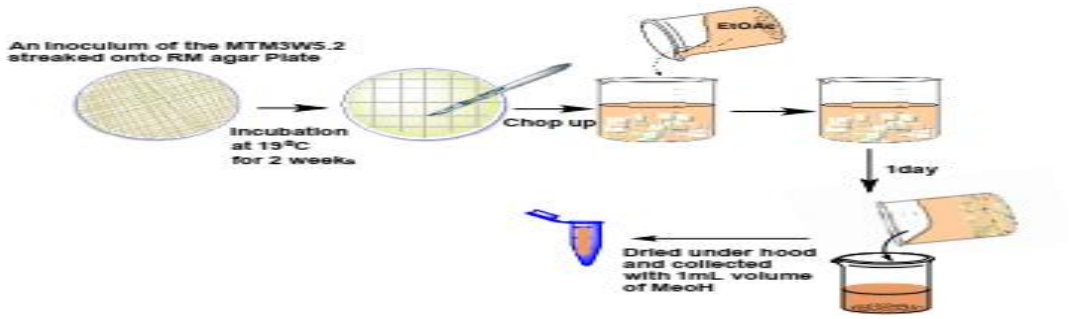


Figure (3-1) : Agar extraction method. An inoculum of *Streptomyces* sp. Was streaked on M-H agar plate and incubated at 19 C for two weeks. The agar plate was then chopped into small pieces, and metabolites were extracted using ethyl acetate.

3.10 : Methods used for Characterization of Produced Antibiotics

There are three methods have been used for purification and identification of antibiotics that extraction from *Streptomyces spp* in this

study. These included, Ultraviolet spectroscopy (UV) and Infrared spectroscopy (IR) . The uses of these methods are explained as the following

3.10.1: Ultraviolet (UV) Spectroscopy

After extraction of antibiotics from *Streptomyces spp* fermentation broth culture when treated with ethyl acetate. The extracted powder was re dissolved with the ethyl acetate to form liquid state and to be measured by spectrophotometer for known the λ_{max} that give idea about the wavelengths of absorption in range from 200 nm to 800 nm. The peaks that formed from measured the structure of materials which can be correlated with the types of bonds in a given molecule and are valuable in determining the functional groups within a molecule (Taware *et al*., 2014). This assay was done in Babylon University / College of Science / Department of Chemistry.

3.10.2: Infrared (IR) Spectroscopy

After antibiotics have been extracted from *Streptomyces spp* fermentation broth culture after seven days from incubation period by treated with ethyl acetate. Grinded appropriate quantity of extracted powder with purified salt such potassium bromide finely this for remove scattering effects from large crystals and this powder mixture and pressed in mechanical press to form then layer and this for beam of the spectrometer can pass through which (Paun *et al*., 2013).

3.10.3 Gas Chromatography - Mass Spectroscopy

GC–MS analysis was performed using a GC-Mass Agilent gas chromatograph (USA) equipped with an Elite -I , fused silica capillary column diameter (30 m * 250 nm) . An electron ionization system with ionizing energy of (70 V) was used . Helium gas (He) was used as the carrier gas at a

constant flow rate of (1 mL /min) and an injection volume of (1 mL) was employed . The injector temperature was 190C and the ion - source temperature , 240 C˚ . The temperature was programmed from 50C (for 1 min) to 240C , with an increase of 12.5C/ min , then 5C/min to 280C , ending with a 5 min at 240 C˚

3.11: Identification of pathogenic Gram negative bacteria

The Gram negative bacteria (*Escherichia coli, Klebsella pneumoniae*) were diagnostic and obtained from the laboratory of Al Noor Children's Hospital - Babil Governorate .

3.12 Minimum Inhibitory Concentrations and Minimum Bactericidal Concentrations (MICs & MBCs) Determination

The minimum inhibitory concentrations (MICs) of the Actinomycetes isolate were determined by a serial dilution technique. MICs were defined as the lowest concentration of an antimicrobial that inhibits growth of a microorganism after their incubation for overnight. To determine MIC:

1. An appropriate amount (0.1mg antimicrobial plus 10 ml respective solvent) of antimicrobial extract was dissolved in respective solvent to prepare an antimicrobial solution containing 10 µg/ml.

2. Two fold dilutions of the antibiotic solution in Mueller Hinton broth were prepared and describe below: (a) Ten sterile tubes were placed in a rack and were labeled each 1 through 8 and first one labeled as antibiotic control) and last one was labeled as G.C (growth control).

(b) 1 ml of Mueller Hinton broth was added in each test tube.
(c)1 ml of antimicrobial solution was added to test tube No 1 and A.C.
(d) With a sterile micropipette and tips, after adequate mixture 1 ml was transferred from tube No. 1 to tube No. 2.
(e) After a through mixing, 1 ml was transferred with a separate micro pipette from tube No 2 to tube No 3.
(f) This procedure was repeated through the next-to-next up to the tube No. 7. Except tube No G.C. (using fresh pipette for each dilution). From tube no 8 1 ml was removed and discarded. The last tube (tube G.C) received no antimicrobial agent and was served as a growth control. First A.C labeled test tube was served as antibiotic control.
3. Each tube was inoculated (including the growth control except antibiotic control) with 1 ml of the culture of respective organism. The final concentration of antimicrobial agent in this test tube was half of the initial dilution series because of the addition of an equal concentration of inoculums in Mueller Hinton broth.
4. The tubes were incubated at 37ºC for 24 hours.
5. The tubes were examined for growth and were determined the MIC of tested antibiotics, which is bacteriostatic for the test organism. The tubes were examined for visible growth (cloudy) and was recorded growth as (+) and no growth as (-).
6. For determination of minimum bactericidal concentrations (MBC), the concentration which was bactericidal, was then found by sub cultured the

contents of selective tubes into a series of Mueller Hinton broth, which did not contain any antibiotic and started from last two non-visible tube to the first two visible tube (direction tube No. 1 to tube No. 7). Then was inoculated into Mueller Hinton agar containing Petri plate by 0.1 sterile micropipette and separate 0.1 ml sterile tips in drop method

7. The plates were incubated at 37ºC for 24 hours. (Reiner, 1982) .

Table(3-12) Determining of MIC and MBC

Tube No.	AC	1	2	3	4	5	6	7	G.C
1) Mueller Hinton broth (1ml)	1	1	1	1	1	1	1	1	1
2) Antibiotic solution (1ml)	1	1	1	1	1	1	1	1	0
3) Bacterial suspension (1)ml	0	1	1	1	1	1	1	1	1
4) Final Volume 2 ml	2	2	2	2	2	2	2	2	2
5) Final antibiotic concentration µg/ml	5	5	2.5	1.25	0.62	0.31	0.15	0.078	0

3.13 Exploration the safety of secondary metabolite produced by evaluating its cytotoxicity property.

Cytotoxicity assays are used for drug screening and cytotoxicity tests of chemicals usually by determining the number of viable cells remaining after a defined incubation period.

3.13.1. Cytotoxic Effect of extract Isolated from Two *Streptomyces* isolates (st6, st12) on Normal and Tumor Cell Lines.

This *in vitro* method was performed to investigate the possible cytotoxic effect of crude extract on tumor cell lines(cell line exhibiting epithelial morphology that was isolated from the skin of a 54-year-old, female patient with malignant melanoma (A375). and normal cell line (Dermal Fibroblast Normal Human Neonatal (HdFn) .

3.13.2 Cell Line Maintenance

When the cells in the vessel formed confluent monolayer, the following protocol was performed (Freshney, 2010):

A- The growth medium was aspirated and the cell sheet washed with PBS.

B- Two to three ml trypsin/EDTA solution was added to the cell. The vessel was turned over to cover the monolayer completely with gentle rocking. The vessel allowed incubation at 37°C for 1 to 2 minutes, until the cells were detached from the vessel.

C- Fresh complete RPMI medium (15-20 ml) was added and cells were dispersed from the wedding surface into growth medium by pipetting.

D- Cells were redistributed at required concentration into culture vessels, flasks or plates whatever needed and incubated at 37°C in 5% CO_2 incubator.

Cell concentration was achieved by counting the cells using the haemocytometer and applying the formula:

Total Cell Count/ml: cell count x dilution factor (sample volume) x 10

3.13.3 MTT Protocol

The cytotoxic effect of external and internal crude extracts was performed by using MTT ready to use kit (Intron Biotech):

A- Kit contents:

• MTT solution 1 ml x 10 vials.

• Solubilization solution 50 ml x 2 bottle.

B- Protocol:

• Tumor cells ($1x10^4$ – $1x10^6$ cells/ml) were grown in 96 flat well micro-titer plates, in a final volume of 200µl complete culture medium per each well. The microplate was covered by sterilized parafilm and shacked gently.

• The plates were incubated at 37°C, 5% CO_2 for 24 hrs.

• After incubation, the medium was removed and two fold serial dilutions of the desired compound (25, 50, 100, 200 and 400 μg/ml) were added to the wells.

• Triplicates were used per each concentration as well as the controls (cells treated with serum free medium). Plates were incubated at 37°C, 5% CO_2 for selected exposure time (24 hrs).

• After exposure, 10 μl of the MTT solution was added to each well. Plates were further incubated at 37°C, 5% CO_2 for 4 hrs.

• The media were carefully removed and 100μl of solubilization solution was added per each well for 5 min.

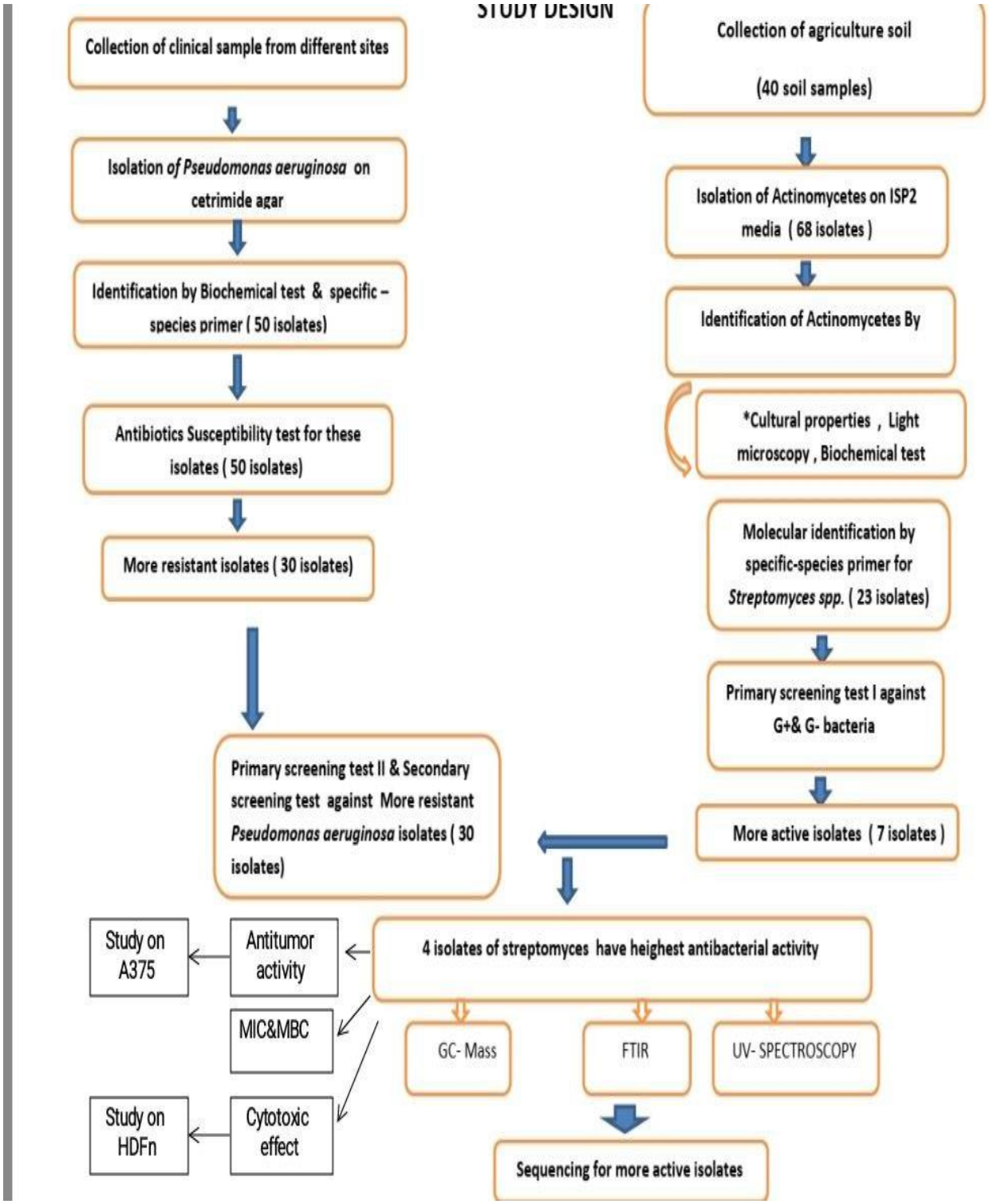
• The absorbance was determined by using an ELISA reader at a wavelength of 575 nm. The data of optical density was subjected to statistical analysis in order to calculate the concentration of compounds required to cause 50% reduction in cell viability for each cell line, through the following equation:

cell viability % = [(mean absorbance of treated samples/mean absorbance of non-treated sample) ×100]

3.14 Statistical Analysis

Data were expressed as mean± standard deviation and statistical significances were carried out using Graph Pad Prism version 6 (Graph Pad Software Inc., La Jolla, CA)(Buysse et al., 2004).

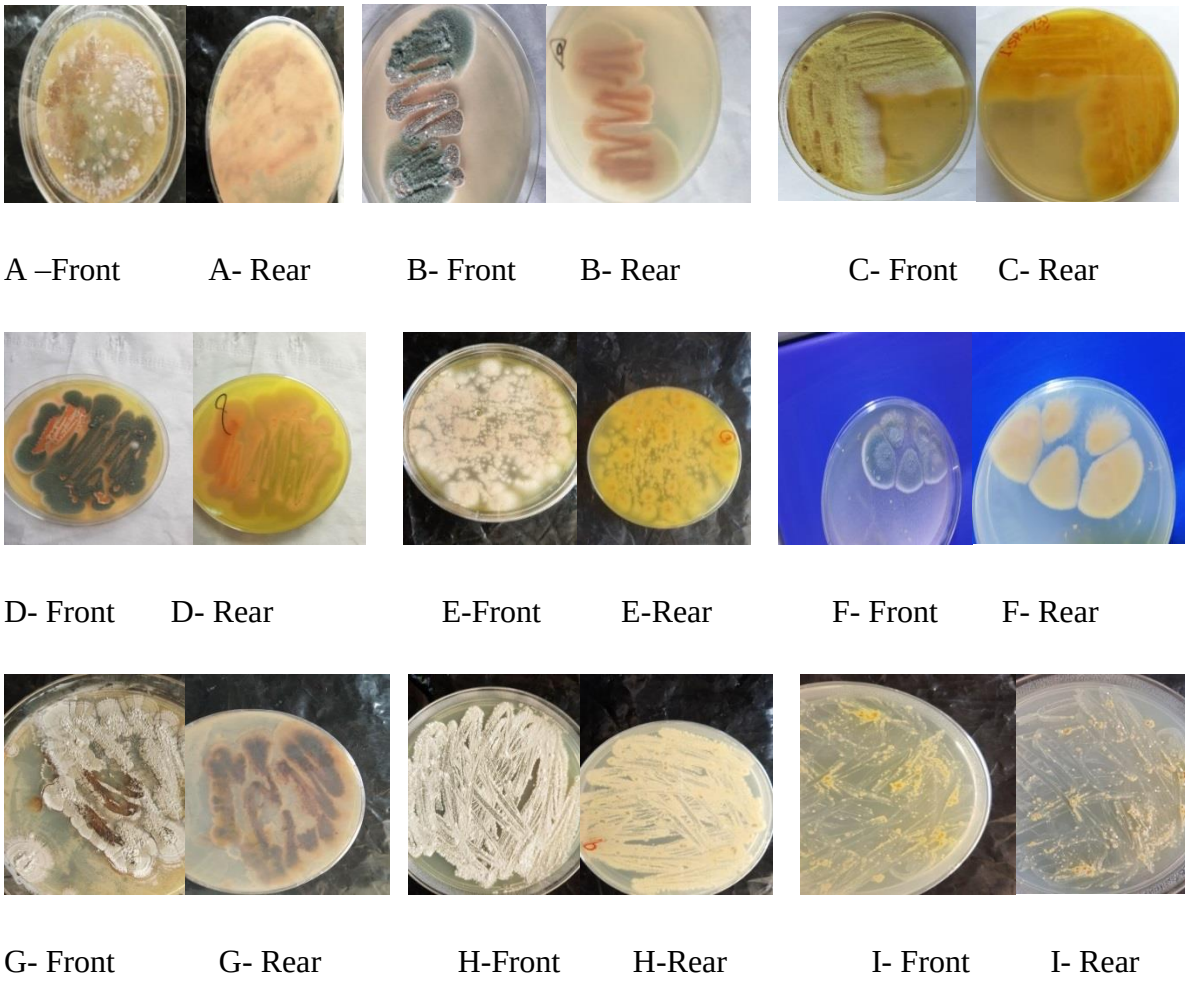
Study Design :



4. Chapter Four: Results and Discussion

4.1: Isolation of *Actinomycetes*

The soil samples were collected randomly from ten location sites of Agricultures areas in Babylon city **Table(4-1)** , the pH of soils sample ranged from (6.5-8.5) **.** and the temperature of soils ranged (26-31) C˚ . Out of 40 soil samples were obtained 68 colonies of different morphological types of Actinomycetes . The color of selected suspect colonies ranged from white, creamy , gray , green ,black to yellow. The morphology and size of the colonies was about 1-10 mm in diameter with a relatively smooth surface at the beginning of the growth, while it was developed to an aerial mycelium that appeared as granular, powdery and soft. Figure (4-1)



A –Front A- Rear B- Front B- Rear C- Front C- Rear

D- Front D- Rear E-Front E-Rear F- Front F- Rear

G- Front G- Rear H-Front H-Rear I- Front I- Rear

Figure (4-1) : Different types of Actinomycetes

These findings were in agreement with those of barbyd (2019), who determined the pH of soils that isolated the Actinomycetes from it , ranged from (6.5 – 8.5) and the temperature between (27- 32) C˚. (Barbyd ,2019) . Other related studies have similarly documented that the optimal pH for the growth of actinomycete is the pH of 7. Moderate growth which was significantly less compared to the growth at pH 7 were observed at pH of 6 and 8 while the least growths were found at the pH of 5 and 9. (Attimarad *et al*. 2012)

Table(4-1) : Numbers and percentage of Actinomycetes isolates obtained from 8 location sites of Agricultures samples (5 samples each)

Site of Collection	**Actinomycetes NO.**	**Samples (%)**
Hilla	8	11.76%
Al-Mahaweel	9	13.23%
Al-Nile	10	14.70%
Al-Mussaiab	7	10.29%
Al –Sadda	5	7.35%
Al-Hashmia	14	20.58%
Al-Hamza	7	10.29%
Al-Kasim	8	11.76%
Total	68 isolates	100%

In order to obtain pure colonies culture of locally isolated strains, 68 colonies were obtained from the isolation processes. These colonies were subcultured on ISP2, and the sub-culturing process was repeated many times. More

of them share a lot of similarities morphologically based on the color of the aerial mycelium, especially when they are purified on plates. However, for additional research and primary screening processes. Biochemical test results show that all isolates are positive to the Catalase test , all are negative for Oxidase test and positive for Gram stain . All isolates have earthy odor .

The results were consistent with the findings of Zhou *et al.* (2007) and Portillo *et al.* (2009) regarding the isolation process, which showed that each plate frequently contained one or a small number of colony types , ranging from two to four colonies, and that the actinomycetes diversity only showed a small number of distinct colony types from similar habitats.

4.1.1 Cultural Characterization of *Streptomyces* Isolates

Visual observation of both morphological and microscopic characteristics using light microscopy, Gram-stain properties were performed. The result showed there were 23 isolates related to *streptomyces spp.* .All morphological characters were observed on International Streptomyces Project type 2 (ISP2) were used for classification and differentiation as follows : Aerial mass color which the mass color of mature sporulating aerial mycelium was observed following growth on ISP2 plates. The aerial mass was classified according to the Bergey's manual of systematic bacteriology in the following color gray , white, red, yellow, green, blue, and violet . Also by Substrate mycelium. Distinctive colors of the substrate mycelium were recorded . The observed colors were beige, black, blue, brown , orange , purple , pink , yellow , yellow-greenish .

Also spore chain morphology that according to the shape of the spore chains observed under light microscopy, the isolates were grouped as follows: Rectus-Flexibilis , spores in straight or flexuous chains, and Spira , spore chains in the form of short gnarled or compact coils or extended, long and open coils.

These results aggreement with Kitouni *etal.* Results which they found the abundance of *Streptomyces* in ecosystems compared to the other genera of actinomycetes.(Kitouni *et al*.,2005)

However, a number of workers have been employed by the International Streptomyces Project Two (ISP-2) to maintain and research cultural traits for Streptomyces species, such as the colors of aerial mycelium and substrate mycelium (Mantada *et al*., 2013 ; Pordeli *et al*.,2013). The ISP-2 medium is a commercially available formulation for the growth and characterization of Streptomyces. This agar medium includes specific ingredients like yeast and malt extract, which are vital and rich in nutrients like nitrogen, amino acids, and vitamins, as well as dextrose, which serves as the source of carbon for the growth of these bacteria. In addition, ISP-2 medium is consistent for good growth for these bacteria in contrast to other media that are varied. Additionally, on ISP-2 medium, more cultural habits for Streptomyces spp. were demonstrated, such as the colors of different aerial mycelia and substrate mycelia, compared to those looking homogenous on other media. This evidence indicates ISP-2 is an effective medium for enhancing the cultural traits of several Streptomyces species (Zin *et al*., 2011). Therefore, Our focus is on studying the morphological characteristics of *Streptomyces spp*. colonies and the colors of aerial and substrate mycelia for the (ISP-2).

4.1.2 Molecular Confirmation of *Streptomyces spp.* Isolates identification by amplification of 16S rRNA gene

After identification of *Streptomyces* by morphological features , these isolates then subjected to molecular identification depended on amplification of 16S rDNA gene by polymerase chain reaction. PCR performed for amplification of genomic DNA by using the16S rRNA primers for identification of *Streptomyces*

spp. isolates , and the product of the polymerase chain reaction was approximately (500 bp) **. Figure (4-2)**

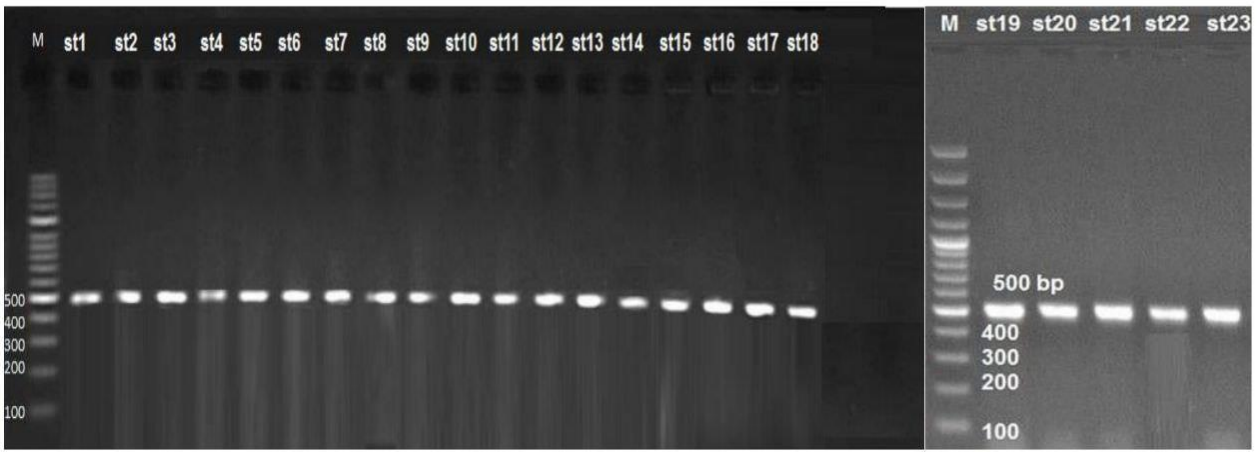


Figure (4-2): Agarose gel electrophoresis for amplified (500 bp) specific- species streptomyces PCR product for 16S rRNA. Bands were fractionated by electrophoresis on a 1% gel (1 h., 80V/cm) and visualized under U. V. light after staining with safe red.

4.2 Isolation of Clinical *P. aeruginosa* Isolates

The results of isolation of different clinical samples (150) distributed as UTIs patients 50 (33.3%) , burn infection patients 50 (33.3%) , wounds infection 30 (20%) while 20 (13.3%) for ear swab, The results revealed high percentage of *P. aeruginosa* 50 isolates distributed as UTIs patients 14/50 (28%) , burn infection patients 26/50 (52%), wounds infection 6/50 (12%) while 4/50 (8%) for otitis media (table 4-2).

Table (4-2): Types numbers and percentage of clinical samples collected from the patients

Clinical samples	NO. of samples	NO. of *P.aeruginosa* isolates	Percentage
UTIs	50 (33.33 %)	14	28 %

burn	50 (33.33%)	26	52 %
Wound	30 (20 %)	6	12 %
Ear swab	20 (13.33%)	4	8 %
total	150 (100%)	50	100%

According to this table, *P. aeruginosa* was highly isolated from burns samples (54%), which is the same as the findings of Fazeli and Momtaz (2014) and Saleh *et al*. (2012), who found that *P. aeruginosa* accounted for (50% and 51.21% respectively) among burns patients.

4.2.1 Identification of *P. aeruginosa* on different media.

On culture mediums (blood agar, MacConkey agar, and cetrimide agar), the morphological characteristics of colonies were examined. The colonies on the blood agar were sticky-textured, white to gray or dark-colored bacteria that were able to hemolyze blood. While *P. aeruginosa* on Macconkey agar were a pale color due to their inability to ferment the lactose sugar present in this culture medium and had a smell similar to fermented grapes, the positive result for P. aeruginosa on blood agar was typically displayed beta hemolysis and blue or green pigment (Jawetz *et al*., 2019) , Cetrimide agar had a mucoid appearance on it, was smooth in shape with flat sides and an elevated center, was creamy in color, and smelled fruity. Cetrimide agar is a selective medium for the isolation and presumed identification of *P. aeruginosa* that contains peptone, $MgCl_2$,K_2SO4,, cetrimide, agar, and add rehydrated contents of one vial of nalidixic acid selective supplement (FD130), which inhibits other microbial flora, grows with Pseudomonas spp., and produces blue-green pigment that is a combination of (Aryal *et al*., 2015). This outcome is comparable to earlier investigations conducted in Iraq (AL-Rubaye *et al*., 2015), which initially identified *P. aeruginosa* using cetrimide agar and other

media. The difference in collection time, location, and hospital unit could all have an impact on the percentage of *P. aeruginosa* isolates (intensive care unit is more implicated than other units).

4.2.2. Detection of *P. aeruginosa* using biochemical methods

Some biochemical tests were performed for more confirmation of *P.aeruginosa* isolates Table (4-3) . It has given in biochemical tests; a positive result for oxidase and catalase tests, while have given negative results to the urease test. The isolates showed negative results for indole, methyl red (MR) and Voges-Proskauer (VP) tests, but have given positive result on Simmon's citrate agar for citrate utilization test, these isolates could utilize the citrate as a carbon sole source. In Kligler iron agar have given alkaline slant and did not change the bottom, H_2S negative without gas production due to the fact that they are strictly aerobic and negative to Gram's stain. The isolates showed ability to grow on cetrimide agar at 42˚C for 24 hrs. (Macfaddin, 2000).

Table (4-3) : Biochemical tests for identification of *P. aeruginosa* isolates

Test	**Results**
Gram-stain	G- rods
Catalase test **,** Oxidase test	(+)
Growth at 42°c	(+)
H2S production	(-)
Indole test	(-)
Kligler's iron agar	Alkaline / Alkaline
Methyl-red	(-)
Voges-Proskauer	(-)

Pigments production	(+)
Simmon's citrate	(+)
Urease	(-)
Motility	(+)

Abbreviations : (+), positive test; (-), Negative test

4.2.3 Molecular Confirmation of *P. aeruginosa* identification

Molecular techniques to identify *P. aeruginosa* more precisely Cetrimide agar (Selective Medium) was used to detect *P. aeruginosa*, and the results were validated by PCR using a species-specific primer for the *P. aeruginosa* specific gene 16S rRNA . The results showed that all isolates were 100% *P. aeruginosa* (Figure 4-3). A practical, inexpensive, and accurate method that enabled the identification and quantification of *P. aeruginosa* in a shorter amount of time was devised by combining the chromogenic agar and PCR procedures. Cetrimide agar for *P. aeruginosa* has been shown in numerous studies to be a suitable medium for direct isolation and identification with excellent sensitivity and specificity (Laine et al., 2009).

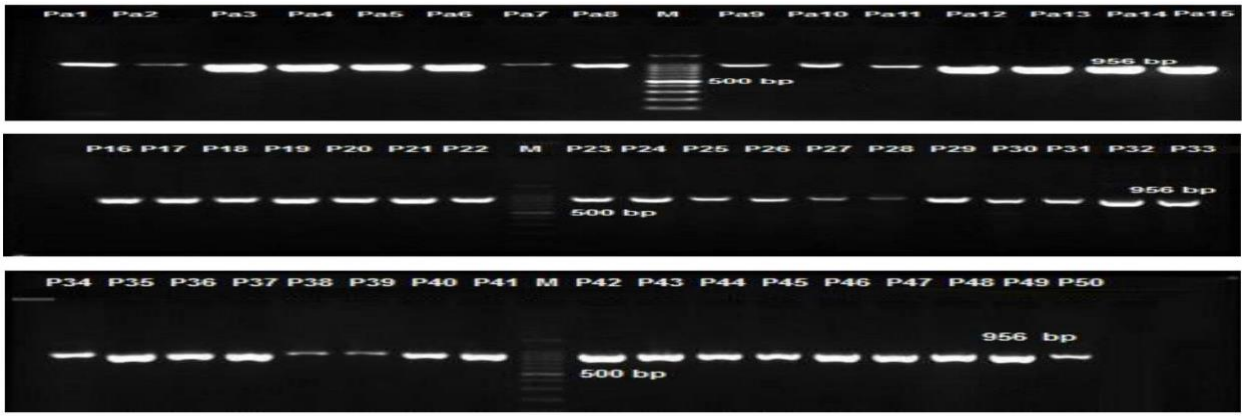


Figure (4-3): Agarose gel electrophoresis for amplified (956 bp) *P. aeruginosa* specific gene 16S rRNA . Bands were fractionated by electrophoresis on a 1% gel (1 h., 80V/cm) and visualized under U. V. light after staining with safe red.

4.2.4 Antibiotic Susceptibility Test

Fifty identified *P. aeruginosa* isolates (Pa1 to Pa50) were evaluated against 17 common antibiotics, as shown in Figure (4-4) and Table (4-4).

Table (4-4) Antibiotic Sensitivity of *P. aeruginosa* Isolates

Antibiotic disc	No. (%) of resistant isolate	No. (%) of intermediate isolate	No. (%) of sensitive isolate
Amikacin (AK)	35 (70%)	2(4%)	13 (26%)
Azteronam (ATM)	50 (100%)	0(0%)	0(0%)
Ceftazidime (CAZ)	14 (28%)	15 (30%)	21(42%)
Ceftriaxone (CTR)	24(84%)	0(0%)	8(16%)
Cepfepime (CEF)	50 (100%)	0(0%)	0(0%)
Ciprofloxacin(CIP)	24(48%)	17(34%)	9(18%)
Doripenem (DOR)	19(38%)	0(0%)	31(62%)
Gentamicin (GN)	45 (90%)	1(2%)	4 (8%)
Imipenem (IPM)	50 (100%)	0(0%)	0(0%)
Levofloxacin (LEV)	21 (42%)	3(6%)	26 (52%)
Meropenem (MEM)	21 (42%)	7(14%)	22 (44%)
Netilmicin(NET)	50 (100%)	0(0%)	0(0%)
Norfloxacin(NOR)	20 (40%)	13 (26%)	17 (34%)
Ofloxacin(OFL)	50 (100%)	0(0%)	0(0%)
Piperacillin (TPZ)	50 (100%)	0(0%)	0(0%)
piperacillin-tazobactam	13(26%)	8(16%)	29(58%)

(P/T)			
Tobramycin (TOB)	50 (100%)	0(0%)	0(0%)

The majority of isolates exhibited antibiotic resistance, particularly β-lactam antibiotics. All 50 isolates of *P. aeruginosa* test were resistant to tobramycin, piperacillin, cefepime, imipenem, ofloxacin, aztreonam, and netilmicin.**.** Antibiotic such as the tobramycin, piperacillin, cefepime, imipenem, ofloxacin, aztreonam, and netilmicin all exhibited 100 % resistance, whereas norfloxacin (40 %), piperacillin-tazobactam (13 %), levofloxacin (21 %), amikacin (35 %), meropenem (21 %), ciprofloxacin (42 %), doripenem (19 %). Table (4.4) shows the phenotypic of antibiotic susceptibility of bacterial isolates in this study .

The study revealed that *P. aeruginosa* is 100% resistant to piperacillin in 50/50 samples. According to Vitkauskien *et al.* (2010), and Hussein *et al*. (2018), who reported rates of 37.0%, 59.61%, and 67.96% respectively, this result is completely inconsistent. Although Al-Marzoqi (2013) and Corehtash (2015) reported resistance rates of 100% and 85.4%, respectively, this result is comparable to or near to those results. Beta lactam-beta-lactamase inhibitors combination antibiotics also showed resistance to piperacillin-tazobactam 13/50 (26%) . The resistance to cefepime were 50/50(100%) his result was close with the results of Othman *et al.* (2014) who reported a resistant rate of *P. aeruginosa* of ceftazidime which were 100% .

The results of the current study are consistent with the results of Al Shwaikh and Alornaaouti (2018) as the isolates of *P. aeruginosa* showed high resistance to Ceftriaxone, Amikacin , piperacillin and was resistant to both ciprofloxacin (43%) and tobramycin (100%) and gentamicin (87%) and resistant to both ofloxacin and imipenem resistant (100%). The majority of isolates exhibited antibiotic resistance., particularly β- lactam antibiotics. The results showed high resistance to

beta lactams mainly piperacillin and this is mediated by beta lactamases due to that when use piperacillin-tazobactam the resistance was dropped from 100% to 26%. Beta-lactamases regard as intrinsic mechanism of resistance leading to inactivating of beta lactam rendering them inactive. Beta lactamase inhibitor like tazobactam (An irreversible inhibitor of a wide variety of bacterial beta-lactamases) can improve many beta lactams like piperacillin once combined with them. Piperacillin/tazobactam is the most widely used β-lactam-β-Lactamase inhibitor combination for treating *P. aeruginosa* infections (Al Muqati *et al*.,2021)

Resistace to carbapenems showed that 38% , 42% and 100% for doripenem, meropenem and imipenem, receptivity, as shown in Figure (4.). Imipenem result was close to that of Fazeli *et al*. (2017) who reported a rate resistance 98.7%, but different from Savari (2016) and Vitkauskienė (2010) who recorded resistance 22% and 24%, receptivity. Meropenem result is compatible with Gad *et al*. (2007) who reported 22%, and far from Coetzee *et al*. (2013) which reported extremely higher rate (93.4%). Carbapenems (Imipenem and Meropenem) antibiotics are members of a β-lactams family, mainly used to treat *P. aeruginosa* infections. Aminoglycosides resistance which icluded, gentamicin 45/90 (90%), tobramycin 50/50 (100%), amikacin 35/50(70%), netilmicin 50/50 (100%) as shown in Figure (4-4). Gentamicin resistance rate recorded in this study was (90%), this result is far from that documented by Vitkauskienė *et al*. (2010) who reported (37%). For tobramycin resistance rate was 100%, this result far from 15.9 and 3.3% which found by Al-derzi. (2012) and Coetzee *et al*. (2013), respectively, but high resistance with Aljanaby and Aljanaby (2018) who reported a rate of 78.8% and Othman *et al*. (2014) who reported a rate of 76.2%. For Amikacin results demonstrated a resistance rate of 70%. This result was close to that of Aljanaby and Aljanaby (2018) (77.4%) and Corehtash *et al*. (2015) (82%). Incompatible with the findings of Alramahy and Aladily, (2017) (26%) and Juhi *et*

al. (2009) (30%), Tobramycin, amikacin, and gentamicin are aminoglycosides antibiotic.

Resistance to flouroquinolones showed 24/50 (48%), 50/50 (100%), 21/50 (42%), to ciprofloxacin, ofloxacin and levofloxacin, respectively, as shown in Figure (4-4). For ciprofloxacin, this result is compatible with the data reported by Alderzi. (2012) who recorded that (23.9%) of isolated were resistance to ciprofloxacin, but disagree with that reported by Othman *et al*. (2014) who recorded (61.3%) resistance. For levofloxacin (42%), this rate is near to the results of Yayan *et al*. (2015) (30.6%) and Lila *et al*. (2017) (36.1%), but disagrees with Khadim and Marjani (2019) (57.14 %) and Hussein *et al*. (2018) (60.19%). Flouroquinolone antibiotics such as ciprofloxacin and levofloxacin interfere with DNA replication by inhibiting DNA gyrase and topoisomerase IV (Pang *et al*., 2019). Ciprofloxacin and levofloxacin resistance can arise through the acquisition of mutations in genes encoding the target proteins of ciprofloxacin and regulators of efflux pumps, which leads to overexpression of these pumps leading to increases the expulsion of ciprofloxacin from *P. aeruginosa* cells and occurs through mutations in regulatory genes of efflux pumps (Rehman *et al*., 2019).

P. aeruginosa shows resistance to a wide range of antibiotics, comprises aminoglycosides, quinolones and β-lactams. The resistance may be intrinsic (low outer membrane permeability, coding for efflux pumps and the making of antibiotic-inactivating enzymes), acquired (either horizontal transport of resistance genes or mutational alteration) and adaptive (involves formation of biofilm which provide as a diffusion barrier to edge antibiotic access to the bacterial cells) resistance (Breidenstein *et al*., 2011).

4.3 Screening test

4.3.1 Primary screening I results for *Streptomyces* isolates against pathogenic bacteria

Twenty three of *Streptomyces* isolates were tested by perpendicular streak method against Gram Gram negative (*Escherichia coli, Klebsella pneumoniae, Pseudomonas aeruginosa*) to select the isolates which have higher antibacterial activity (Table: 4-5)

Table: 4-5 : Diameters of inhibition zones given by *Streptomyces* isolates against pathogenic bacteria

Diameters (mm) ± SE			
Streptomyces spp. Symbol	Pathogenic gram negative bacteria		
	E. coli	*K. pneumoniae*	*P. aeruginosa*
St-4	12±0.33	9±0.1	15±0.4
St-5	5±0.4	4±0.2	5±0.01
St-6	11±0.2	15±0.3	9±0.3
St-8	4±0.3	4±0.3	15±0.3
St-11	23±0.1	11±0.1	10±0.2
St-12	16±0.2	11±0.4	13±0.1
St-14	12±0.5	11±0.1	7±0.01

Out of 23 *streptomyces* isolates only 7 *Streptomyces* isolates (St-4 , St-5, St-6, St-8 , St-11 , St-12 , St-14) were found to have antibacterial activity against pathogenic G-ve bacteria . As showen in Table (4 -6)

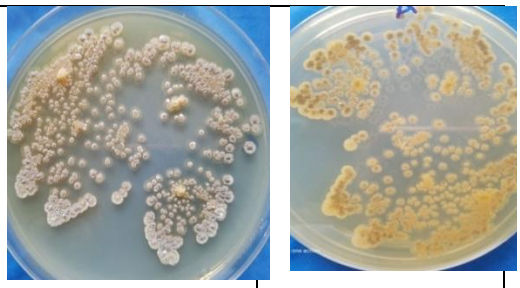
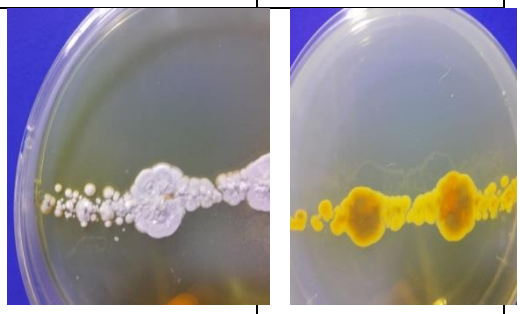
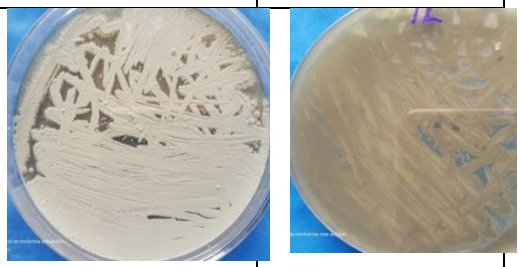
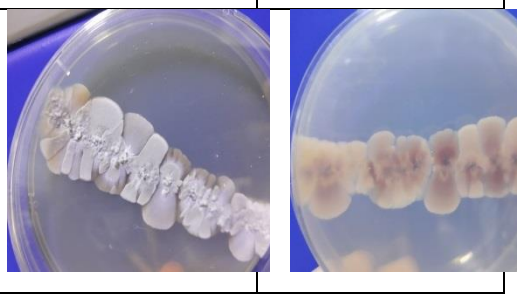
Studies of a wide range of species indicate that the genome contains a Central regionpresent in all species, is the region that contains essential genes responsible for DNA replication, cell division, and genes responsible for trophic metabolism, and a peripheral region responsible for secondary metabolites and the production

of antibiotics and exogenous enzymes, this explains the difference in their ability to secrete antibiotics and the ability of one type of them to secrete more than one secondary metabolite (Xu *et al*., 2017). Also *Streptomyces spp.* contains several types of plasmids that have a role in the production of antibiotics and resistance to them . (Sevillano *et al*., 2013)

Also the result showed that 7streptomyces isolates have more antimicrobial activity against Gram negative bacteria (*Escherichia coli, Klebsella pneumoniae, Pseudomonas aeruginosa*) as a result for the nature of cell wall of G-ve bacteria which have Lipopolysaccharide (LPS) and phospholipid membrane that prevent the enterance of many antibiotics to inside cell and the ability of G-ve bacteria to less the permeability of cell membrane by decrease porins and increase efflux pumps. (Friedman *et al.*,2016)

Table(4-6):Morphological characteristics of isolated *Streptomyces spp.* growing on ISP2 , after 7-14 days at 28±1°C

Isolate no.	Colony texture	Aerial mycelium color	Substrate (reverse) color	Culture shape	
				Front shape	Rear shape
St4	Filamentous	White	Brown		
St5	leathery	Grey	Green		

St6	Filamentous	Purple	Yellow	
St8	Powder	White	Yellow	
St11	Powder	White	Brown	
St12	Powder	Grey	Green	
St14	leathery	Grey	Brown	

The current primary screening test results were in agreement with those of Pan *et al* (2013), who recovered 95 *Stretopmyces* from soils on Signy Island in Malaysia; 46 of these shown antibacterial activity against Gram positive and negative test bacteria (Pan *et al*,. 2013).

This results supported those of Abussaud *et al*. (2013), who obtained 28 isolates of *Stretopmyces spp.* from soils in the north of Jordan and studied their antibacterial activity. They reported that only 8 of these isolates had antibacterial activity.

The study results of Maleki *et al*. (2013), however, who isolated 140 *Streptomyces spp.* isolates from soils in northwest Iran and observed that only 12 of them had antibacterial activity against gram positive and gram negative pathogenic bacteria, disagreed with the findings of the present primary screening test (Maleki *et al*., 2013).

4.3.2 Primary screening II results for *Streptomyces spp.* isolates against *Pseudomonas aeruginosa*

Streptomyces isolates that have antimicrobial activity against pathogenic G+ve and G-ve bacteria testes against clinical isolates of *Pseudomonas aeruginosa* by perpendicular streak method that showed resistance against (17) comercial antibiotic . **Table 4-7**

Table 4-7: Diameters of inhibition zones given by *Streptomyces spp.* isolates against *Pseudomonas aeruginosa*

No.	**Isolate NO. of *Pseudomonas aeruginosa***	**Diameters (mm)**						
		***Streptomyces spp* Isolates**						
		St4	St5	**St6**	St8	St11	**St12**	St14
1.	Pa5	-	-	**10**	-	6	**18**	8
2.	Pa6	7	5	**16**	6	8	**13**	12
3.	Pa7	11	9	**12**	9	13	**16**	5
4.	Pa8	-	-	-	-	-	-	-

5.	Pa9	-	7	**13**	10	-	**11**	-
6.	Pa11	6	12	**11**	12	10	**14**	6
7.	Pa12	4.5	18	**18**	7	11	**16.5**	8
8.	Pa13	-	-	**-**	6	9	**12**	7
9.	Pa14	-	4	**9**	5	-	**11**	-
10.	Pa15	6	-	**11**	-	-	**14**	-
11.	Pa16	6	-	**11**	-	7	**8**	-
12.	Pa17	-	-	**9**	15	6	**11**	-
13.	Pa18	8	9	**19**	-	12	**20**	15
14.	Pa19	-	-	**-**	-	-	**-**	-
15.	Pa20	11	-	**16**	8	-	**17**	-
16.	Pa22	8	11	**10**	-	-	**12**	8
17.	Pa24	10	-	**16**	11	18	**20**	10
18.	Pa26	8	-	**12**	10	-	**-**	-
19.	Pa28	7	11	**4**	-	8	**-**	-
20.	Pa30	6.5	9	**4**	-	-	**8**	-
21.	Pa31	-	-	**-**	-	-	**-**	-
22.	Pa32	5	9	**16**	-	4.5	**18**	8
23.	Pa33	-	-	**-**	-	-	**-**	-
24.	Pa39	12	10	**-**	-	6	**15**	-

25.	Pa40	9	8	**15**	11	13	**13**	9
26.	Pa42	8	-	**-**	8	8	**-**	-
27.	Pa43	8	11	**6**	8	9	**6**	11
28.	Pa44	10	8	**10**	9	8	**18**	-
29.	Pa46	-	-	**9**	5	9	**11**	6
30.	Pa48	8	-	**13**	-	-	**10**	-

Results in this table showed that diameters of inhibition zones between zero and 20 mm against *P. aeruginosa* isolate. The isolates (pa6 , pa7, pa11 , pa12 , pa40 , pa43) inhibited by all *Streptomyces spp*. Isolates .The isolates (pa8 , pa19, pa30 , pa33) did not effected by any *Streptomyces spp*. Isolates.

4.3.3 Secondary screening results for *Streptomyces* isolates against *p. aeruginosa*

The secondary screening test for 7 *Streptomyces spp.* cultures extracellular extracts against *p. aeruginosa* was done . And the results were exhibited that extracts of isolates st6 &st12 were have the heighest antibacterial activity against most isolates of *p. aeruginosa* (tables 4-8) while cultures extracts of isolates st4 &st11 had moderate antibacterial activity and st5, st8 and st14 had weakly antimicrobial activity (table 4-8)

Table: 4-8. Diameters of inhibition zones given by *Streptomyces* isolates against *Pseudomonas aeruginosa*

No.	Isolate NO. of *Pseudomonas aeruginosa*	Diameters(mm)						
		Streptomyces spp. Isolates						
		ST 4	ST 5	**ST 6**	ST8	ST 11	**ST12**	ST 14
1.	Pa5	8	7	**20**	11	8	**22**	9
2.	Pa6	8	11	**17**	5	8	**18**	9
3.	Pa7	11	9	**27**	7	12	**30**	10
4.	Pa8	-	-	**20**	-	8	**25**	-
5.	Pa9	11	7	**23**	7	8	**12**	7
6.	Pa11	6	10	**15**	7	10	**20**	11
7.	Pa12	13	11	**18**	7	8	**20**	8
8.	Pa13	11	-	**20**	12	12	**22**	11
9.	Pa14	-	-	**9**	0	-	**13**	9
10.	Pa15	-	-	**21**	-	-	**18**	-
11.	Pa16	6	-	**17**	-	6	**-**	8
12.	Pa17	-	10	**17**	12	-	**18**	-
13.	Pa18	8	18	**19**	11	12	**20**	6
14.	Pa19	-	-	**21**	-	8	**13**	-
15.	Pa20	11	8	**16**	8	9	**22**	10
16.	Pa22	8	-	**10**	-	-	**25**	8
17.	Pa24	22	10	**20**	11	18	**26**	10

18.	Pa26	8	-	**16**	-	-	**12**	-
19.	Pa28	10	12	**22**	-	18	**-**	-
20.	Pa30	-	-	**-**	-	-	**22**	-
21.	Pa31	-	13	**22**	-	-	**18**	-
22.	Pa32	-	-	**26**	-	-	**21**	8
23.	Pa33	-	-	**-**	-	-	**-**	-
24.	Pa39	22	10	**20**	-	21	**20**	-
25.	Pa40	12	8	**22**	9	9	**30**	12
26.	Pa42	18	-	**-**	8	8	**22**	-
27.	Pa43	-	-	**-**	-	-	**-**	-
28.	Pa44	10	8	**15**	9	8	**18**	15
29.	Pa46	12	-	**20**	-	9	**20**	-
30.	Pa48	14	-	**23**	-	-	**18**	-

Streptomyces spp. Showed different antimicrobial activity against *P. aeruginosa* but st6 and st12 have heighest effect, st4 and st11 had moderate effect , st5 , st8 and st14 had the lowest effect on these isolates .

Results in this table showed that diameters of inhibition zones between zero and 22 mm against *P. aeruginosa* isolate. The isolates (pa5 , pa6, pa7, pa11 , pa12 , pa18, pa 20, pa 24, pa40 , pa44) inhibited by all *Streptomyces spp*. isolates , so it was selected for MIC and MBC test .

In this regarded, Kumar *et al* (2010) *Stretopmyces* strains were collected from the wasteland alkaline and garden soils in India and tested their extracts against

P. aeruginosa cultures, results showed that the diameters of inhibition zones ranged between (0) and over (20.5) mm. Deepthi *et al* (2012) who collected *Stretopmyces* spp from Coringa mangrove forest soils in India and examined their antibacterial activity against *P.aeruginosa* cultures and found that inhibition zones ranged between (0) to (20) mm (Deepthi *et al*.,2012).

Streptomyces spp isolates had different activity in primary and secondary screening tests against same clinical *P. aeruginosa* isolates, according to the comparison of the primary and secondary screening tests for *Streptomyces spp*. isolates. The current findings of the primary and secondary screening tests against pathogenic Gram negative bacteria were in agreement with the findings of Bizuye et al. (2013), who isolated Actinomycetes from soils and studied the primary and secondary screening tests against these pathogenic Gram negative bacteria and discovered different activity in primary and secondary screening tests against these pathogenic bacteria.

According to Bushell's (1993) findings, there were some differences between the primary and secondary screening tests for Actinomycetes against pathogenic G+ve and G-ve bacteria. These variations could be the result of different Actinomycetes' morphology and activity when grown in solid and liquid media as filamentous mycelia and fragmenting mycelia, respectively, the concentration of extract or the chemical modification of the active compounds to make them inactive in broth culture media .

4.4 Characterization of antibacterial produced by *Streptomyces spp.* isolates by :

4.4.1 UV spectroscopy

following antibiotic extraction from *Streptomyces spp*. the maximum wavelength for these antibiotics was measured using ultraviolet light, it was discovered that it

ranged from 210 nm to 250 nm Figure (4-4). When determining the functional groups within a molecule, the peaks that developed from measuring the structure of materials and correlating them with the types of bonds in a particular molecule are valuable. (Taware *et al*.,2014).

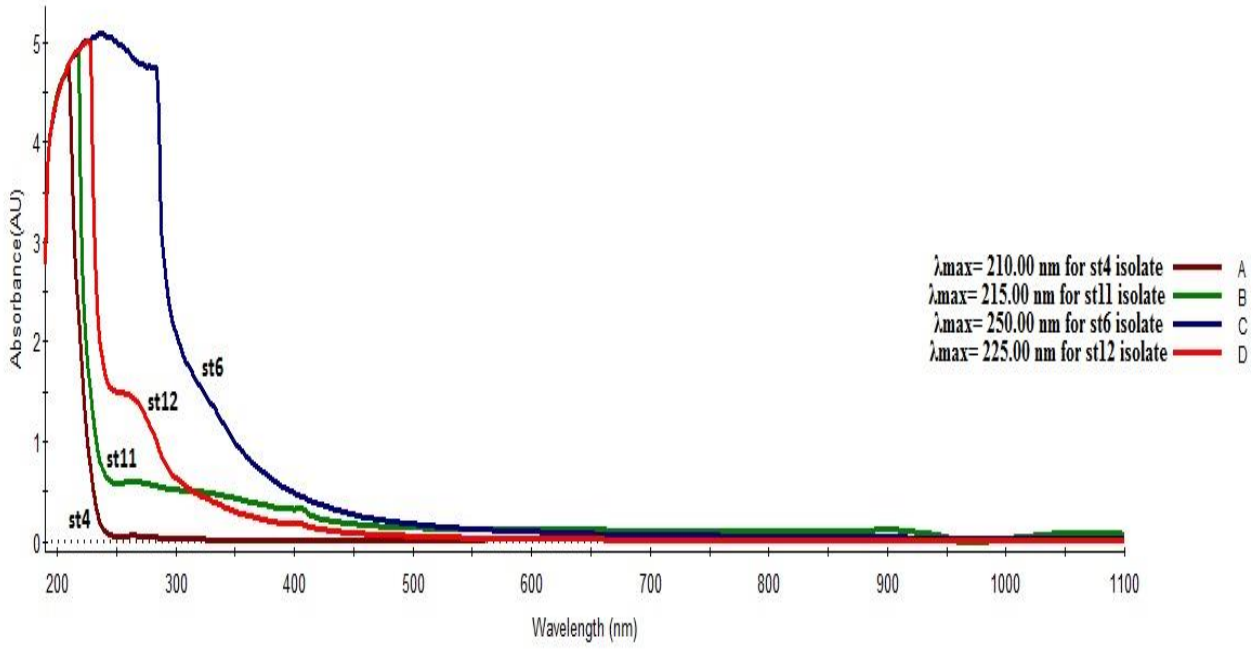


Figure (4-4): Ultraviolet spectrum for antibacterial extracts of st4, st6 ,st11 and st12 isolates

These findings were in agreement with those of Abu-Khumrah (2014), who determined the maximum wavelengths for two antibacterial extracts for Actinomycetes isolated from soils in Babylon city to be 210 nm and 247 nm (AbuKhumrah., 2014). The current findings were also in agreement with Swaadoun et al. (1999), who examined the maximum absorbance of antibiotic extracts from isolates of Actinomycetes spp. and discovered that the range of maximum absorbance for these was 215 to 320 nm.

All antibiotics are made up of peptide chains, and the majority of peptide antibiotics have a maximal UV absorbance range between 200 and 400. But according to Sudha and Masilamani (2013), the majority of peptide

antibiotics show UV maximal absorption at wavelengths between 210 and 230 nm and 270 and 280 nm. Additionally, according to Khanna et al. (2011) and Ababutain et al. (2012), antibiotics exhibit UV maximum absorbance at 223-225 and 332 nm and a peak at 269 nm.

The UV absorption spectrum of the purified active compound indicated the presence of R-COOH with a maximum absorption at λmax of 206 nm, C D O group with a maximum absorption at λ max of 218 nm, conjugated polyene with a maximum absorption at λmax of 225 nm, CHCl3 solvent with a maximum absorption at λmax of 237 nm (CHCl3), aromatic ring with a maximum absorption at a λmax of 256 nm, C D O group with a maximum absorption at λmax of 428 nm, (conjugated double bond system) with a maximum absorption at λmax of 434 nm and polypyrrole with a maximum absorption at λmax of 446 nm. (El-Naggar *et al*.,2017)

4.4.2 FT-IR spectroscopy

Infrared Red (FT-IR) spectroscopy was used for known the important chemical functional groups present in produced antibiotic (Figures **4-5 , 4-6 , 4-7 , 4-8)**

The figure show several bands of absorption each one refer to specific functional chemical group and these explained as the following; Absorption band 3735.04 cm -1 -3418.72 cm -1 refer to Alcohol , phenol (O-H) group (H-bonded) or N-H group (primary and secondary amines and amides, stretch) . Absorption band -2921.63 cm -1 -2852.97cm -1 refer to C-H group (Aldehyde) or (Alkane, stretch) and Alcohol , phenol (O-H) group Carboxylic acids . Absorption band -2352.25 cm -1 refer to Alkene (C=C) . The absorption band at -1749.34cm -1 refer to C=O (Aldehyde) . Absorption band at -1650.75 cm -1 refer to C=C group (Alkene). Absorption band at -1555.04 cm -1, -1459.59cm -1 refer to (Aromatic) group or Nitro (R-NO2) group. Absorption band at -1378.66 cm -1 refer to C-H group (-CH2- bend) . Absorption band at - 1241.80 cm -1 , - 1182.44 cm -1 ,- 1141.48 cm -1 , - 1057.10 cm -1 refer to C-O group (several chemicals) or C-N group (Amine) . Absorption band at -830.99 cm -1 - refer to C-H group (out of the plane bend) . Absorption band - 726.28 cm -1 refer to C-X group (Chloride). Absorption band -630.15 c m-1 refer to C-X group (Bromide , Iodine) . (Pavia *etal*.,2001)

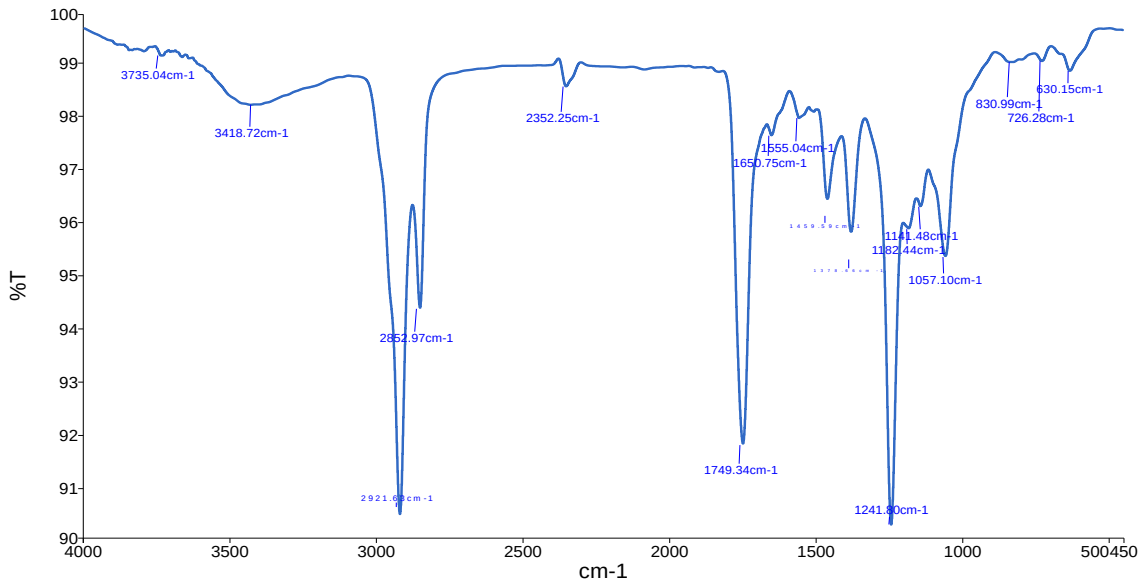


Figure (4-5) Infrared Red spectrum for *Streptomyces spp.* isolates st4 extracellular antibacterial extract.

The figure(4-6) show several bands of absorption each one refer to specific functional chemical group and these explained as the following ; Absorption band 3849.69 cm-1 refer to O-H group (free). Absorption band - 3397.66 cm-1 refer to Alcohol , phenol (O-H) group (H-bonded) . Absorption band - 2082.80 cm-1 refer to (X=C=Y) group Allenes, Ketenes, Isocyanates , Isocyathiocyanate . Absorption band at -1638.13 cm -1 refer to C=C group (Alkene) . Absorption band at -1558.18cm -1, -1455.51cm-1 , -1415.57cm-1 refer to (Aromatic) group or Nitro (R-NO2) group . Absorption band at - 1247.24 cm -1 , -1076.18 cm -1 , -1019.15 cm -1 refer to C-O group (several chemicals) or C-N group (Amine) . Absorption band at -675.16cm -1 refer to (C-X) group (Chloride) . Absorption band at -608.81cm -1 refer to (C-X) group (Bromide , Iodide) . Absorption band - 563.52 cm -1 ,-556.99 cm -1 ,-549.57 cm -1 ,-543.86 cm -1 ,-538.12 cm -1 , -531.82 cm -1 ,-525.12 cm -1 ,-519.57 cm -1 ,-512.94 cm -1 ,-506.38 cm -1 ,-499.75 cm -1 ,-493.53 cm -1 ,-487.23 cm -1 ,-480.70 cm -1 ,-473.90 cm -1 ,-467.93 cm -1 ,-461.49 cm -1 refer to (C-H) group . (Pavia *etal*.,2001)

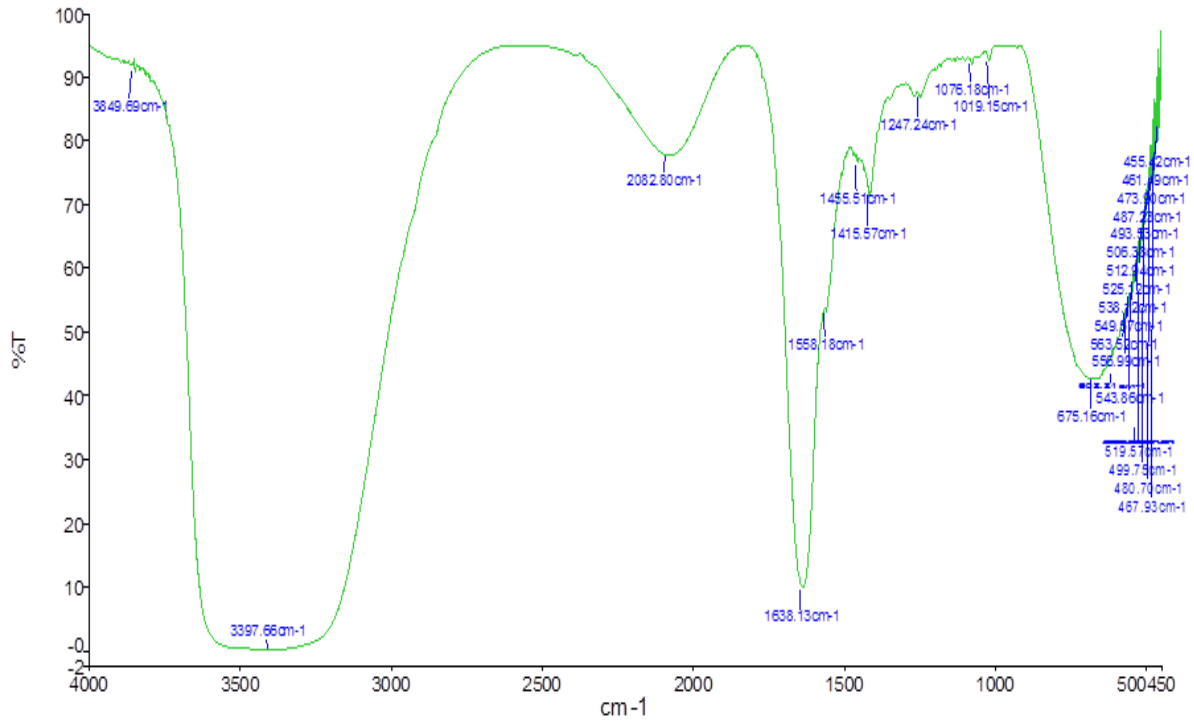


Figure (4-6) Infrared Red spectrum for *Streptomyces spp.* isolates st6 extracellular antibacterial extract.

The figure (4-7)show several bands of absorption each one refer to specific functional chemical group and these explained as the following ; Absorption band - 3402.96 cm-1 refer to Alcohol , phenol (O-H) group (H-bonded) or N-H group (primary and secondary amines and amides, stretch) . Absorption band -2923.08 cm-1 , -2852.97cm-1 refer to C-H group (Aldehyde) or (Alkane, stretch) and Alcohol , phenol (O-H) group Carboxylic acids . Absorption band -2356.09 cm -1 refer to Alkene (C=C) . The absorption band at -1741.68 cm -1 refer to C=O (Aldehyde) . Absorption band at -1459.29 cm -1 refer to (Aromatic) group or Nitro (R-NO2) group . Absorption band at -1379.43 cm -1 refer to C-H group (-CH2- bend) . Absorption band at -1244.66 cm -1 , - 1107.52 cm -1 , - 1057.18 cm -1 refer to C-O group (several chemicals) or C-N group (Amine) . Absorption band -730.27 cm -1 refer to C-X group (Chloride). Absorption band -630.84 c m-1 refer to C-X group (Bromide , Iodine) . (Pavia *etal*.,2001)

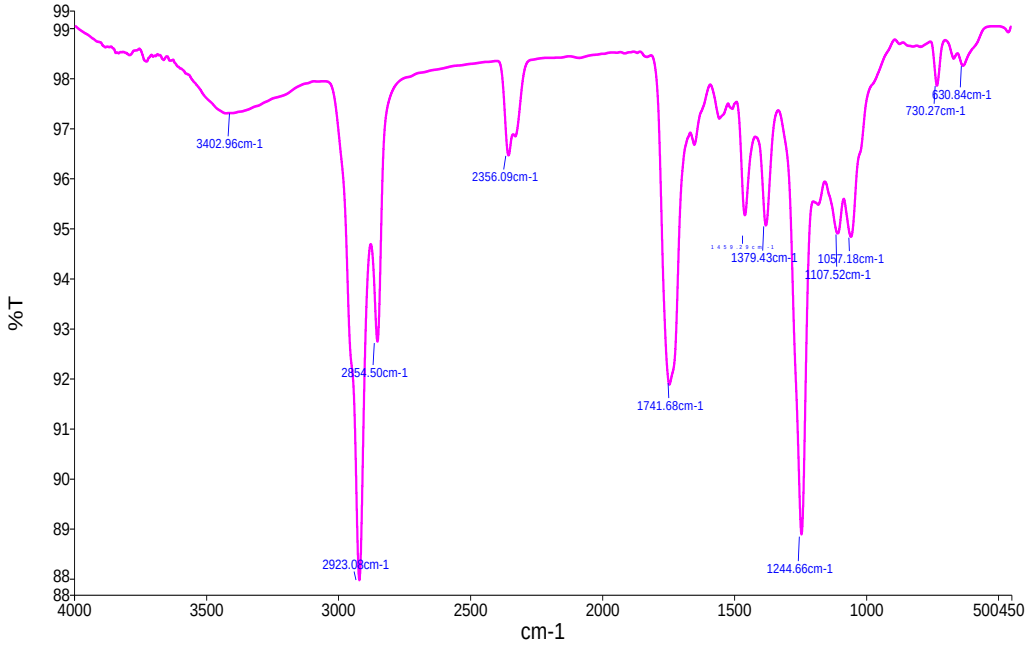


Figure (4-7) : Infrared Red spectrum for *Streptomyces spp.* isolates st11 extracellular antibacterial extract .

The figure(4-8) show several bands of absorption each one refer to specific functional chemical group and these explained as the following ; Absorption band - 3898.53 cm-1 -3850.93cm-1 refer to O-H group (free) . Absorption band - 3393.01cm-1 refer to Alcohol , phenol (O-H) group (H-bonded) , Absorption band -2087.81 cm-1 refer to (X=C=Y) group Allenes, Ketenes, Isocyanates , Isocyathiocyanate . The absorption band at -1843.00 cm-1 ,-1828.67 cm-1 refer to C=O (Aldehyde) . Absorption band at -1651.27 cm -1 ,-1636.82 cm -1 ,-1634.29 cm -1 refer to C=O group (Amide) or C=N group (Imines and oximes) . Absorption band at - 1575.18 cm -1,-1557.96 cm -1 ,-1506.02 cm -1 ,-1471.16 cm -1,-1455.89 cm -1 , -1414.23 cm -1 refer to (Aromatic) or Nitro (R-NO2) group . Absorption band at -1347.22 cm -1 ,-1268.11 cm -1 ,-1248.17 cm -1 ,-1180.02 cm -1 ,-1116.33 cm -1 ,-1101.11 cm -1 ,-1076.21 cm -1 ,-1019.00 cm -1 refer to C-O group (several chemicals) or C-N group (Amine) . Absorption band - 927.93 cm -1 ,- 729.37cm -1 refer to (C-H) group . Absorption band at -661.81cm -1 refer to (C-X) group (Bromide , Iodide) . (Pavia *etal*.,2001)

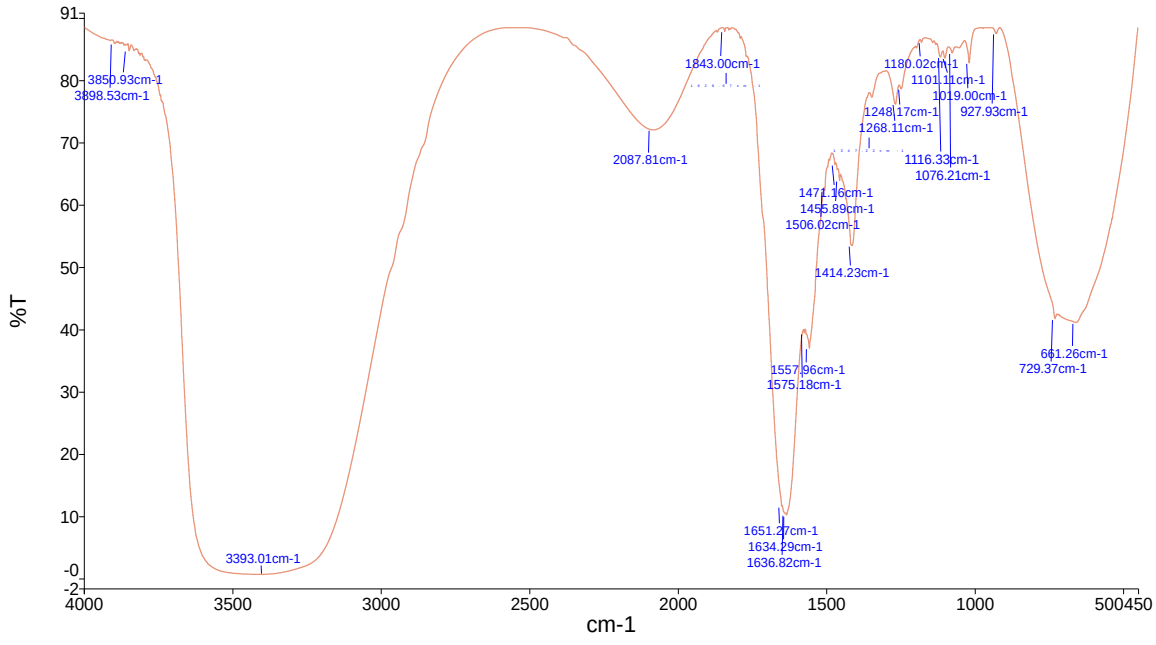


Figure (4-8) Infrared Red spectrum for *Streptomyces spp.* isolates st12 extracellular antibacterial extract.

4.4.3 Analyzing Ethyl Acetate Extract of Streptomyces spp. by Gas Chromatography Mass Spectrometer (GC-MS)

The results represent the GC-MS chromatogram of the ethyl acetate extract with all its separated peaks obtained during the sample running, with a flow rate of 1.51 ml/minutes for the entire process. On the other hand, by the means of (NIST) library and depending on peaks height, retention time, the molecular formula and the molecular weight of all 17 compounds were identified as summarized in Table (4.9) for st4 isolate , 17 compounds,Table (4.10) for st6 isolate , 11 compounds ,Table (4.11) for st11 isolate and 14 compounds ,Table (4.12) for st12 isolate . These identified compounds may play as the major constituents alone or with minor constituents offered (provided) as antimicrobial bioactive compounds, as described formerly by Jalaluldeen et *al.* (2015). Furthermore, different numbers of chemical compounds with different retention times and abundances were obtained from the profiles of the fractions in (GC-MS) supporting the fact that actinomycetes species contain large amounts of chemical compounds with their different performing abilities (functions). The whole achieved documents about ethyl acetate extract st4 isolates with GC-MS were found through analyzing all peaks with some of their chemical info in Table (4-9) , the figure show the relatioship between retation time and peak area as presented in Appendices , Appendix 2,3,4,5 .

Table (4-9): GC-MS analysis profiles were applied for ethyl acetate extract of st4 isolate

NO.	Compound	Molecular Formula	Molecular Weight	Retention time (min)	Peak Height
1.	1-Chloro-2-	$C_3H_5ClO_2S$	140.59	2.36	1629432

	(methylsulfonyl) ethylene				
2.	Carbonic acid , hepatyl ester	$C_9H_{18}O_3$	174.24	2.36	1629432
3.	Divinyldithiophosphinic acid	$C_4H_7PS_2$	150.2	2.36	1629432
4.	N,N –Bis (2-chloroethyl) oxamide	$C_6H_{10}Cl_2N_2O_2$	213.06	2.36	1629432
5.	Methyl 4- pentynote	$C_6H_{10}O_2$	114.14	13.28	540990
6.	2- Cyclobutene -1-carboxamide	C_5H_7NO	97.12	13.28	540990
7.	Borinic acide , diethyl- , 1-methyl-2- propynyl ester	$C_5H_{13}BO$	99.967	13.28	540990
8.	Spirohexan-5-one	C_6H_8O	96.13	13.28	540990
9.	16- Hexadecanoyl hydrazide	$C_{16}H_{34}N_2O$	270.45	13.28	540990
10.	Methyl 4-pentynoat	$C_6H_{10}O_2$	114.14	13.28	540990
11.	2- Cyclobutene -1-carboxamide	C_5H_7NO	97.12	13.28	540990
12.	12- Tridecynoic acid , methyl ester	$C_{14}H_{24}O_2$	224.34	13.28	540990
13.	Tricyclo(6.3.3.0) tetradec-4-ene , 10,13-dioxo-	$C_{14}H_{18}O_2$	218.29	13.35	269935
14.	Spiro(2,4)heptan , 1,5-dimethyl-6- methylene-	$C_{10}H_{16}$	136.2340	14.70	456638
15.	3-octyne , 5-methyl-	C_9H_{16}	124.22	14.70	456638
16.	Dihydromyrcene	$C_{10}H_{18}$	138.25	14.70	456638
17.	3-Nonyne	C_9H_{16}	124.22	14.70	456638

The highest peak 1629432 was obtained for 1-Chloro-2-(methylsulfonyl) ethylene in retention time 2.36 min and the lowest peak 456638 was obtained for Dihydromyrcene in retention time 14.7 min

Table (4-10): GC-MS analysis profiles were applied for ethyl acetate extract of st6 isolate

NO.	Compound	Formula	Molecular weight	Retention time (min)	Peak Height
1.	Butanoic acid , 3,3-dimethyl-, methyl ester	C4H8O2	88.11	13.28	592515
2.	2-pentanethiol	C5H12S	104.22	2.76	4138772
3.	(3-Hydroxy-5-methoxycarbonylmethyl-cyclohexyl)- acetic acid , methyl ester	C12H20O5	244.28	13.28	592515
4.	3- pyrrolidinol	C4H9NO	87.12	13.28	592515
5.	Diazoprogesterone	C21H28N2O2	340.5	14.69	494069
6.	Cyclohexane, 1-nitro-	C6H10N2O3	158.16	14.69	494069
7.	2,3-pentadiene, (z)	C5H8	68.12	14.69	494069
8.	4-Tridecen-6-yne , (E)-	C13H22	178.31	14.69	494069
9.	Bicyclo(2.2.1) hepta-2,5dien-7-ol	C7H8O	108.14	14.69	494069
10.	1,8- Nonadiyne	C9H12	120.19	16.30	1254127
11.	5-Hexynenitrile	C6H7N	93.13	10.32	149976
12.	Octanoic acid , methyl ester	C9H18O2	158.2380	13.28	592515
13.	1,1- Dicyanoethane	C4H4N2	80.0880	14.68	494069
14.	Cyclohexane , 1-nitro-	C6H9NO2	127.	14.68	494069

			14		
15.	Spirohexane -5- one			14.68	494069
16.	1,3-pentadiene	C5H8	68.12	14.68	494069
17.	Alantolactone, 4.alpha., 4A. alpha .- epoxy	C15H20O3	248.32	14.68	494069

The highest peak 4138772 was obtained for 2-pentanethiol in retention time 2.76 min and the lowest peak 494069 was obtained for Alantolactone, 4.alpha., 4A. alpha .- epoxy retention time 14.68 min

Table (4-11): GC-MS analysis profiles were applied for ethyl acetate extract of st11 isolate

NO.	Compound	Formula	Molecular weight	Retention time (min)	Peak Height
1.	Silane , dimethoxymethyl-	C9H20O4Si	220.34	2.76	8039796
2.	Acetic acid , fluoro-, ethyl ester	$C_4H_7FO_2$	106.0956	2.76	8039796
3.	Silsnol , dimethyl	C2H6Si	58.15	2.76	8039796
4.	Nonanoic acid , methyl ester	$C_{10}H_{20}O_2$	172.3	13.28	500622
5.	Decanoic acid	$C_{10}H_{20}O_2$	172.2646	13.28	500622
6.	Decanoic acid , methyl ester	$C_{11}H_{22}O_2$	186.2912	13.28	500622
7.	3-Hydroxy-5-methoxycarbonylmethyl - cyclohexyl) – acetic acid , methyl ester	$C_{12}H_{20}O_5$	244.28	13.28	500622
8.	Octanoic acid , 8-	$C_9H_{18}O_3$	174.24	13.28	500622

	hydroxyl-, methyl ester				
9.	4-Cyclopropylnorcarane	$C_{10}H_{16}$	136.234	14.67	518866
10.	Limonene	$C_{10}H_{16}$	136.23	14.67	518866
11.	Cyclohexane , 1,3-dimethyl-2- methylene , cis	C_9H_{16}	124.2233	14.67	518866
12.	3- Dodecyne	$C_{12}H_{22}$	166.3031	14.67	518866
13.	2H-pyran, 5,6 – dihydro-2- methyl-	$C_6H_{10}O$	98.1430	14.70	518866
14.	Bicyclo[4.1.0] heptane, -3-cyclopropyl, -7- carbethoxy, trans-	$C_{13}H_{20}O_2$	208.30	14.70	518866
15.	1,3 – pentadiene	C_5H_8	68.12	14.70	518866

The highest peak 8039796 was obtained for Silane , dimethoxymethyl- in retention time 2.76 min and the lowest peak 518866 was obtained for 1,3 – pentadiene with retention time 14.7 min .

Table (4-12): GC-MS analysis profiles were applied for ethyl acetate extract of st12

NO.	Compound	Formula	Molecular weight	Retention time (min)	Peak Height
1.	Glycerine	C3H8O3	92.09	2.76	7678439
2.	Hexadecanoic acid ,methyl ester	C17H34O2	270.4507	13.28	385380
3.	Pentadecanoic acid , 14-methyl , methyl ester	C17H34O2	270.4507	13.28	385380
4.	Nonanoic acid , 9-oxo- , methyl ester	C10H18O3	186.25	13.28	385380
5.	D-phenylalanine	C9H11NO2	165.19	13.28	385380

6.	Bicyclo(2.2.1)heptan-3-one, 6,6-dimethyl-2methylene-	C10H14O	150.22	10.32	163513
7.	5-Hexenal, 4-methylene-	C7H10O	110.15	10.32	163513
8.	2-phenyl-3-butyn-2-ol	C10H10O	146.19	10.32	163513
9.	2,5-octadiene	C8H14	110.20	10.32	163513
10.	Bicyclo(4.1.0)heptane,-3-cyclopropyl, -7-carbomethoxy , trans-	C12H18O2	194.27	10.32	163513
11.	Trifluoroacetyl-.alpha.fenchol	C12H17F3O2	250.26	14.70	482598
12.	3-Hepten-1-yne ,(z)-	C7H10	94.15	14.70	482598
13.	2,3-Dimethyl-1,4-pentadiene	C7H12	96.17	14.70	482598
14.	-cis -myrtanol	C10H18O	154.25	14.70	482598

The highest peak 7678439 was obtained for Glycerine with retention time 2.76 min and the lowest peak 163513was obtained for 2,5-octadiene in retention time 10.32 min

Most of these compound have antibacterial activity against several pathogenic bacteria included G+ve & G-ve bacteria .

Shaaban & Fahmi (2021) found that hexadecenoic acid methyl ester with the highest antimicrobial effect against clinical pathogenic bacteria among them *Pseudomonas aeruginosa* that resist to 7 antibiotic disk.

Nonadecane, which contributes to the bacteriocin's significant antibiofilm activity and cytotoxic and antibacterial effects, is a chemical found in a culture of Aeromonas sp. and Enterobacter sp. By using GC-MS analysis, (Selvin *et al*. 2016) discovered hexadecanoic acid and hexadecanoic acid methyl ester from

Nocardiopsis dassonvillei MAD08. They serve as an antioxidant , nematicide , insecticide , lubricant , antiandrogenic , flavoring agent, hemolytic, and 5-alpha reductase inhibitor in addition to these other functions . N-hexadecanoic acid , squalene , and tetracosane are additional components of the Streptomyces extract that were identified by GC-MS . Squalene possesses antibacterial , antitumor , anti-cancer , and immunostimulant properties. while tetracosane has antioxidant properties . Actinobacteria have been related to some of these fatty acids. Due to their safety, general mode of action , and lack of mechanisms of resistance to their effects, fatty acids offer opportunities as antimicrobial agents in both the food industry and medicine. (El-Naggar *etal*.,2017)

Pyrrolidine and its derivaters are used widely by medicinal chemists to obtain compounds for the treatment of human diseases and to obtain compound with high antimicrobial activity . (Li Petri *etal*.,2021)

Several studies have attributed the antioxidant effect to phenol, 1,1- Dicyanoethane , phenol 4,6- di(1,1- dimethylethyl)-2-methyl, 1-hexadecene, hexadecanoic acid methyl ester, 9- octadecenoic acid (Z) methyl ester, hexadecanoic acid, Nonanoic acid , methyl ester.(FAJA , 2018)

Pentadecanoic acid, 14-methyl-,methyl ester (Fatty acid methyl ester) reported as Antimicrobial, antifungal (Chandrasekaran et al.2011)

4.5 Estimating minimum inhibition concentrations(MIC) & minimum bactericidal concentrations (MBC) of selected *Streptomyces spp.* Isolates Extracellular

Crude extract of isolate st6 and crude extract of isolate st12 were was used to determine its MICs and MBCs against MDR *P. aeruginosa* pathogenic bacteria. The concentrations were used ranged 0.078, 0.156, 0.312, 0.625, 1.25, 2.5 and 5 µg/ml, and the Mueller Hinton broth was used as a culture medium broth.

Table (4.13) : Minimum inhibition concentrations and minimum bactericidal concentrations extract of isolate st6 against MDR *P. aeruginosa* pathogenic bacteria.

No.		Extracellular crude extract of isolate st6 concentrations µg/ml								
		AC	5	2.5	1.25	0.625	0.312	0.156	0.078	GC
1	Pa 5	-	-	-	-	MIC MBC	+	+	+	+
2	Pa 6	-	-	-	-	MIC MBC	+	+	+	+
3	Pa 7	-	-	-	-	MBC	MIC	+	+	+
4	Pa 11	-	-	-	-	MBC	MIC	+	+	+
5	Pa 12	-	-	-	-	MBC MIC	+	+	+	+
6	Pa 18	-	-	-	-	MIC MBC	+	+	+	+
7	Pa 20	-	-	-	-	MIC MBC	+	+	+	+
8	Pa 24	-	-	-	-	MIC MBC	+	+	+	+
9	Pa 40	-	-	-	-	MIC MBC	+	+	+	+
10	Pa 44	-	-	-	-	MBC	MIC	+	+	+

A.C = Antibiotic Control G.C = Growth Contro

- No growth + growth

The data presented in Table (4-13) summarize minimum inhibition concentrations and minimum bactericidal concentrations the crude extract of isolate st6 against MDR *P. aeruginosa* .The MIC values for microbial isolates were ranged between 0.625-0.312 µg/ml. The MIC of isolates of *P. aeruginosa* (Pa 5 , Pa 6 , Pa 12 , Pa 18 , Pa 20, Pa 24 , Pa 40) was 0.625µg/ml while the MIC was 0.312 µg/ml for (Pa 7 , Pa 11 , Pa 44) isolates . The MBC values for all the microbial isolates were 0.625 µg/ml . This indicated that the crude extract was very strong against MDR *P. aeruginosa* .

Table (4.14) : Minimum inhibition concentrations and minimum bactericidal concentrations extract of isolate st12 against MDR *P. aeruginosa* pathogenic bacteria.

No.		Extracellular crude extract of isolate st12concentrations µg/ml								
		AC	**5**	**2.5**	**1.25**	**0.625**	**0.312**	**0.156**	**0.078**	**GC**
1	Pa 5	-	-	-	-	MIC MBC	+	+	+	+
2	Pa 6	-	-	-	-	MBC	MIC	+	+	+
3	Pa 7	-	-	-	-	MIC MBC	+	+	+	+
4	Pa 11	-	-	-	-	MIC MBC	+	+	+	+
5	Pa 12	-	-	-	-	MIC MBC	+	+	+	+
6	Pa 18	-	-	-	-	MBC	MIC	+	+	+
7	Pa 20	-	-	-	-	MIC MBC	+	+	+	+

8	Pa 24	-	-	-	-	MIC MBC	+	+	+	+
9	Pa 40	-	-	-	-	MBC	MIC	+	+	+
10	Pa 44	-	-	-	-	MIC MBC	+	+	+	+

A.C = Antibiotic Control G.C = Growth Control

- No growth + growth

The data presented in Table (4-14) summarize minimum inhibition concentrations and minimum bactericidal concentrations Extracellular crude extract of isolate st12 against MDR *P. aeruginosa* .The MIC values for microbial isolates were ranged between 0.625-0.312 µg/ml. The MIC of isolates of *P. aeruginosa* (Pa 5 , Pa 7 , Pa 11 , Pa 12 , Pa 20, Pa 24 , Pa 44) was 0.625µg/ml while the MIC was 0.312 µg/ml for (Pa 6 , Pa 18 , Pa 40) isolates . The MBC values for all the microbial isolates were 0.625 µg/ml . This indicated that the crude extract was very strong against MDR *P. aeruginosa* .

The nature of the test organism used, the inoculum size, the composition of the culture medium, the incubation duration, and aeration are all elements that might alter the activity, hence the MIC and MBC are not constant for a particular agent (Pandey *et al*. 2004) . Our results were agreed with those Xie *et al.* (2007), Mukai *et al*. (2006) and Ababutain *et al*. (2012) and which were intended for the activities of the bioactive metabolites against both gram positive and negative bacteria.

All the tested bacterial pathogens after inhibiting them by the activity of the extract don't have the ability to regrow on prepared nutrient agar. In conclusion, the isolate st6 and st12 have bacteriostatic and bactericidal activities on the bacterial pathogens.

Our results were agreed with the finding of Sabahi, (2018) which found that the MIC for two *Streptomyces spp.* Isolates were 0.625µg/ml and 0.321µg/ml against MDR *P. aeruginosa* and The MBC values were 0.625 µg/ml . But disagree with Iranian study which done by Ramazani *et al*,. they found that the MIC was 1.25 µg/ml and MBC was 2.5 µg/ml . (Ramazani *et al* .,2013)

4.6 : Anticancer Activity of Crude Extract of st6 & st12 Isolates

Determining the antitumor activities of crude extracts was carried out using MTT assay . This assay was performed to investigate the possible cytotoxic effect of crude extract on tumor cell lines(cell line exhibiting epithelial morphology that was isolated from the skin of a 54-year-old, female patient with malignant melanoma (A375). and normal cell line (Dermal Fibroblast Normal Human Neonatal (HdFn) . through using different concentrations 400, 200, 100, 50, 25 After incubating the cell lines with the mentioned concentrations for 72 hrs, at 37C the optical densities were recorded at 570nm in Five replicates trials . The results presented in Table (4.15) summarize the mean percent cell viability with its standard deviation for cell lines (cancer & normal) used to determent antitumor activity of crude extract of st6 isolate .

The results listed in Table (4.15) for cancer cell (A375) , only highlighted the concentrations 400, 200µg/mL that gave less rate of viable cells after treated with extract 30.054±1.39 , 39.27±2.27 which mean that have high inhibition rate . it can be noticed that the inhibition rate of (A375) cells was increased by increasing the concentration of crude extract st6 isolates . For cells line used as formerly mentioned by increasing the concentration of the crude extract, their cell viability rate was decreased which means that the rate of grown cancer cell lines will be inhibited (decreased) and could not convert MTT compound to form insoluble formazan products . Concentration comparing with pattern of normal (HdFn) cells viable , especially at concentrations 50 , 25 µg mL-1 which gave the maximum

rate of viability 86.959±1.44 , 93.82±0.77 respectively . In these concentrations (HdFn) cells able to form insoluble formazan products which remained as alive cells when treated with extract.

Table (4-15): The cytotoxic effect of Crude Extract st6 on HdFn and A375 Cell Line .

Concentratio nµg mL^{-1}	Mean viability (%) ± SD		Inhibited cell (%) ± SD	
	HdFn	A375	HdFn	A375
400	55.86±5.08	30.054±1.39	44.14±5.08	69.94±1.39
200	64.12±2.42	39.27±2.27	35.882.42	60.73±2.27
100	75.887±1.63	51.157±2.53	24.12±1.63	48.84±2.53
50	86.959±1.44	65.12±0.99	13.05±1.44	34.88±0.99
25	93.82±0.77	73.264±2.04	6.18±0.77	26.74±2.04

The results presented in Figure (4.19) summarize the effects of different extract concentrations on A375 cells and Hdfn, the concentration 400 µg/mL exhibited the highest result for both types of cell. The IC50 (inhibits 50% 0f cell) for cancer cell was 99.16 µg/ml and IC50 for normal cell Hdfn was 111.1 µg/ml

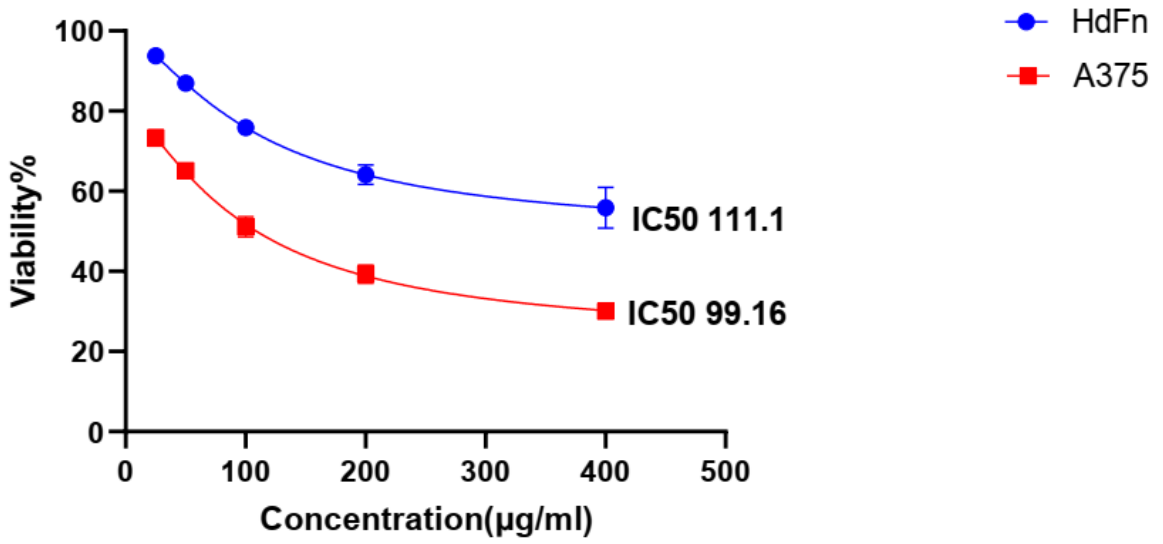


Figure (4-9): Cell survival curve (mean±SD%) of A375 cells and Hdfn after treatment with cerd extract st6 using MTT *in vitro* assay at 37ºC, 5% CO2 for 24 hrs.

Table (4.16) summarize the mean percent cell viability with its standard deviation for cell lines (cancer & normal) used to determent antitumor activity of crude extract of st12 isolate .

The results listed in Table (4.16) for cancer cell (A375) , only highlighted the concentrations 400, 200µg/mL that gave less rate of viable cells after treated with extract 29.39±2.31 , 38.233±0.94 which mean that have high inhibition rate . It can be noticed that the inhibition rate of (A375) cells was increased by increasing the concentration of crude extract st12 isolates . For cells line used as formerly mentioned by increasing the concentration of the crude extract, their cell viability rate was decreased which means that the rate of grown cancer cell lines will be inhibited (decreased) and could not convert MTT compound to form insoluble formazan products . Concentration comparing with pattern of normal (HdFn) cells viable , especially at concentrations 100, 50 , 25 µg mL-1 which gave the maximum rate of viability 86.42±1.91, 92.592±2.14 , 95.216±0.57 respectively .

In these concentrations (HdFn) cells able to form insoluble formazan products which remained as alive cells when treated with extract .

Table (4-16): The cytotoxic effect of Crude Extract st12 on HdFn and A375 cell line.

Concentration µg mL^{-1}	Mean viability (%) ± SD		Inhibited cell (%) ± SD	
	HdFn	A375	HdFn	A375
400	66.39±1.52	29.39±2.31	33.61±1.52	70.61±2.31
200	74.76±1.31	38.233±0.94	25.24±1.31	61.77±0.94
100	86.42±1.91	51.08±3.08	13.42±1.91	48.92±3.08
50	92.592±2.14	64.930±1.3	7.40±2.14	35.07±1.3
25	95.216±0.57	73.341±1.27	4.79±0.57	26.66±1.27

The results presented in Figure (4.10) summarize the effects of different extract concentrations on **A375 cells and Hdfn**, the concentration 400 µg/mL exhibited the highest result for both types of cell. The IC50 (inhibits 50% 0f cell) for cancer cell was 99.16 µg/ml and IC50 for normal cell **Hdfn** was 157.6 µg/ml

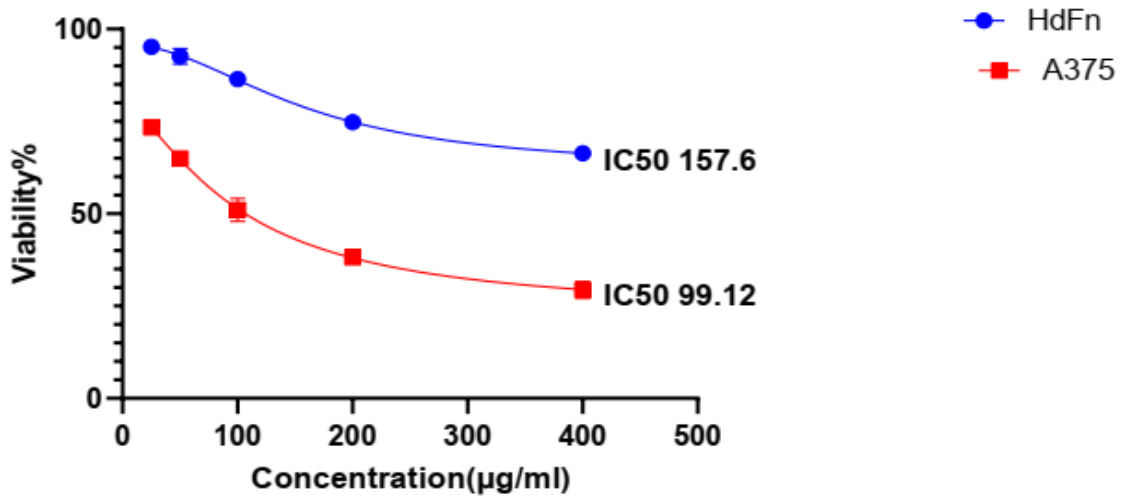


Figure (4-10): Cell survival curve (mean±SD%) of A375 cells and Hdfn after treatment with cerd extract st12 using MTT *in vitro* assay at 37ºC, 5% CO2 for 24 hrs.

However the IC50 theoretically calculated depend on the concentration (in dose-dependent pattern) , type of cells , number of duplicates , inhibition percentage , the difference between cell concentration and if there were normal cells used for comparison with the result of cancer cell .

In general all compound considers toxic but depend on concentration rate (dose)

4.7 : Sequencing of PCR products

To understand the genetic variation and characterization of *Streptomyces spp* in the current study, two positive samples were sequenced after amplified 1500bp of *16s rRNA* coding region by PCR . Nucleic acid sequencing was conducted to emphasize their specificity and introduce the ultimate means to detect the *Streptomyces spp*. In this study; amplicons have *Streptomyces spp* isolated sequences of conserved regions. After received the sequences from Macrogene company three format editing the sequence by using Geneious program show(figure 4-11).

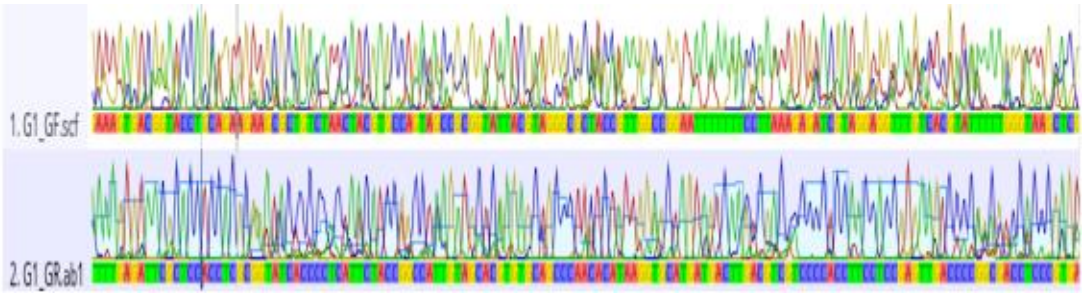


Figure 4-11: The sequence of two PCR products to local *Streptomyces* spp isolates 16s rRNA after edited by using Geneious program.

Consequently, two local *Streptomyces* spp isolates were analyzed and compared with a reference strain available in the Genbank database of National Center Biotechnology Information (NCBI). After using Basic Local Alignment Search Tool (BLAST) program which is available at the NCBI, the result of sequencing appeared 98% compatibility for one isolates and 96% for other isolate

with reference *Streptomyces* spp, *16s rRNA* gene and alignment with references *Streptomyces* spp from NCBI isolate comparison with Algeria isolate 94% under accession numbers [MN826256.1] and 93% Thailand isolate under accession numbers [DQ663190.1] as shown in (figures from 4-12 to 4-13).

Isolate ST6 as matched with *Streptomyces* sp. strain HH1 16S ribosomal RNA gene, partial sequence

Sequence ID: MN826256.1Length: 1489Number of Matches: 1

Range 1: 73 to 527

Alignment statistics for match #1				
score	Expect	Identities	Gaps	Strand
682 bits(369)	0.0	428/457(94%)	2/457(0%)	Plus/Plus

```
Query  1    TCGCTGGCGATTAGTGCCGAACGGCGGCAGTAACACGCGGGCAATCTGCCCTGCGCTCTG  60
Sbjct  73   TCGGTGGGGATTAGTGGCGAACGGGTG-AGTAACACGTGGGCAATCTGCCCTGCACTCTG  131

Query  61   GTAGAAGCCCTGGAAACGGGGGCTAATACCGGATACTGAGCCAGGATCAAACTCTAAATG  120
Sbjct  132  GGACAAGCCCTGGAAACGGGGTCTAATACCGGATACTGAGCCA-CTTGGGCATCCAAGTG  190

Query  121  GTTCGAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAA  180
Sbjct  191  GTTCGAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAA  250

Query  181  TGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACT  240
Sbjct  251  TGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACT  310

Query  241  GAGACACGGCCCAGACTCATACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAA  300
Sbjct  311  GAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAA  370

Query  301  GCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGC  360
Sbjct  371  GCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGC  430

Query  361  AGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCTGTCTAACTACGTGCCAGTA  420
Sbjct  431  AGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCA  490

Query  421  GCCGCGGTATTACGTAGGGCGCTACCGTTGGCCGGAA  457
Sbjct  491  GCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAA  527
```

Figure 4-12: Sequences of *Streptomyces* spp isolate (No. st6) with *Streptomyces* sp. The differences in the nucleotides of this study query and the subject were found in positions (76, 80, 89, 97, 98, 100, 110, 127, 133, 135, 153, 175, 176, 178, 179, 180,181, 182, 185, 188, 329, 471, 473, 489, 500, 513, 515, and 521).

Isolate ST12 as matched with *Streptomyces* sp. 3150 16S ribosomal RNA gene, partial sequence . Sequence ID: DQ663190.1Length: 1492Number of Matches: 1 Range 1: 975 to 1410

Alignment statistics for match #1				
score	Expect	Identities	Gaps	Strand
634 bits(343)	2e-179	410/442(93%)	6/442(1%)	Plus/Plus

```
Query  1     ACGTGCCAGAGTATGGGCGTCCCGTTAGTGGGTGAGGTGTACAAGATGGTGCATGGCTGT  60
             ||||  ||||||  ||||||||  |||  ||  ||  |||  ||||||||  ||  ||||||||||||||||
Sbjct  975   ACGT-CCAGAG-ATGGGCGCCCCCTT-GT-GGT-CGGTGTAC-AGGTGGTGCATGGCTGT  1028

Query  61    AGTCGGCTCGTGTCGTGAGATGTTGGGTTAAGTGAAGCAAAGAGAGCAACCGATGTCCGG  120
              |||  ||||||||||||||||||||||||||||||    ||||  |||  ||||||  ||||||  |
Sbjct  1029  CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCG  1088

Query  121   TGTTGCCAGCAGGACCTTGTGGTGCTGGGGACTCACGGGAGGTCGCCGGGGTCAACTCGG  180
             ||||||||||||| ||||||||||||||||||||||||||||||||  ||||||||||||||||||
Sbjct  1089  TGTTGCCAGCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGGCCGCCGGGGTCAACTCGG  1148

Query  181   AGGAAGGTGGGGACGACGTCAAGTCATCATGCACCTTATGTGTTGGGCTGCACACGTGCT  240
             ||||||||||||||||||||||||||||||||||  ||||||||  ||||||||||||||||||
Sbjct  1149  AGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCT  1208

Query  241   ACAATGGCCGGTAGAATGAGGGGTGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGT  300
             ||||||||||||| ||||||  | ||||||||||||||||||||||||||||||||||||||
Sbjct  1209  ACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGT  1268

Query  301   CTCAGTTCGGATTGGGGTGTGCAACTGGACCCCATGAAGTCGGAGTCGCTAGTAATCGCA  360
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Sbjct  1269  CTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCA  1328

Query  361   GATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCATGTCAC  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||  |||||
Sbjct  1329  GATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCAC  1388

Query  421   GAAAGTCGGTGACACCCGAAGC  442
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Sbjct  1389  GAAAGTCGGTAACACCCGAAGC  1410
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Figure 4-13: Sequences of *Streptomyces* spp isolate (No. st12) with *Streptomyces* sp. The differences in the nucleotides of this study query and the subject were found in positions (479, 986, 994, 998, 1001, 1004, 1008, 1009, 1017, 1020, 1029, 1033, 1062, 1063, 1064, 1069, 1073, 1080, 1081, 1087, 1102, 1131, 1181, 1190, 1222, 1229, 1230, 1232, 1287, 1295, 1383, and 1899).

Comparison nucleotide sequences from the *16s rRNA* gene of 2 Iraqi *Streptomyces* isolates from Babylon Province all these isolates were analyzed by (NCBI program through BLASTN with different strains showed that 100% of

local *Streptomyces* isolates belonged *Streptomyces spp*. The identity in nucleotide sequence 94% to one isolate (st6) with China isolate under accession numbers[MN826256.1.1] and 93% to another (st12) with China isolate under accession numbers [DQ663190.1].

The two sequences of the 16 srRNA region from *Streptomyces spp*. obtained in this study were deposited in the GenBank sequence database under the accession numbers [OQ119140.1 and OQ119141.1]. Appendix 6 , 7

Jaber , In his study used molecular identification by 16S rRNA sequence homology for 6 isolates which revealed that actinomycetes isolates had 98% - 99% similarity with *Streptomyces spp.* all isolate were deposited at NCBI . (Jaber , 2020)

Another study done by Singh, *et al.* that they selected 5 isolates of Actinomycetes which most active microbial strains were identified using 16S rRNA sequence homology and designated as *Streptomyces xanthophaeus* MTCC 11938, *Streptomyces variabilis* MTCC 12266, *Streptomyces xanthochromogenes* MTCC 11937, *Streptomyces levis* EU 124569, and *Streptomyces* sp.(Singh, *et al.*2016*)*

The primers that were used in this study were the two universal primers, the forward primer 27F and the reverse primer 1492R. The reason why these primers were considered as universal was because they are known to align very well to the highly conserved regions of the 16S rRNA gene and these conserved regions are shared amongst bacterial species (Frank *et al.*,2008) . Fedriksson *et al*. (2013) mentioned that these two primers were considered as good primers because they are known to amplify almost the whole of the 16S rRNA gene sequences of bacteria. The results clearly showed that the primers were able to align to the sequences of the gene of the bacteria. The PCR bands were clear, indicating no contamination from primers .

The gene is also known to be highly conserved and this is because the gene does not mutate over a long period of time. This gene is known as an orthologous gene meaning that it performs the same function throughout evolution or history and therefore enables us to identify different species that have a common ancestor. And for this reason, 16S rRNA sequencing is better known for its ability to identify newly isolated pathogenic bacteria and bacteria that cannot be cultured in the laboratory . (Clarridge, 2004)

4. 8 : Phylogenetic analysis

Streptomyces spp sequences from the *16s rRNA* studied in two *Streptomyces spp*. isolates are displayed in the phylogenetic tree constructed by the neighbor-joining method .The optimal tree with the sum of branch length = 0.435 is shown. The tree is drawn to scale , with branch lengths (above the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 10 nucleotide sequences. There were a total of 339 positions in the final dataset (figure 4-15). Separate clusters of related *Streptomyces* strains can be observed among isolates from the Iraqi soil sample in the Babylon province of *Streptomyces* spp. However, distinct clusters of highly related sequences were observed in *Streptomyces spp*. , suggesting high prevalence and endemic circulation of *Streptomyces* belonged to *Streptomyces spp*. in this area. In phylogenetic tree analysis, the first isolate(st6) showed nearly to the China, Thailand, Indian, and Turkey isolates. Greater percenear to China isolate under accession number [MN826256.1] *Streptomyces spp*. strain HH1, and less percent with another country Thailand accession numbers [ID: **LC487846.1**], Turkey accession numbers [ID: **OQ2444493.1**], and Indian accession number [ID: **KF956675.1**]. The second isolate (st12)showed nearly to the China, Morocco, India, and Japan isolates. Greater percentage near China isolate under accession

number [ID: **DQ663790.1**] *Streptomyces* spp strain 3150, and less percent another country Morocco accession numbers [ID: MT538264.1], India under accession number [ID: **MH299820.1**], and Japan accession numbers [ID: LC5177768.1].

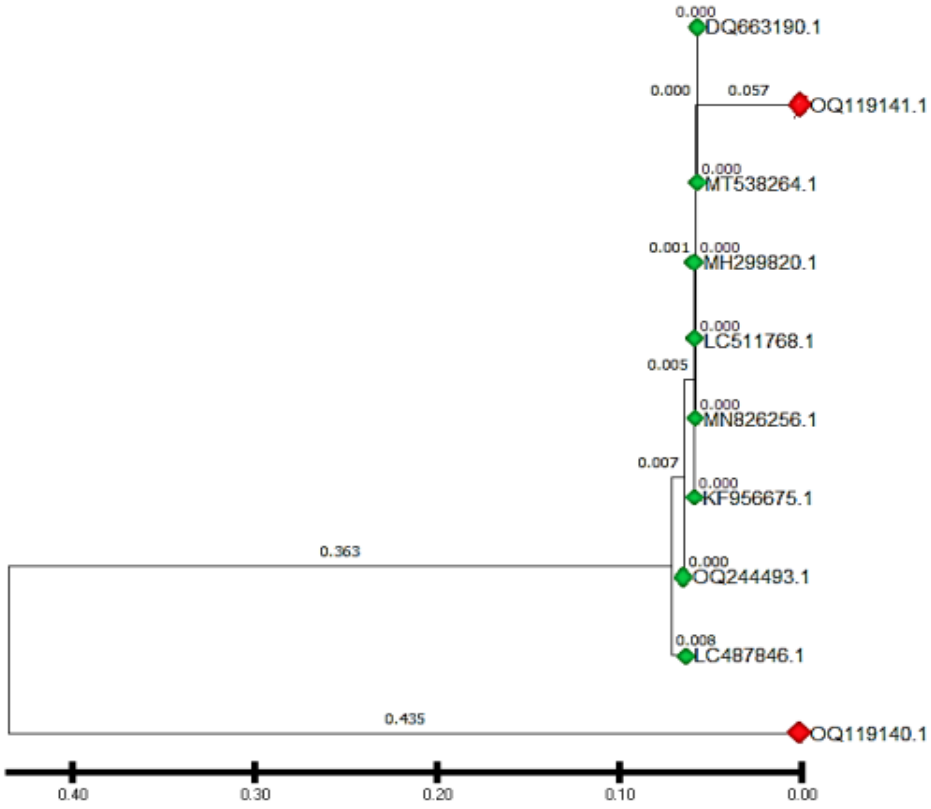
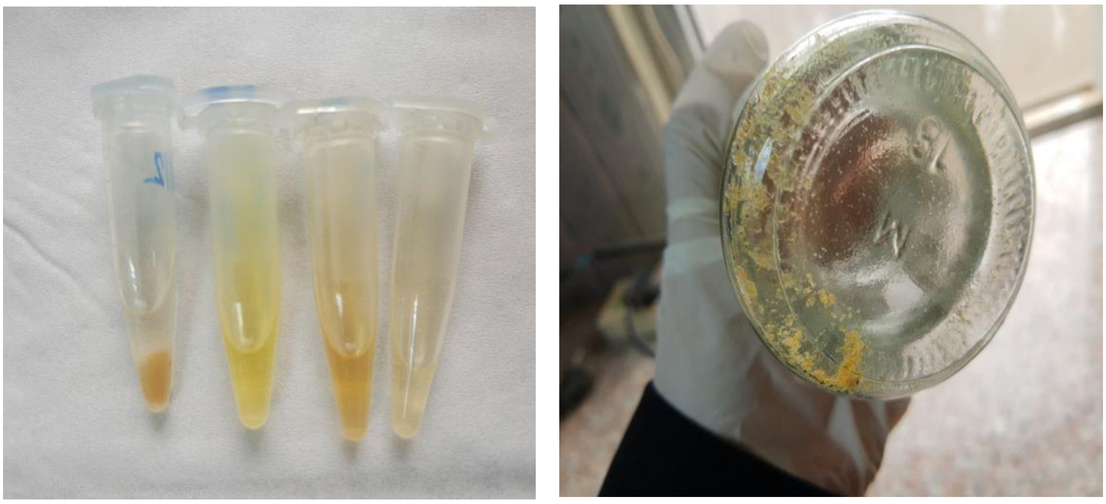


Figure 4-14: Phylogenetic tree including two local *Streptomyces* spp isolates sequences in the *16s rRNA* and 8 isolates from China, Thailand, Turkey, Indian, Moroco , and Japan clustering based on neighbor-joining method by using MEGA X program version 10.1.8.

Appendix: 1



A-*Streptomyces spp.* ethyl acetat extract B- *Streptomyces spp.* ethyl acetat extract after drying

Figure 1 : Ethyl acetat extract

Appendix : 2

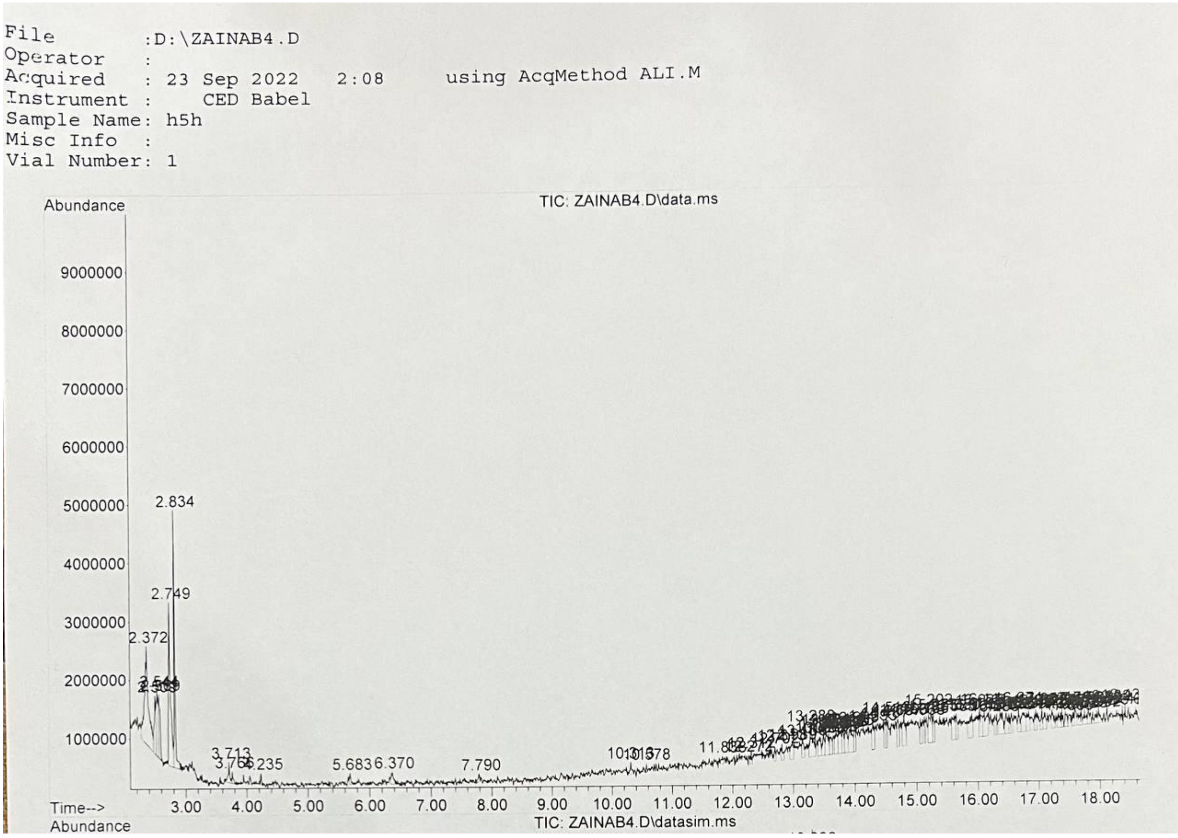


Figure 2 : GC-MS Chromatogram of Ethyl Acetate st4 Extract

Appendix: 3

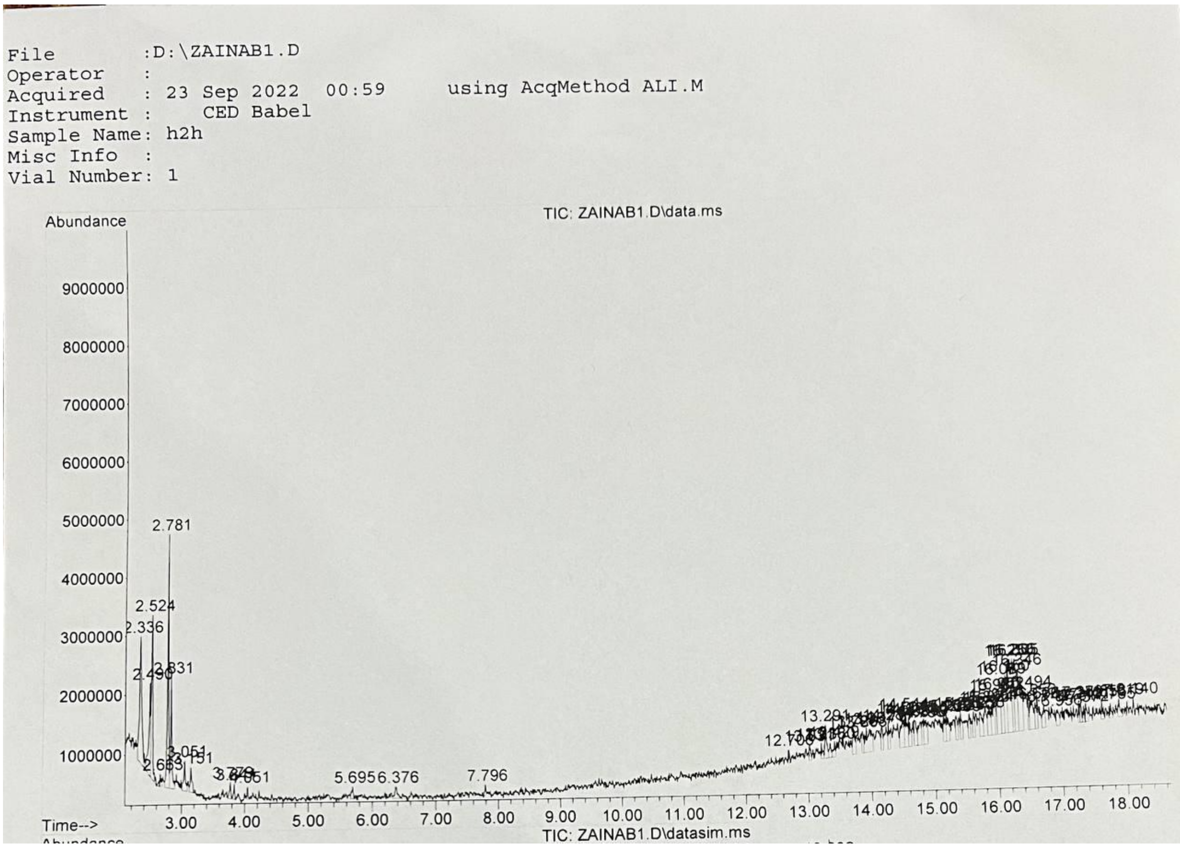


Figure 3 : GC-MS Chromatogram of Ethyl Acetate st6 Extract

Appendix:4

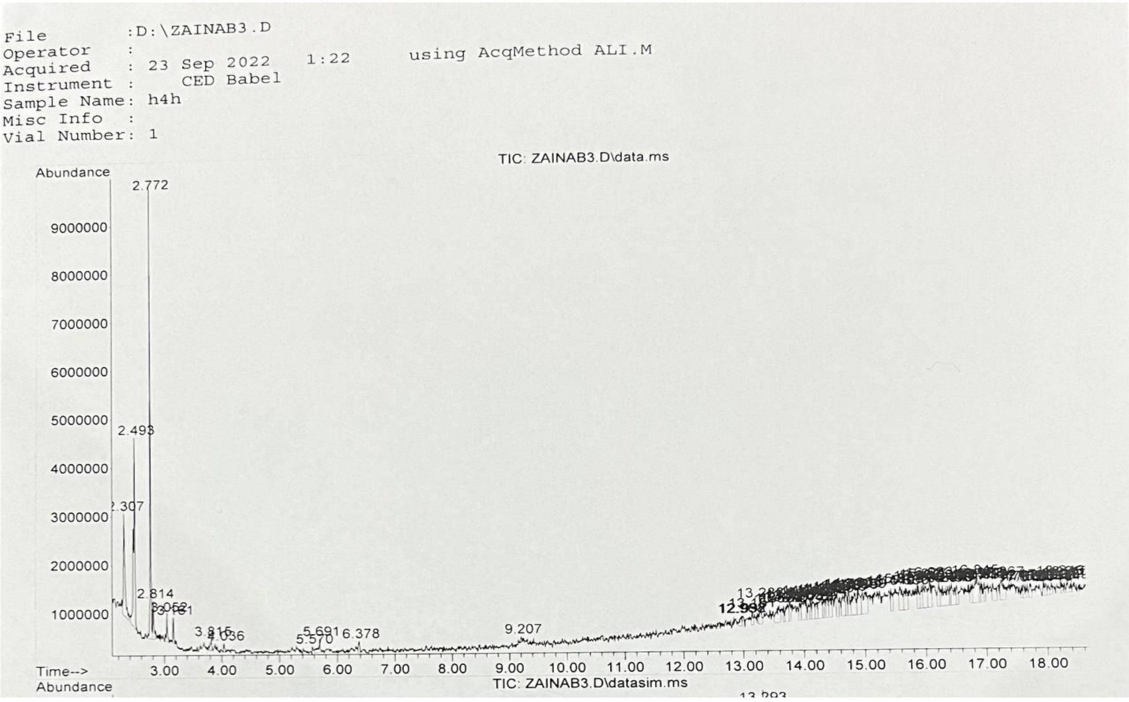


Figure 4 : GC-MS Chromatogram of Ethyl Acetate st11 Extract

Appendix:5

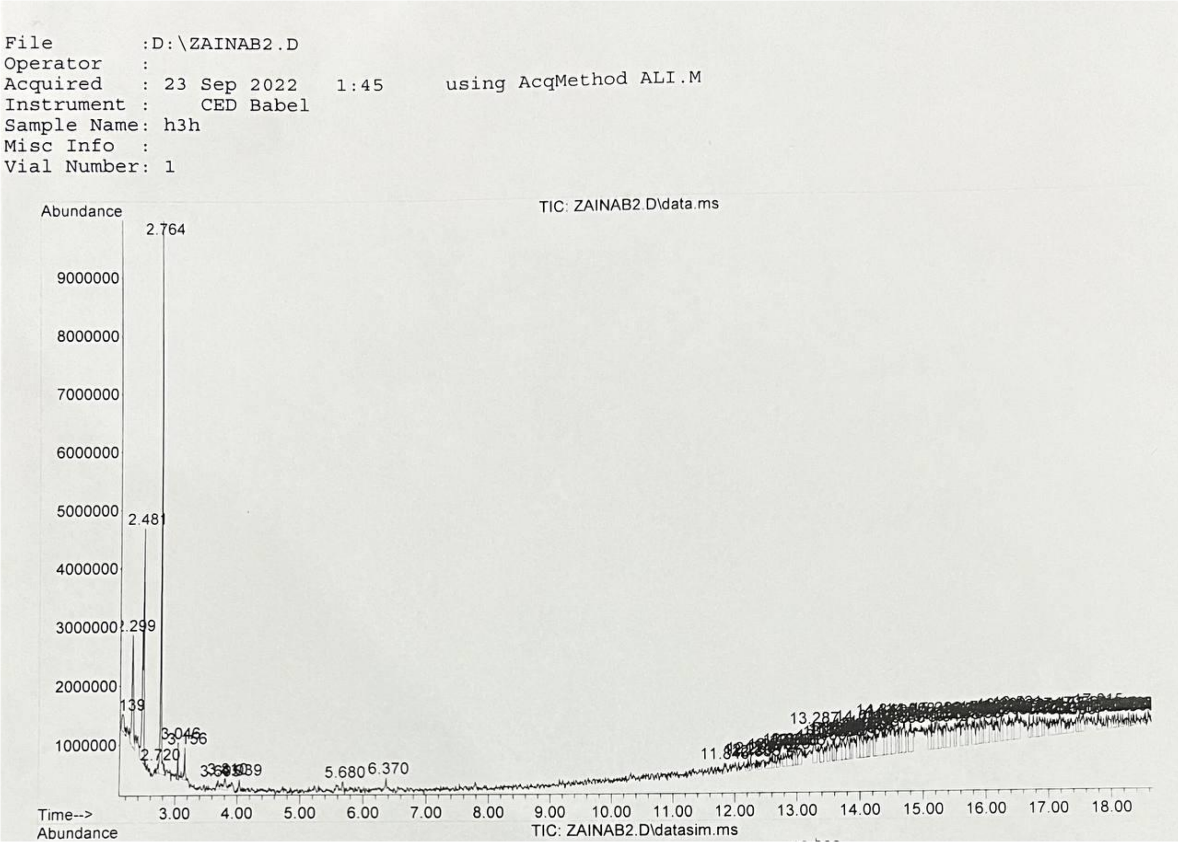


Figure 5 : GC-MS Chromatogram of Ethyl Acetate st12 Extract

Appendix:6

National Library of Medicine
National Center for Biotechnology Information

Streptomyces sp. strain ZHA1 16S ribosomal RNA gene, partial sequence

GenBank: OQ119140.1

```
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DEFINITION  Streptomyces sp. strain ZHA1 16S ribosomal RNA gene, partial
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ACCESSION   OQ119140
VERSION     OQ119140.1
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  ORGANISM  Streptomyces sp.
            Bacteria; Actinobacteria; Streptomycetales; Streptomycetaceae;
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REFERENCE   1  (bases 1 to 457)
  AUTHORS   Abed,Z.M. and Jarallah,E.M.
  TITLE     Antibacterial Activity of Streptomyces spp. Crude Extract on
            Multidrug Resistant Psedomonas aeruginosa Isolated from Clinical
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  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 457)
  AUTHORS   Abed,Z.M. and Jarallah,E.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (24-DEC-2022) Biology, University of Babylon, 60 street,
            Babylon, Babylon 00964, Iraq
COMMENT     Sequences were screened for chimeras by the submitter using
            Geneious 2022.2.1.

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//
```

FOLLOW NCBI

Figure 6 : St6 isolate which deposited in the GenBank sequence database

Appendix : 7

National Library of Medicine
National Center for Biotechnology Information

Nucleotide | Nucleotide | Advanced

GenBank | Send to:

Streptomyces sp. strain ZHA2 16S ribosomal RNA gene, partial sequence

GenBank: OQ119141.1

FASTA Graphics

Go to:

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DEFINITION  Streptomyces sp. strain ZHA2 16S ribosomal RNA gene, partial
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ACCESSION   OQ119141
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  ORGANISM  Streptomyces sp.
            Bacteria; Actinobacteria; Streptomycetales; Streptomycetaceae;
            Streptomyces.
REFERENCE   1  (bases 1 to 442)
  AUTHORS   Abed,Z.H. and Jarallah,E.M.
  TITLE     Antibacterial Activity of Streptomyces spp. Crude Extract on
            Multidrug Resistant Psedomonas aeruginosa Isolated from Clinical
            Samples
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 442)
  AUTHORS   Abed,Z.H. and Jarallah,E.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (24-DEC-2022) Biology, University of Babylon, 60 street,
            Babylon, Babylon 00964, Iraq
COMMENT     Sequences were screened for chimeras by the submitter using
            Geneious 2022.2.1.

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//
```

FOLLOW NCBI

Figure 7 : st12 isolate which deposited in the GenBank sequence database

Conclusions

1- Many isolates were obtained from screening for Actinomycetes *spp*. from various soils. Identified the *Streptomyces spp*. By specific –species primer . These isolates had a wide range of bioactive activities, which revealed their potential for antibacterial and anticancer activity.

2-*Streptomyces spp.* produced antibacterial agents with different chemical structures when analyzed by the spectral methods included ultraviolet spectroscopy (UV) and infrared spectroscopy(FT-IR) .

3-GC-Mass chromatograph analysis of ethyl acetate extract observed the presence of large numbers of biologically active compounds, with different biological prosperities.

4-The cytotoxic effect of crude extracts was performed on normal cell and cancer cell by using MTT and There are attractive biological features which achieve the powerful anticancer activities against cancerous cell line.

Recommendations

1- Screening protocol (survey) must include more and more soil types and conditions such (aquatic environment, mountain area and soils within extreme condition), and within different depths for collecting the suitable soils, in order to increase the chance for obtaining the novel isolates of Actinomycetes, as well as, the novel bioactive compounds .

3-Study gene expression on pathogen bacteria isolates

4- Detection of biological activity of extracts compounds towards another microorganism such as fungi, virus.

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Ababutain, I. M.; Abdul Aziz, Z. K. and AL-Meshhen, N. A. (2012). Lincomycin antibiotic biosynthesis produced by *Streptomyces sp*. Isolated from Saudi Arabia soil II-extraction, separation and ourification of lincomycin Cana. J. Pu and ApplSci. 6 (2): 1905-1911.

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