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Production, Purification, Characterization of Antibiotic from Locally Isolated of *Actinomycetes spp.* and Studying its Cytotoxicity and Antitumor Activity

A Dissertation

Submitted to the Council of the College of Science, Al-Nahrain University, in Partial Fulfillment of the Requirements for the Degree of Doctorate of Philosophy in Science, Biotechnology

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Dedications

I dedicate the outcome of my efforts to

- ✓ Who always encouraged me to do the best, My supervisions
- ✓ Who I never forget him, the soul of my father
- ✓ Who their words of encouragement and push for tenacity ring in my ears. My mother
- ✓ The most greatest and patient wife, My lovely wife
- ✓ The fruit of my life and most loved ones, My sons Dwarozh and Bawk,
- ✓ My beloved and dear in my life, my brothers and sisters.
- ✓ Everyone who contributed to the progress of science and service to the humanity

Saman Amin

Summary

Recently, the increase of the pathogens resistant to recent antimicrobial agents has become a health problem. Accordingly, the current study aimed at the isolation of local *actinomycetes* spp. that is capable of producing novel bioactive compounds, as well as investigating their biological (antimicrobial and anticancer) activities, in addition to, characterizing and partial purifiying the antimicrobials metabolites. Forty soil samples were collected in Garmian area, during the period from 2nd to 15th Feb. 2015. Out of the forty soil sources were screened, only (65%) of them harbored potential actinomycetes, and 55 suspected colonies with different morphotypes were isolated.

All isolates were tested for antimicrobial activities by primary screening against reference strains. Among all suspected actinomycetes candidates, only six isolates KH14, KH16, KH18, KA19, KA38 and KA39 were able to inhibit the growth at least two of tested microorganisms. Optimizing media composition for enhancing the production of antimicrobial metabolites, five different broth media ISP1, ISP2, ISP4, ISP5 and Glycerol yeast extract broth were used and ISP2 revealed the best results (inhibition zone) among all media used. Furthermore, five different organic solvents were used to obtain the maximum extraction of extracellular antimicrobial metabolites.

Results exhibited the extraction by n- butanol, methanol, ethyl acetate, n-hexane and chloroform, for recording zones of inhibition as 46.6%, 33.3%, 20%, 13.3% and 6.6% respectively against the tested microorganisms. Comparisons of antimicrobial activities between extra and intracellular crude extracts were carried out. In general, the extracellular exerted more potent antimicrobial activity than the intracellular.

Ten different synthetic antibiotic discs were used to compare their activities with the extracellular crude extract. Interestingly, the extract, when compared their activities, show a higher inhibitory activity against the pathogens than synthetics discs activity. The results of the optimization experiments, such as growth kinetic, pH and salt tolerance NaCl revealed that the 3 days of incubation at pH 8 and 5% of NaCl tolerance especially for KH14 isolate. Minimum inhibitory concentration against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* shows that 64, 500 and 500µl/ml respectively. The extracellular crude extract exhibited a strong inhibitory activity against Gram-positive bacteria and a moderate inhibitory activity against both Gram-negative bacteria and yeast. In addition to the antimicrobial actions, *DPPH* assay has been performed to determine the antioxidant activity. The extracellular crude extract from KH14 isolates showed IC_{50} about5.24 mg/ml which scavenged 91% of formed DPPH free radicals.

Determining the nature of the antimicrobial compounds was analyzed by TLC, which indicated the presence of four separate spots, only one of them revealed the antimicrobial activities, as well as their position was determined by bioautography, with different solvents (mobile phase) exhibited different R_f values. HPLC-MS analysis of the scraped active spot and ethyl acetate extract indicated the presence of many compounds. In addition, the results showed that most of them have a similar mass, which becomes noticeable in chromatogram. UV absorbance designated the presences of only two peaks at(221.85)and (264.85)nm from the scraped spot, while three distinguished peaks were shown at 222.85, 264.85 and 442.85 nm from ethyl acetate extract. Furthermore, the similarity between the two extracts on the basis of UV absorbance was achieved by the second peak of both extracts which is 264.85nm, and this was considered

as an antimicrobial absorbency. GC-MS analysis results for ethyl acetate extract of KH14 displayed 12 peaks, their molecular weight and molecular formulas were determined depending on NIST library.

The antitumor activities were determined by MTT assay, which indicated that KH14 possesses a powerful effect against used cancerous cell lines, especially against MCF-7 by inhibiting 75.84% (400 µg concentration) of cancer cells after 72 hours of incubation, while against the rest of other cell lines, PC3and A375with moderate activities. High content screen assay was carried out for six cellular multi-parameters, against MCF-7 cell line. Besides, the comparison was carried out between the vehicle and extracellular crude extract of KH14.

Statistical analyses within different (*P* values) revealed significant differences between vehicle samples with extracellular crude extract concentrations exactly in case of cell viability, nuclear morphology, cell membrane permeability and mitochondrial membrane potential with different concentrations, while both total nuclear intensity and cytochrome c did not show any significant differences with the negative control (vehicle).

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List of Abbreviations

Abbreviation	Full Name
A375	Human malignant melanoma, skin cancer
ANOVA	Analysis of variance
D. W	Distilled water
DMSO	Dimethyl sulphaoxide
DPPH	Diphenylpicrylhydrazil
EDTA	Ethylene diamine tetra acetic acid
EMEM	Eagles Minimum Essential Medium
FBS	Fetal bovine serum
GC	Gas Chromatography
HCS	High content screen
HPLC	High performance liquid Chromatography
IC_{50}	Half maximal inhibitory concentration
KA	Kalar
KE	Kefri
KH	Khanaqeen
MCF-7	Breast cancer cell
MIC	Minimum Inhibition Concentration
µg	Microgram
µl	Microliter
nm	Nanometer
PC3	Human Prostate cancer cell
R_f	Retention factor
rpm	Rotation per minute
SDS	Sodium Dodecyl Sulphate
TBE	Tris-Borate-EDTA
TLC	Thin layer chromatography
UV	Ultra Violet

Chapter One: Introduction and Literature Review

1. Introduction and Literature Review

1.1 Introduction

Actinomycetes are remarkable, bodacious and attractive filamentous Gram-positive bacteria and their DNA has high G+C (>55%) conten, and possesses the accurate aerial hyphae. They belong to the phylum Actinobateria that represents one of the major taxonomic units amongst the 18 main lineages which are now documented inside the bacterial domain with many fluctuates among the soil category (Athlete *et al.*, 1981; Olano *et al.*, 2009). Bergey's Manual for classification and according to their principle for classification, actinomycetes is divided into eight different families which are summarized by Holt, (1994) and by the way they include 63 genera as mentioned by Nisbet and Fox, (1991).

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 (Babalola *et al.*, 2009; Hozzein and Goodfellow, 2011). On the other hand, a number of organisms that are accidentally placed in disqualified groups are currently classified in the correct way due to the introduction to molecular techniques (Zhi *et al.*, 2009). However, recently, the classification of the species and phylogenies has frequently resulted from 16S rRNA and the use of (PCR) for sequencing and analyzing it (Wood *et al.*, 2007; Zhi *et al.*, 2009).

The life cycle of actinomycetes in general possesses three main separate appearances at the level of microscopic categorization specifically vegetative mycelium (growth), aerial mycelium baring cuffs of spores and the characteristic agreement of spores and finally the spore arrangement, while the last two features provided the most investigative information for identification (Anderson *et al.,* 2001; Taddei *et al.,* 2006). 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.,* 2010; Kekuda *et al.,* 2012).

Actinomycetes are frequently documented as producers of antibiotics and other useful secondary metabolites as explained by Challis and Hopwood (2003). Comprehensively, till now about 70-80% of the commercially obtained antibiotics and antimicrobial secondary metabolites has been isolated and characterized from numerous actinomycetes species (Khanna *et al.,* 2011). These secreted secondary metabolites are characterized as an opulent source of biologically active compounds such as antibiotics, enzyme, agrochemicals, antiparasitics, immune suppressants and antitumoragents Berdy (2005). However, over the previous decade, the search for antibiotics and other secondary bioactive metabolites in actinomycetes species and other microorganisms has become less popular due to the retreating achievement for discovering novel strains, as an outcome, screening for new potential chemicals compounds increased, but it didn't give way for much accomplishment, which is not remarkable since actinomycetes have evolved their capabilities over many years (Baltz, 2008).

About 80% of the rationally proceeding antibiotics are produced by actinomycetes and amongst the most abundant producers are *Streptomyces* and *Micromonospora* (Stackebrandt *et al.*, 1997). Furthermore, the study of Raja and Prabakarana (2011) and Suthindhiran and Kannabiran (2009) 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 tetracycline's, which have a broad variety of biological activities.

In addition, a large number of the antibiotics used in clinical are straight natural products and /or semi synthetic derivatives from natural microorganisms and from their secondary metabolites (Thakur *et al.*, 2007). To achieve this excited isolation of novel actinomycetes from natural income and characterization, separation of their secondary metabolites is still a precious attempt (Rahman *et al.*, 2011). However among all isolated actinomycetes strains, *Streptomyces* are everywhere present in the natural environment but the largest numbers of them are found in soil, Outstanding to their antagonism activity with other different microorganisms (Ceylan *et al.*, 2008). *Streptomyces* are the most well recognized genus of actinomycetes family which all the time has been notified because of their capability to manufacture and exude a large diversity of bioactive secondary metabolites. On the other hand the isolated and purified compounds from soil actinomycetes have a wide spectrum of biological actions such as antibacterial, plant growth hormone, cytotoxic, immunosuppressive, antifungal, antimiotic, neurotoxic, antineoplastic and antiviral activities (Black *et al.*, 1982; Schulz *et al.*, 2009).

Aims of the Study

This study is intended to investigate the following aims:

1. Isolating and identifying the actinomycetes species in the Garmian area from farming and none farming soil, as well as detecting the most powerful antimicrobial producer strains of actinomycetes by primary screening program, and studying their morphological characteristics.

2. Evaluating both the effects of the composition of media on antimicrobial production, and differnt organic solvent ability to extract antimicrobial compounds, as well as, comparing the antimicrobial activities between extracellular crude extracts with synthetic antibiotic discs, furthermore, determining the minimum inhibition concentration (MIC) against reference strains used.

3. Evaluating some growth conditions such as (best days for production of secondary metabolites, optimum pH and NaCl salt tolerance), for achieving maximum products. Besides it, estimating the extracellular crude extracts antioxidant activity of by DPPH assay.

4. Determining and separating the antimicrobial bioactive compounds by thin layer chromatography (TLC), and their R_f values with different mobile phases, furthermore demonstrating the position of bioactive compounds via bioautography.Furthermore, Analyzing and partially purifying the ethyl acetate extract by both high liquid chromatography mass spectra (HPLC-MS) and gas chromatography mass spectrum (GC-MS).

5. Determining the antitumor activity of the extracellular crude extract via MTT assay against three different cancer cell lines, as well as evaluating the cytotoxicity of extracellular crude extract through High Content Screening (HCS) multi-parameters assay.

1.2 Literature Review

1.2.1 General Backgrounds of Actinomycetes

Actinomycetes are aerobic, able to form spore, gram-positive bacteria, and belong to the order Actinomycetales which is characterized by possessing both substrate and aerial mycelium growth at their entire growth (Lechevalier and Lechevalier, 1981). The high (G+C) ratio of their DNA contained (>55%), which are phylogenetically linked to the facts of 16S ribosomal cataloguing and DNA: rRNA combination study (Goodfellow and Williams, 1983; Korn-Wendisch and Kutzner, 1992). It reprsents one of the major taxonomic groups among the 18 main lineages presently documented within the bacterial domain (Ventura *et al.*, 2008).

The name "Actinomycetes" was originally derived from the Greek word "attacks" means (a ray) and "mykes" means (fungus), therefore having similar characteristics of both fungi and bacteria (Das *et al.,* 2008). Furthermore, they possess sufficient characteristic features that help to define them into 'Kingdom of bacteria'. The actinomycetes are prospective producers of antibiotics, therapeutic constructive compounds, and the bioactive compounds in the secondary metabolites. These include antibiotics, immunosuppressive agents, antitumor agents, and enzymes. Beside, these metabolites are known to possess antibacterial, antioxidant, antifungal, anti-cancer, neuritogenic, anti algal, anti-malarial, and antiinflammatory and anti-helmintic activities (Kekuda *et al.*, 2010; Ravikumar *et al.,* 2011).

They reveal a range of life cycles which are exclusive among the prokaryotes and emerge to play a main role in the cycling of the organic matter in the soil environment (Veiga *et al.,* 1983). Actinomycetes have proven their capability to produce a multiplicity of the bioactive secondary metabolites and

depending on this basis, the discovery of novel antibiotic and none antibiotic guide molecules throughout the microbial secondary metabolite screening is appropriate gradually more important. Furthermore, actinomycetes (Actinobacteria) are extremely imperative to human medicine, food production and agriculture. The key reason for this activity is their capability to cooperate with other organisms, an area which has mainly been unnoticed in the previous 150 years of their study. In humans, actinobacteria are expected to framework around one-third of the gut microbiota, most remarkable bifidobacterium species, which are valuable to human health and nourishment (Miao and Davies, 2010).

Finally a significant constituent in the drug innovation based on the microbial extract is the isolation of undiscovered groups of microorganisms that are at the same time excellent producers of the bioactive secondary metabolites. Many environmental niches immobile till now remain uncultivated. This needs to be studied for a better variety of new actinomycetes. Unusual strains of actinomycetes in general produce diverse compounds. In addition to achieving this rationale, concentrated hard work can be augmented for the isolation and screening of novel strains to find out new compounds (Chaudhary *et al.*, 2013).

1.2.2 Ecology and Habitat of Actinomycetes

Actinomycetes are the most abundant microorganisms that appear as thread-like filament structure in the nature, and they are cultivated as hyphae like fungi in appearance which is responsible for the typically "earthy" smell of recently turned well soil (Sprusansky *et al.*, 2005). The actinomycetes live in a variety of habits in environment (George *et al.*, 2012), are characterized as un ubiquitous group of microorganisms broadly dispersed in usual ecosystems approximately around all the world (Srinivasan *et al.*, 1991).They are mainly earth (soli) inhabitants (Kuster, 1968), but also have been broadly scattered in a varied range of aquatic ecological unit, including sediments obtained from

marine (Walker and Colwell 1975; Colquhoun *et al.*, 1998), yet from maximum depth Mariana Trench (Takami *et al.,* 1997; Pathom-aree *et al.,* 2006).

Their presence in extreme environmental conditions, especially in cryophilic regions, has also been documented (Raja *et al.,* 2010). For instance, soils were taken from Antarctica (Moncheva *et al.,* 2002) and even from desert soil (Diraviyam *et al.,* 2011). It has been established that in a proportional survey, actinomycetes inhabitants are the largest in soils source and they from a surface layer which slowly decreases as depth increases. Character of actinomycete strains are found in all soil layers (Takahashi and Omura, 2003).

Information on the actinomycete variety on limestone habitats are still insufficient, while some studies have been approved out on the microbial population of the caves, especially the limestone caves (Schabereiter-Gurtner *et al.*, 2002; Ortiz *et al.,* 2013). Actinomycetes are the most widely distributed group of microorganisms in nature (Takizawa *et al.,* 1993). The strong odor in the air when rain falls after a dry magic charm of weather is due to production of geosmin by the soil actinomycetes (Gust *et al.,* 2003). In natural habitats, *Streptomyces* species are common populations and are usually a main component of the whole actinomycete inhabitants. Upto date, there are over 600 *Streptomyces* species cited in the literature review (Euzéby, 2014). Other actinomycete genus such as *Actinoplanes, Catenuloplanes, Amycolatopsis Micromonospora Kineospora, Dactylosporangium, Microbispora* and *Nonomuraea* are frequently very complicated to isolate and grow due to their slow growing and they are called "rare actinomycetes" (Hayakawa, 2008). In addition, actinomycetes are often heterotrophic in the environment. Most of them are strict saprophytes, while some from parasitic or mutualistic associations with plants and animals. Actinomycetes are commonly believed to have a role in the recycling of nutrients, and they are aerobic and some like actinomycetes are

anaerobic. Some species such *Frankia* needs very specific growth media and incubation situation; furthermore, many actinomycetes are growing on the ordinary bacteriological media used in the laboratory such as nutrient agar, blood agar, trypticase agar, starch casein agar and brain heart infusion agar. Sporoactinomycetes on the other hand, need very special media to ensure differentiation and the improvement of spores characteristic and pigment production (Attwell and Colwell, 1984; Chavan Dilip *et al.,* 2013).

1.2.3 Life Cycle of Actinomycetes

Actinomycetes represent as an important model of bacterial development; they display an extraordinary multifaceted life cycle with dissimilar cell types (spores, vegetative and reproductive mycelia), and with the morphological changes severely associated to the physiological segregation. 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 growth of their vegetative parts leads to the formation of a mycelium which consists of a ramifying arrangement of hyphae that enter a wet substrate by the extension of hyphal tips and sub-apical branch, then followed with reproductive growth often accompanied with the formation of filamentous aerial hyphae that finally undertakes differentiation into chains of uni-genomic spores. The multipart developmental life cycle includes apoptosis phenomena that construct this bacterium with a multicellular prokaryotic model (Manteca *et al.,* 2010; Rioseras *et al.,* 2014).

In general, the life cycle of *Streptomycetes* is complex and is classified as being neither multicellular nor unicellular (Maguelez *et al.,* 2000). They

generally describe the life cycle of *Streptomyces* as in (Figure 1.1) which starts with the spore germination resulting in the dispersal of filaments into the solid medium to give an outward appearance as vegetative mycelium. Then the vegetative mycelium develops to form sporophores which expand vertically on the way to the surface and aerial mycelium formed. In addition, the poly-nucleated aerial is formed from aerial mycelium when spirals and then the filaments are partitioned. The final sheaths become spores and then the cycle begins repeating. The secondary metabolites of microbial are almost organic compounds in nature which include antibiotics, toxins, pigments, antitumor agents and enzymes which are not directly concerned with the normal growth, development or duplicate of the producing microorganism (Martin *et al.*, 2005). Microorganisms on the other hand produce their secondary metabolites throughout the stationary phase of the grown life cycle. Waksman (1957) described the life cycle of filamentous actinomycetes was regularly occuring as either spores form or minute fragments of mycelium. When there are favorable growth conditions, germination occurs by the formation of branching threads or rods that develop into unicellular mycelia. The formed hyphae are usually non-septated, and within the solid media substrate the vegetative mycelia grow, at the same time the aerial mycelia stick out from the vegetative growth. The majority of actinomycetes are reproduced either from special sporulation bodies such as in *Streptomycetes*, or from the hyphal tips part of the formed mycelium in the case of none sporulating genus such as *Dietzia*, *Mycobacterium*, *Gordonia*, *Rhodococcus* and *Nocardia*.

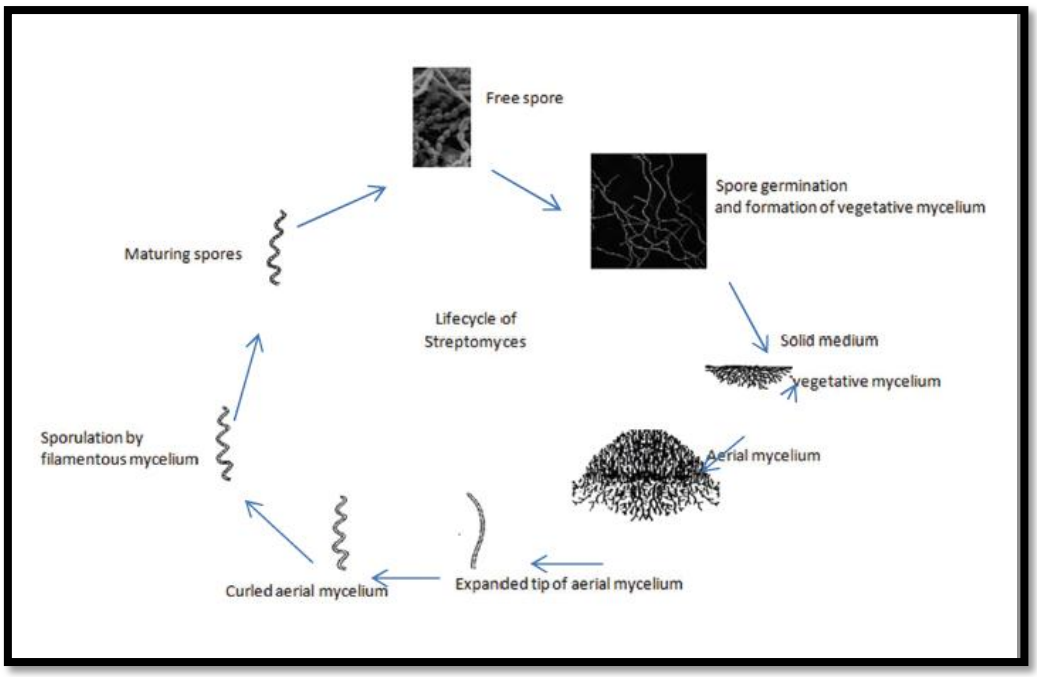

Figure 1.1: Life cycle of actinomycetes in general, especially Streptomycetes, from (Maguelez *et al.*, 2000).

1.2.4 Structure, Composition and Taxonomy of Actinomycetes

Actinomycetes are differentiated by the forming of branching threads or rods at normal state. The hyphae are generally found to be non- septated; under definite particular conditions, septa may be seen in some different forms. The sporulating mycelium may be branched or none branched, straight or spiral shaped (Chaudhary *et al.*, 2013). The spores of actinomycetes are spherical, cylindrical or oval. Moreover, actinomycetes produce early micro colonies which are composed of branching filaments system, after 24 to 48 hrs the fragment parts changed into diptheroids, short chain and coccobacillary forms (Waksman, 1940). The actinomycetes cell wall is an inflexible structure that maintains the cell wall of actinomycetes shape and maintains the shape of the cell which prevents bursting of the cell through the high osmotic pressure (Goodfellow *et al.*, 1998; Manuselis and Mahon, 2007).

The wall consists of a large mixture of different compounds, including peptidoglycan, teichuronic acid, teichoic and polysaccharides. The peptidoglycan components consist of glycan as a chain of irregular N-acetyl-d-muramic acid (NAM), diaminopimelic acid and N-acetyl-d-glucosamine (NAG) and (DAP), which is unique in the cell walls of prokaryotic microorganisms. The teichoic and teichuronic acids are chemically bonded to peptidoglycan (Davenport *et al.*, 2000; Manuselis and Mahon, 2007). The chemical composition of their cell wall is like that of gram positive bacteria, but because of their well morphological developed and cultural characteristics, actinomycetes have been considered as a group which separate is from the other ordinary bacterial groups (Cummins and Harris, 1954; Das *et al.*, 2008)

Studies on various cell well structures of the actinomycetes spores during germination have been limited to the *Streptomyces* (Kalakoutswl and Agre 1973). The *Streptomyces* forms endospores which proceed in a similar way to those of *Bacillus*, a fresh wall layer being synthesized within the cortex of the spore and extending to form the germ-tube wall. In a study of *Streptomyces* species, the spores had a two-layered wall and the inner one was extended to form the new germ-tube wall. It is not clear if this layer is lately formed throughout germination or if it is created by reorganization of wall material existing in the latent spore. Moreover, when grown on a solid surface, the actinomycetes branches form a complex of hyphae growing both on the surface and undersurface of the agar. The one which grows on the surface is called “aerial hyphae” while the one growing undersurface is identified “substrate hyphae”. The present septa normally divide the hyphae into long cells (20 mm) possessing numerous bacterial chromosomes. These aerial hyphae that expand above the substratum reproduce asexually. Most of actinomycetes species are non motile, but when the motility is present, it is confined to flagellated spores

(Sharma *et al.,* 2014). Actinomycetes are classified as actinobacteria which are characterized with Gram-positive and their high guanine-plus-cytosine DNA content about (69–73 mol %); with extensive of substrates branching and aerial mycelia present. Furthermore, the taxonomy of actinomycetes is summarized in (figure 1.2), (Williams *et al.,* 1983; Williams *et al.,* 1989).

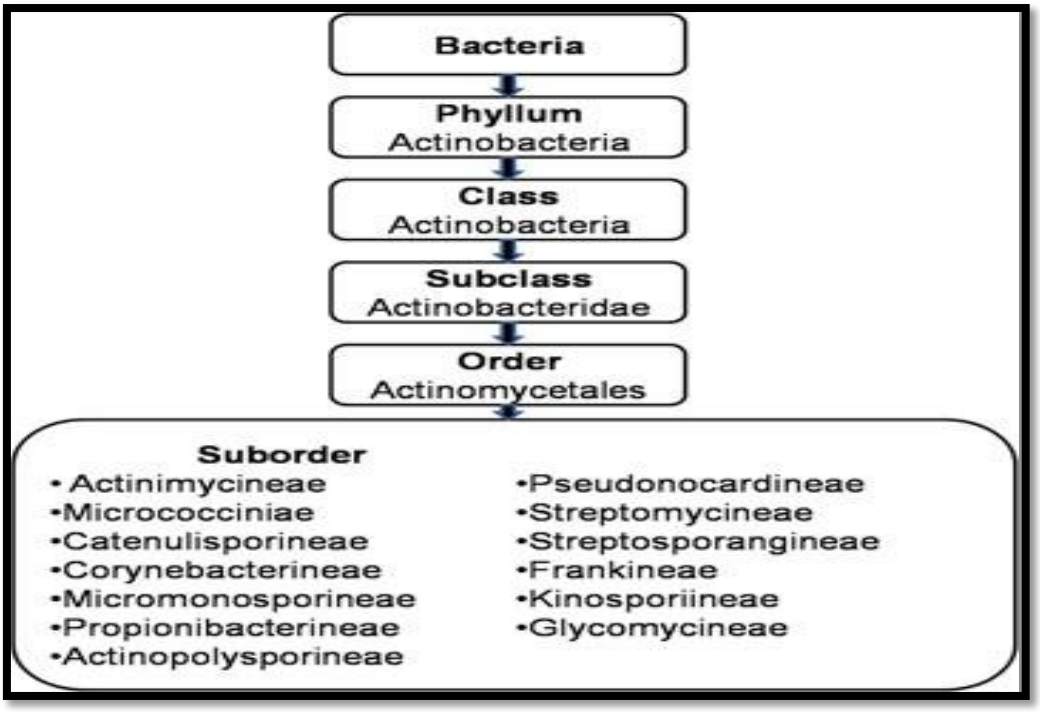

Figure 2.1: Taxonomy of the actinomycetes with all 13 suborders, (Mahajan and Balachandran, 2015)

1.2.5 Isolation of Actinomycetes

The practice has revealed that the discovery of previously unknown and important natural bioactive compounds takes place when novel selection systems are used. The isolation of actinomycetes from mixed micro flora found in the nature is difficult because of their features with a slow growth relation to that of the other soil bacteria; however, there are several technologically important options used for the isolation of actinomycetes (Chavan Dilip *et al.,* 2013).

1.2.5.1 Choice of Substrate

Isolation of actinomycetes from soils, freshwater and marine environment has been reported. There should be some differences between organisms obtainable in marine and earthly environments. In the course of screening of actinomycetes isolated from the shallow sea area, some antagonistic actinomycetes, such as xanthomycin producing actinomycetes, have been isolated more frequently than from terrestrial soil. A small number of these actinomycetes were found to be late and produce either new antibiotics or new bioactive substances under especially devised conditions. Thus, the isolation of actinomycetes from marine areas gives us another foundation for discovering new actinomycetes and novel antibiotics (Chavan Dilip *et al.,* 2013).

1.2.5.2 Selective Media

Bacteriostatic and fungistatic chemical agents such as phenol and sodium propionate have been mixed into the isolation media to inhibit or reduce the growth of bacteria and molds and thus favor actinomycetes growth. But such amendments at allowable concentrations frequently let the growth of contaminants and at higher levels may also prevent actinomycetes growth. Chitin agar with mineral salts is extra efficient than that without mineral salts for isolating actinomycetes from water. Chitin agar exhibited selectivity superior to that of other media for isolating actinomycetes from water and soil (Carlsen *et al.,* 1996).

1.2.5.3 Incubation

The popular of antibiotics producing actinomycetes species grow most excellent between 25 to 30^{o}C. Thermopiles are incubated at 40 to 45^{o}C and psychrophiles at 4 to 10^{o}C. Incubation times for the isolation of plates are typically from 7 to 14 days. Longer incubation times have often been unnoticed because of the slow growing actinomycetes would be inappropriate candidates

for trade and industrial fermentation, however, the early growth of some species of bacteria can modify the nutrient environment of the isolation plate by supplying with growth factors. For the isolation of new actinomycetes species incubation time may be extended to one month (Chavan Dilip *et al.,* 2013).

1.2.5.4 Colony Selection

Choosing the colony for the isolation process is the major time consuming scheme. It depends upon the aims of the screening plan, and there might be much duplication of the colonies in the screening protocol. For the isolation of the microorganisms, extra rational ways must be applied. The regular researchers now select candidate colonies by using a stereomicroscope and transferring growth with the help of a sharp wooden stick. Tiny colonies can be distinguished and chosen. The rough wooden points carry enough spores or hyphal fragments to provide a successful transfer. The site of sample collection, enrichment techniques objective, knowledge about the secondary metabolite of an isolate and the aim of culture media formulation would lead to the isolation of new and potential isolate strains (Collins *et al.,* 1995).

1.2.6 Identification of Actinomycetes

Numerous actinomycetes species are growing on the ordinary bacteriological media, used in the laboratory such as nutrient agar, blood agar, trypticase agar, starch casein agar and brain heart infusion agar. Sporoactinomycetes require special media to allow segregation and the improvement of characteristic spores shape and pigment production. However, a number of these media do not exist commercially and must be prepared in the laboratory using colloidal chitin, decoctions of plant materials and soil extract. Pale, hard colonies and shiny *Streptomyces* species on nutrient agar can be distorted into light yellow colonies with a chalky white aerial mycelium and spirals of arthrospores when the organism is sub-cultured in a more appropriate

growth medium, such as oatmeal or inorganic salts starch agar. Outgrowths from a spore or fragments of mycelium expand into hyphae that enter the agar (substrate mycelium) and hyphae that branch frequently and become covered together on the surface of the agar to form a leathery and tough colony. The composition of the medium determines the density and the regularity of the colony. *Nocardio* from actinomycetes reveal fragmentation, the hyphae breakup into rods and cocci and form elastic or friable colonies (Ayakkanu and Chandramohan, 1971; Chavan Dilip *et al.,* 2013).

In actinomycetes classification and characterization, phenotypic and phylogenetic methods are involved as a polyphasic taxonomic approach (Tan *et al.,* 2007). These include morphological, biochemical and physiological characterization in phenotypic approach; while molecular methods are in phylogenetic characterization part.

According to Shirling and Gottlieb (1966) methods for *Streptomyces* isolation and characterization, the essential observation should concern the cultivation of cultures on various media; yeast extract – malt extract agar (ISP2), inorganic salts – starch agar (ISP4), oatmeal agar (ISP3) and glycerol – asparagine agar (ISP5). Growth and morphology of *Streptomyces* species are seen when cultures become mature with serious spore mass in order to determine aerial spore mass color, substrate mycelium and diffusible pigment color production (Williams *et al.,* 1993). Beginning differentiation could be completed by color grouping when dealing with a great quantity of different isolates. However, this group of actinomycetes fell into the same group with comparable morphological and physiological properties based on their color alignment.

Morphological observation deals with sporophores morphology as it is well thought-out stable and evidently defined feature for actinomycetes classification. However, this is only suitable without the incidence of strain

degeneration appropriate to subculture or improper maintenance (Pridham *et al.*, 1958). In some strains of certain genera such as *Streptomyces*, the colony becomes covered with free, straight hyphae bounded by a hydrophobic cover that grow into air left from the colony (aerial mycelium). These hyphae are originally white, but form diverse colors when spore creation begins. Colonies appear velvety or powdery and readily distinguished from the more characteristic bacterial colonies. *Streptomyces* species own chain of spores on its aerial mycelium, which are normally absent from the substrate mycelium. These spores are arthrospores, usual segments of hyphae with a thicken spore wall bounded by a hydrophobic sheath that may stand hairs or spin (Burman, 1973; Chavan Dilip *et al.*, 2013). The principles of identification are the isolation of colonies an aerobically grown on blood agar or egg containing media, followed by identification through biochemical tests with the modern taxonomic tables and molecular methods will greatly assist in the identification of the most challenging actinomycetes species (Sarkonen *et al.*, 2001).

1.2.7 Optimizing Production of Bioactive Compounds from Actinomycetes

Enhancing the production of any bioactive compound from actinomycetes species was normally achieved by a variety of physicochemical, molecular, nutritional, immobilization and mutational regulated processes. Regulation and operating of physicochemical and nutritional variations for the maximum production is a fundamental and classical technique to augment the production. In addition, genetic engineering, mutations, and immobilization techniques are effective tools to enhance the production. Modern advances prepared in software and bioprocess tools results a number of highly integrated software based techniques to attain the maximum production of the end product in any bioprocess or fermentation. Response Surface Methodology (RSM) with an

appropriate statistical plan is one of the modern and important techniques to achieve this goal (Chang *et al.*, 2002; Aghaei Kohazani *et al.*, 2012)

A large number of actinomycetes species especially Streptomycetes have the ability to grow on nutrient agar, Muller-Hinton agar and trypticase soy agar with calcium chloride for the production of desired bioactive compounds (Busti and Yushi, 2006), as well as each of the carbon and nitrogen sources. Furthermore, oxygen, temperature pH, ions and some other precursors play pivotal roles and can affect the production of bioactive compounds from isolated actinomycetes species (Rafieenia, 2013).

Media composition has the most important impact on the production of antibiotics and other bioactive compounds, especially relating with both glucose and phosphate, known as suppressors for the production of some metabolites process. However, strategies to realize novel biologically active secondary metabolites are extremely reliant on the culture conditions. In order to determine how these bioactivities of a broad collection of actinomycetes changed according to growth conditions, and to investigate chemical or growth supplements ability to trigger antimicrobial production, the productivity of the collected species grown under disparate conditions and with the addition of various additives was assessed; starch (1% w/v) ,Peptone (0.8% w/v) and also with pH 10 in particular were found to be the most efficient conditions for activating the production of antibiotic among the 40 assessed conditions. All the conditions are without difficulty available at a low cost, with minimum batch to batch variation (Sanchez *et al.*, 2010; van Wezel and McDowall, 2011).

The enhanced production related to the improvement in yield amount or value of yield greater than the former obtained by some strategies is followed. Commonly fermented yield can be enhanced by a suitable fermented design, optimization of process parameters, media optimization and recombination in

microorganisms (Ren *et al.,* 2013). Furthermore, improvement of the strain used in the study was done by different strategies like immobilization and mutation (Haq and Ali, 2006).

1.2.8 Importance and Diversity of Secondary Metabolites Produced by Actinomycetes

Secondary metabolites are the byproduct of actinomycetes microorganisms which are organic compounds that thought to be indirectly involved in normal growth curve, reproduction and development of the producing microorganism (Martin *et al.,* 2005). Microbial secondary metabolites have potential applications in many fields such antimicrobial agents, enzyme technology, pigment production, antitumor agents against cancer cells and production of toxins. Approximately, 7000 compounds were reported in the glossary of natural products from actinomycetes secondary metabolites (Jensen *et al*., 2005). Therefore, actinomycetes are the vast attention to industry manipulation because of their capability of producing significant secondary bioactive metabolites. *Streptomyces* is the genus considered as the largest genus for antimicrobial producing agents. Moreover, it accounts for 80% of the actinomycetes derived from natural products (Goodfellow and Fiedler, 2010).

Although thousands of antimicrobial compounds also have been reported, these are thought to be characterized only as a small fraction of the list of bioactive compounds that members of the *Streptomyces* genus were capable of producing it (Watve *et al.,* 2001). However, it has become more and more difficult to discover new secondary metabolites from common actinomycetes as the most screening efforts lead to be the expensive rediscovery of known bioactive compounds, as well as several attempts deal with this trouble include the careful isolation and screening program for the rare actinomycetes

(Goodfellow and Fiedler, 2010).The innovation of the aminoglycoside gentamicin produced by *Micromonospora echinospora* and *Micromonospora purpurea* stimulate the interest in screening for non-streptomycete genus from actinomycetes genera to obtain the novel antibiotics (Abou-Zeid *et al.*, 1978).

These screening operations were productive and with a huge array of antimicrobial compounds isolated from non-streptomycete species. Several *Amycolatopsis* and *Actinomadura* species were found to produce vancomycin-type glycopeptides. Napthacene-quinone and macrolactam antimicrobial also have been isolated from *Actinomadura* species, whilst a number of macrolide-type antibiotics have been found by *Saccharopolyspora* and *Micromonospora* strains (Moncheva *et al.*, 2002). Separated from antimicrobial compounds, actinomycetes also produce other efficiently significant compounds, including immunomodulators, vitamins and enzymes used in industry as the biocatalysts in wide ranges (Moncheva *et al.*, 2002).

The secondary metabolites produced by some species of actinomycetes in addition to discovering their application in the treatment of many useful fields such cancers and autoimmune disorder were also reported (Genilloud *et al.*, 2011; Lucas *et al.*, 2013). On the other hand Lucas *et al.* (2013) reported that currently there are more than 2,400 different biologically bioactive secondary metabolites produced by *Streptomyces* species, Furthermore Genilloud *et al.*, (2011) described that the scientists and researchers think that there could be quite a lot of more secondary metabolites with other different therapeutic activities to be explored and discovered. Finally, some of the newly discovered bioactive compounds from actinomycetes species are listed in (Table 1.1).

Tabel 1.1: Recently discovered biologically bioactive compounds from actinomycetes

Bioactive compounds	Actinomycetes	Potential use	References
BE43472A	*Streptomyces* strain (N1-78-1)	Antibacterial	Rahman *et al.,* 2010
Citreamicin delta	*Streptomyces vinaceus*	Antitumor	Hopp *et al.,* 2008
Dynemicin	*Micromonospora chersina*	Antitumor	Pei *et al.,* 2010
Lenticulone	*Streptomyces* sp JP 95	Antibacterial	Yun *et al.,* 2009
Lecensimycin D	*Streptomyces lucencis* MA 7349	Antibacterial	Singh *et al.,* 2009
Mediomycin B	*Streptomyces mediocidicus*	antifungal	Cai *et al.,* 2007
Rapamycin	*Streptomyces hygroscopicus*	Immunosuppressant	Nicolaou *et al.,* 2009
Sansamycin A	*Streptomyces* sp SS	Antibacterial	Xi *et al.,* 2007

1.2.9 Application of Actinomycetes Secondary Metabolite Products

The concentration on the actinomycetes in the biotechnological applications is as a result of their naturally great metabolic variety of these organisms and their long involvement with the environment. Actinomycetes are a unique collection of prokaryotes microorganisms having diverse morphological, biochemical, cultural and physiological characters. This group of microorganism is a prospective producer of a large number of biologically bioactive compounds such antimicrobial substances, immunomodifiers, enzyme inhibitors, enzymes and growth promoting compounds for animals and plants **(**Collins *et al.,* 1995; Chavan Dilip *et al.,* 2013) as presented in (Figure 1.3). Among the actinomycetes species, *Streptomyces* is recognized as a main mumber of the antimicrobial derived antibiotics (Boonlarppradab *et al*., 2008).

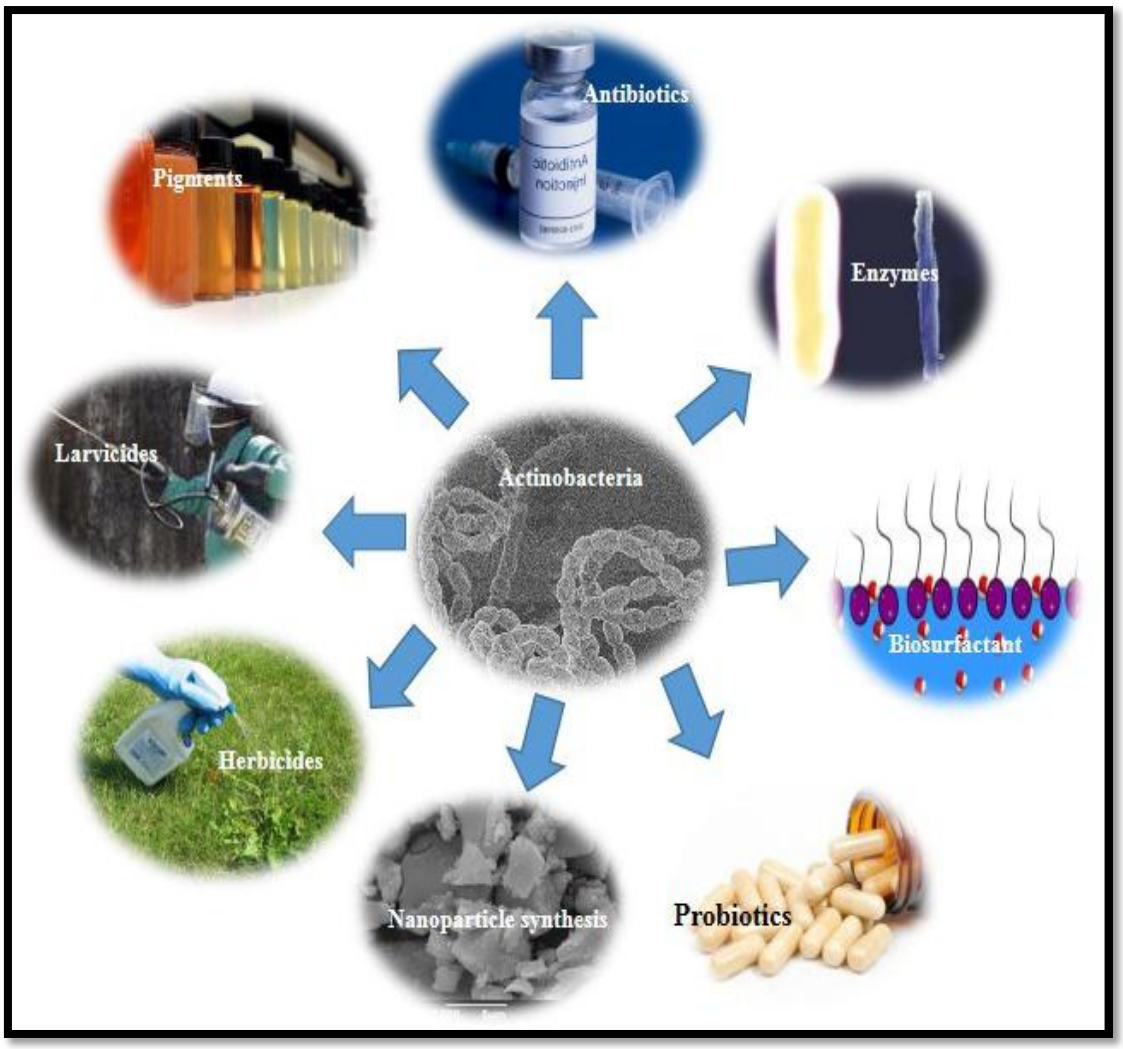

Figure 1.3: Biotechnological applications of Actinomycetes, (Dharumadurai and Yi, 2016).

1.2.9.1 Antitumor Compounds from Actinomycetes

Chemotherapy is one of the major treatments used to battle against cancer; and great quantities of antitumor compounds are in natural origin as a whole or their derivatives, mostly formed and produced by microorganisms, especially, actinomycetes are the producers of a huge number of natural products with different biological bioactive properties, as well as antitumor properties (Olano *et al.,* 2009). These discovered antitumor compounds belong to numerous structurally different classes such as anthracyclines, indolocarbazoles, enediynes,

macrolides, isoprenoides and non-ribosomal peptides, and they exhibit antitumor activities by producing apoptosis through one of the appropriate mechanisms. Such DNA cleavage is mediated by topoisomerase I or II inhibition. Inhibition of key enzymes is concerned with signal transduction like proteases, mitochondria permeabilization, or cellular metabolism and in some cases by inhibiting tumor-induced angiogenesis (Olano *et al.,* 2009). Furthermore, some antitumor compounds were isolated from a *Streptomyces* strains perform by intercalating with DNA duplex, which leads to harmful effects on rapid proliferating cells by inhibiting the DNA dependent RNA polymerase actions (Wadkins *et al.*, 1998).

In the study of Boonlarppradab *et al.* (2008), they isolated *Streptomyces* from marine sediments that produced two new spiroaminals, marineosins A and B which had significant inhibition of (HCT-116) human colon carcinoma in an *in vitro* assay and showed activities in various cancer cell lines. Conventionally, antitumor compounds for treatment of the cancerous cell are either taken from natural products or produced by chemical modifications of a natural compound product (semi-synthetic antitumordrugs). Natural products are the most constantly successful sources of drug safety; and from all the 175 antitumor discovered drugs between1940s to 2006, only 42% of them are either natural products or compounds derived from them. On the other hand, many antitumor discovered drugs are natural products isolated and purified from microorganisms (in particular from actinomycetes) or growing plants in ecological niches (Gullo *et al.*, 2006; Newman and Cragg 2007; Itokawa *et al.*, 2008).

1.2.9.2 Actinomycetes Application in the Environmental and Ecosystem Fields

Actinomycetes have numerous roles in the environment. Such streptomycetes are saprophytic bacteria that decompose organic matter,

especially complex polymers such as chitin, keratin, hemicelluloses, natural rubber, lignocelluloses, pectin, starch and constantly some synthetic compounds that may enter the soil as contaminants (Goodfellow and Williams, 1983; Crawford *et al.,* 1993). Actinobacteria are also imperative in the rhizosphere relationship, where they may control plant growth and defend plant roots against attack by many pathogenic fungi (Goodfellow and Williams, 1983).

The possible role of actinomycetes as a biological control agent of soil-borne root diseases in produced plant has been investigated, mostly in greenhouse experiments. Furthermore, several *Streptomyces* species (in addition to a few other actinomycete genera) have been revealed to guard different plant species against soil borne pathogenic fungal disease. A number of genra have also been shown to produce insecticidal and herbicidal bioactive compounds (Crawford *et al.,* 1993). Correspondingly, members of the actinomycetes such *Frankia* can fix nitrogen in the soil. They have a wide host variety and have been revealed to show root nodule symbioses with more than 200 species of flowering plants (Clawson *et al*., 2004).

1.2.9.3 Actinomycetes as Agents of Biodegradation and Bioremediation

The published report of Zhou and Zimmermen (1993) summarizes the responsibility of actinomycetes as a manager of decolorization in manufacturing effluents containing synthetic reactive dyes as water-soluble. It exhibited the capacity of actinomycetes in decolorizing between 17% up to 73% of the effluent and by decolourization or adsorption of the dyes to the cells. Azo dyes, anthraquinone, azo-copper complex, phthalocyanine dye and formazan-copper complex and were among the included dye which documented in their report.

Actinomycetes isolated from soil and correlated substrates illustrate a primary biodegradative action, secreting a collection of extracellular enzymes

and exhibiting the ability to metabolize intractable molecules. Moreover, compost is a procedure that heavily depends on such actinomycetes prolific activity, however, among soil actinomycetes; there are examples of diverse strategies, from cycles of quick proliferation and sporulation to the preservation of the populations by extending slow growth and scavenging (McCarthy and Williams, 1992). Actinomycetes are also responsible for pesticides degradation with a variety of different chemical structures, including organochlorines, triazinones, s-triazines, organophosphates, carbamates, acetanilides, organophosphonates, and sulfonylureas (Nawaz *et al.,* 2010). Native soil actinomycetes was reported to degrade the herbicide in the soil, especially Durian (D'Esposito *et al.,* 1998).

Among the actinomycetes species, *Streptomyces* were established to be the main and followed by *Micromonospora, Nocardia, Actinoplanes, Actinomadura* and *Dactylosporangium* with the novel rubber-degrading in *Gordonia* species from fouling water within a tire in a deteriorated automobile (Linos *et al.,* 2002).

1.2.9.4 Potential Enzymes from Actinomycetes

Enzymes are present in nature. A wide diversity of microorganisms survives the secret extracellular enzymes compounds that can demean a lot of polymer forms of compounds. Actinomycetes produce numerous enzymes, which are or could be useful in biotechnology applications, medical therapy and clinical chemistry. As traditional themes, they are recognized as antimicrobial producers and at the present being explored on the huge rate for the production and manufacturing enzymes. A diversity of enzymes of profitable importance are identified to be formed by the actinomycetes such as protease, catalase, amylase, carbohydrases and cellulose (Prakash *et al.,* 2001).

Actinomycetes play pivital role in the providing many enzymes for a lot of biotechnological and manufacturing bioprocesses. Thus, glycosidases of all kinds are exploited for the deprivation of plant biomass and very active proteases serves as additives to detergents or are used in the tanning industry. Glucose isomerases have been successfully used to obtain fructose-heavy syrups. Moreover, mycolytic enzymes and bacteriolytics isolated and purified from actinomycetes may be used for beer and wine clarification or serve as non-toxic food preservatives. Enzymes produced by actinomycetes also seem to be very promising as immobilized preparations for regular clinical diagnostic tests (Abdullah *et al.*, 2008). Actinomycetes enzymes are the most significant yield after antibiotic production. Microbial enzymes are broadly used in food processing techniques, detergent manufacturing, pharmaceutical industries, the textile, medical therapy, molecular biology and bioorganic chemistry (Peczynska-Czoch and Modarski, 1988).

Actinomycetes were best known as antibiotics sources for many years. Recently, they also have been detected to be a potential source of a wide range of important enzymes. Rifaat *et al.* (2005) investigated twenty producing cellulase free-xylanase *Streptomycetes* strains isolated from Egyptian soils. Among them, only two strains were mostly active and identified as *Streptomyces chromofuscus and Streptomyces albus*. The augmentation of the enzyme activity was found when both isolates grown on yeast extract medium. Optimum production of xylanase was recorded after five days of fermentation. Xylanase produced with *Streptomyces chromofuscus* showed an advanced bleaching activity than that from *Streptomyces albus*. The enzyme improved the release of reducing sugars, which enhanced pulp bleach ability. Actinomycetes are able to synthesize enzyme inhibitors with a low molecular weight. The first low molecular weight

enzyme inhibitor was produced by a streptomycetes strain; more than 60 inhibitors were obtained by the researcher, including leupreptins, which inhibit plasmin, papain and trypsin. Antipain inhibits papain; trypsin, chymotrypsin and cathepsnin B. Enzyme inhibitor compounds are found to be probably useful in cancer treatment. e. g. revistin, an enzyme inhibitor isolated from *Streptomyces* species which inhibited reverse transcriptase. Retrostatin and Streptonigrin were synthesized by *Streptomyces* strain inhibit reverse transcriptase (Goodfellow *et al.,* 1988).

1.2.9.5 Immunomodifiers Agents

Low molecular weight compounds have been isolated from filtrated culture of actinomycetes species, which improve immune responses, and these were called "immunomodifiers". Enzymes inhibitor, located on the surface of cell concerned in immunity, may bind to such cells and enhance immune responses. Bestatin was isolated from *Streptomyces olivoreticuli*, phenicine from *Streptomyces lavendulae* and amastatin from *Streptomyces ME* 98-M-3, all of them showed enhancement to the immune responses in mice. Immunosuppressive agents such as FR-900506 which reported by Fujisawa pharmaceutical company, produced by *Streptomyces tsukubaensis* showed strong inhibiton against interleukin-2 production, cytotoxic-T cells mixed lymphocyte reaction, interferon, and platelet activating factor-C induction (Drouin and Cooper, 1992).

1.2.10 Identification and Profiling of Bioactive Compounds Produced by Actinomycetes

Numerous screening programs have been approved and resulted in finding thousands of bioactive compounds from microbes. Therefore, a new method is needed to decrease the probability of rediscovering of recognized compounds

(Busti *et al.,* 2006). Systematic methods or an original technology in the search for new medicine candidate compounds should be applied, so that the processes carried out are ineffective. These methods can be completed with the submission of analysis to recognize and reduce the secondary metabolites which are previously known in the research process as early as possible; this analysis is also called "dereplication" (Newman *et al.,* 2006; Baltz *et al.,* 2008). Dereplication is a method for screening by comparing secondary metabolites or compounds that are previously known to the suspected novel compounds, so there is no duplication of research for the results or the identical compound (Lang *et al.,* 2008). Several methods with spectroscopic analysis play an imperative role in the development of the innovation of antimicrobials, and these analyses can be used untimely in the process to detect the presence of certain compounds in the extract. Numerous spectroscopic approaches have been applied to reach the dereplication importance such as high performance liquid chromatography, HPLC (Tormo *et al.,* 2003), Nuclear magnetic resonance NMR (Lang *et al.,* 2008), and Liquid chromatography–mass spectrometry LC-MS Cremen and Zeng 2002; Genilloud *et al.,* 2011, and finally Infrared radiation (IR) which can also be used for dereplication. This is because the presence of two different molecules with different chemically structures will produce a different IR spectrum (Silverstein *et al.,* 1981).

1.2.10.1 Thin Layer Chromatography (TLC)

Thin layer chromatography is the most familiar and efficient technique method used for the detection, analysis and separation of the bioactive compounds, so it is probably that 60% of the analyzed compounds are performed based on TLC over international. Thus, it is important to know the basic operation and performance of the TLC protocol (Maitland *et al.,* 2010). Thin

layer chromatography has been important for the separation of secondary metabolites, Polyphenol, saponin, alkaloids, flavanone, flavanoid, aromatic amines, amino acids, alcohols, acids, glycols, proteins, amides, antibiotics, peptides, pesticides, bile acids, vitamins and porphyrins in soft drinks. Steroids such as sterols, cholesterol, sterone, estrogen, hormones, bile salt progesterone and synthetic steroids such as oxandrolone, mifepristone, other steroid nandrolone, stanozolol, dromostanolone, are defined as unlawful and are disqualified in sports, especially of Olympic Games which are also analyzed by using TLC (Bhawani *et al.*, 2012).

Thin layer chromatography is generally regarded as a simple, inexpensive and rapid technique for the separation, visual semi quantitative assessment and tentative identification of a broad diversity of substances. Recently, TLC has become important to competitors with HPLC and GC in its capability to determine difficult mixtures and to supply quantitative results. The development of the performance has included improvements in quality of the thin layer chromatography plates and application reagent detection techniques, the preface of new stationary phases and approaches in plate development, as well as the design of densitometry scanning and sample application (Striegel *et al.*, 1996). The low cost and the qualified speed of analysis were considered as the main advantages of thin layer chromatography, and the materials needed to do the TLC are minimal including a development chamber, chromatographic plates, suitable solvents, developed reagents, and orientation materials. The main disadvantages of thin layer chromatography include its minor sensitivity in a comparison to other methods, such as HPLC or GC and it need to a larger sample size (Selvameenal *et al.*, 2009).

1.2.10.2 Bioautography

Large numbers of secondary metabolites are produced during the fermentation process from produced microbes; however, the real bioactive compound fraction among these products needs to be documented. Bioautography helps in the rapid and straight detection of active antimicrobial metabolite compounds in microbial fermented culture broth by applying it on the TLC plate and determination of their R_f values (Rahalison *et al.,* 1991; Navarro *et al.,* 1998). Bioautography is simple, somewhat sensitive, simple to perform and not expensive. It allows bioassay-directed fractionation and position localization of bioactive compounds from complex microbial mixture and a plant extracts mixture (Hamburger and Cordell 1987; Shahverdi *et al.,* 2007). The first time paper chromatography was used followed by bioautography for the estimation of penicillin purity was applied by Goodal and Levi (1946). Bioautography is predominantly important to keep away from the time-consuming for the isolation of inactive compounds. Thin layer chromatography bioautographic methods come together with chromatographic separation and activity determining which facilitate the localization and target-directed isolation of active constituents in a mixture (Shahverdi *et al.,* 2007).

The quantity and number of bioautographic assays have been improved. In general, they can be divided into three main groups as described by Rios *et al.,* (1988). These groups include direct bioautography; where the interested microorganisms directly grow on thin-layer chromatography (TLC) plates, the second one is contact bioautography; where the antimicrobial compounds are transferred from the TLC plate to an inoculated agar plate through direct contact, and finally agar overlay or immersion bioautography; where a seeded agar medium is applied onto the TLC plate. The last technique can be considered as a hybrid of direct and contact bioautography (Islam *et al.,* 2003).

1.2.10.3 High Performance Liquid Chromatography (HPLC)

In distinguishing between thin layer chromatography (TLC) and high performance liquid chromatography (HPLC), both are similar in their expression and with the same of the mobile phase, the stationary phase, and the separation mechanism, and both TLC and HPLC are considered as a complementary technique, while HPLC is considered as more efficient than TLC in separating components. Furthermore, the HPLC system is a closed system which allows a better control of the mobile phase velocity (Striegel and Hill, 1996).

Extra newly high performance liquid chromatography and its supercritical fluid chromatographic methods are based either on derivatives diastereomeric of DAP (El-Waziry *et al.,* 1996; Kudo *et al.,* 1998), chiral mobile state (Wiseman and Nichols 1984) and chiral stationary state (Nagasawa *et al.,* 1993; Medvedovici *et al.,* 1996). All of these methods have not been useful for bacterial culture, while some of them are inappropriate for multifaceted amino acid mixtures, else having a sample preparation process which is uninteresting and prolonged (Kudo *et al.,* 1998).

1.2.10.4 Elucidation of the Structure

Structure elucide isolation and structural elucidation of natural compounds are both considered as an expensive process and time-consuming. However, dereplication is a significant step with the intention to differentiate between unknown and known isolated compounds. Moreover, it is a result of allowing exclusion of existing compounds at an early stage. In addition, there are different modern technologies which until that time compounds observed in semi- purified culture can be recognized, and the advantage of using these techniques is that those compounds are not highly purified (Hostettmann *et al.,* 2001).

This allows researchers to differentiate among known compounds and novel molecules directly from the crude extracts,. Thus, the boring and hard isolation of known compounds can be avoided, and at the same time, a targeted isolation of presenting new compounds or abnormal structures can be undertaken. These techniques include HPLC-MS, HPLC-MS/MS, HPLC-DAD, HPLC-NMR, HPLCMS-NMR, LC–NMR, LC–DAD–MS (Hostettmann *et al.*, 2001; Schneider *et al.*, 2006). Tondeur *et al.*, (1984) used techniques to monitor the toyocamycin synthesis at all stages of fermentation, in which HPLC coupled with electrospray ionization tandem mass spectrometry that confirmed an important dereplication method in the program of screening new antibiotics. Besides, the combination of liquid chromatography with tandem mass spectrometry (HPLC-MS/MS) has led to novel strategies by which bacterial culture extract and plant crude extract are screened to get information as much as possible about known structures with high selectivity and sensitivity (Bringmann *et al.*, 2001; Schneider *et al.*, 2006).

1.2.11Future Prospect of the Actinomycetes

The secondary metabolites discovered from actinomycetes are unrivaled and unmatched in medical significance. Moreover, actinomycete remains as the main source of clinically important antibiotics, most of which are too difficult to be synthesized by combinatorial chemistry (Baltz, 2008; Kekuda *et al.*, 2010). Structurally and functionally diverse bioactive compounds have been isolated from actinomycetes as antibiotics with antibacterial, antiparasitic, antifungal, anti-tumor and anti-viral activity. Popular actinomycetes that are potential drug sources remain uncultivable, and therefore, inaccessible for novel antibiotic discovery. Less than one fraction in 10^{12} of the earths' soil has been screened for actinomycetes (Baltz, 2004). About 1-3% of the Streptomycete antibiotics has

been revealed and the remaining 97-99% needs recent technologies for screening, enrichment and selection of actinomycetes (Clardy *et al.*, 2006; Goodfellow, 2010).

The public health officials consider the current state of available antibiotics to be perilous, as some organisms are close to having complete resistance to all commercially available antibiotics. Fortunately, new antibiotics are constantly being discovered from *Streptomyces*. Also, older drugs that were not deemed suitable for use are being re-examined. In some cases, they are being chemically modified. This can cause them to have new abilities to inhibit other microorganisms (Donadio *et al.*, 2010). No doubt, actinomycetes represent the biggest possibility to obtain further medically, agriculturally and industrially valuable compounds which may serve as direct or indirect drugs leading compounds for structural modifications and templates for the rational drug design and other derivatives. Chemical diversity of bioactive compounds, particularly from uncommon actinomycetes, is promising. However, detection of bioactive actinomycete taxa requires in-depth thinking of their accurate diversity and eco-physiology through which target directed isolation strategies can be implemented (Kurtböke, 2012; Bull and Stach, 2010). As long as the major challenges in biotechnology and biomedicine remain (e.g. emerging diseases, antibiotic resistance, established diseases, environmental pollution and need for renewable energy), microbial income will be of interest to providing sustainable and environmentally friendly solutions. Actinomycetes and their bioactive compounds show an antibacterial and antimicrobial activity against various pathogens and multi-drug resistant pathogens e.g vancomycin resistant enterococci, methicillin resistant *Staphylococcus aureus, Shigella dysenteriae, Klebsiella* sp., *Escherichia coli, Pseudomonas aeruginosa* etc (Selvameenal *et al.*, 2009; Singh *et al.*, 2012).

Chapter Two: Materials and Methods

2. Materials and Methods

2.1 Materials

2.1.1 Apparatus and equipments

The following apparatus and equipments were used in this study:

No.	Equipment	Company/ Origin
1	Autoclave	Lab. Tech/ Korean
2	Centrifuge (macro)	Sigma/ USA
3	Incubator	Sigma/ USA
4	Magnetic stirrer	Lab. Kits/ China
5	Analytical balance	Sartorius / Germany
6	Water bath	Clifton / Great Britain
7	Compound microscope	Zeiss/ Germany
8	Oven	Memmert/Germany
9	Shaking incubator	Lab. Tech/ Korean
10	Eppendorf bench centrifuge	Sigma /USA
11	Deep freeze -80°C	Lab-kits/ China
12	Laminar air flow hood	Lab. Tech / Korean
13	Micropipettes	Eppendorf/USA
14	pH-meter	Martini/Italy
15	Refrigerator	Beko/Turkey
16	Vortex	Lab. Tech/ Korean
17	Spectrophotometer	Lab. Tech / Korean
18	Gas chromatography (GC-MS QP-2010)	Shimadzu Instruments/ Japan
19	High Performance Liquid Chromatography (HPLC-MS)	Waters 2545/USA
20	High-Content Screening Array Scan	Thermo Scientific /USA
21	TLC Aluminum Sheet Of Silica Gel	Merck co, USA
22	U.V Trans illuminator	Cleaver /England
23	Whatman No. 1 filter paper	Sigma /USA

2.1.2 Biological Media and Chemicals

The following chemicals and biological media used in the study.

2.1.2.1 Cultural Media were used in the Study, Lab. prepared

No.	Media name	Abbreviation	Composition	Amount
1.	Tryptone-yeast extract broth (Shirling and Göttlieb, 1966)	ISP1	Tryptone	5 gm
			Yeast Extract	3gm
			Distilled water	1liter
			pH 7.0 to 7.2	
	Preparation condition		121°C, 15 lb and 15minutes	
2.	Yeast extract-malt extract broth (Shirling and Göttlieb, 1966)	ISP2	Yeast Extract	4 gm
			Malt Extract	10 gm
			Dextrose	4 gm
			Distilled water	1 liter
			pH 7. 3	
	Preparation condition		121°C, 15 lb and 15minutes	
3.	Inorganic salts-starch broth	ISP4	soluble starch	10 gm
			K2HP04	1 gm
			MgS04.7HzO	1 gm
			NaCl	1 gm
			(NH4)2S04	2 gm
			CaC03	2 gm
			Trace salt s solution	1 ml
			Distilled water	1 liter
			pH between 7 and 7.4.	
	Preparation condition		121°C, 15 lb and 15minutes	
4.	Glycerol-asparagine broth (Shirling and Göttlieb, 1966)	ISP5	L-asparagine	1 gm
			Glycerol	1 gm
			K2HP04	1 gm
			Trace salt s	1ml

			solution	
			Distilled w a t e r	1 liter
			pH between 7. 0-7. 4.	
	Preparation condition		121°C, 15 lb and 15minutes	
5.	Glycerol yeast extract broth (Abussaud and Saadoun, 1991)	GYE	Yeast extract	2gm
			Glycerol	10ml
			Distal water	1liter
			pH between 7.0 and 7.2	
	Preparation condition		121°C, 15 lb and 15minutes	
6.	Trace salt solution (Shirling and Göttlieb, 1966)		FeS04. 7Hz0	1 gm
			$MnCl_2$. 4H20	1 gm
			ZnS04.7Hz0	1 gm
			Distilled water	1liter

2.1.2.2 Culutral Media were used in the Study (Redy to use)

No.	Media	Abbreviation	Company/Origin
1	Starch casein agar	SCA	BDH/England
2.	Nutrient agar and Nutrient broth	NA and NB	BDH/England
3	Eosin methylene blue	EMB	BDH/England
4	Potato dextrose agar	PDA	BDH/England
5	Saborate dextrose agar	SDA	BDH/England
6	Mueller Hinton agar	MHA	BDH/England
7	Mannitol salt agar	MSA	BDH/England

2.1.2.3 Chemical Materials used in the Study

No.	Chemicals	Company/Origin
1	Sodium dodecyl sulfate (SDS)	Sigma/ USA
2	Ethylene diamine tetra acetic acid (EDTA)	Sigma/ USA
3	Proteinase K	Sigma/ USA
4	TE buffer	Sigma/ USA
5	1,1-Diphenyl-2-Picryl-hydrazil (DPPH)	Sigma/ USA
6	3-(dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide(MTT) Stain	Sigma/ USA
7	Acetone	BDH/England
8	Ethyl Acetate	BDH/England
9	Chloroform	BDH/England
10	n-Hexane	BDH/England
11	n- Butanol	BDH/England
12	Absolute methanol	BDH/England
13	Toluene	Sigma /USA
14	Ethanol	Sigma /USA
15	Glacial Acetic Acid	Fluka/Switzerland
16	Vitamin C	Sigma/USA
17	DMSO	BDH/ England
18	Cyclohexamide	Sigma /USA

2.1.3 Antibiotic Discs and Kits

2.1.3.1 Antibiotic Discs

Antibiotic Discs used in the Study

No.	Antibiotics	Abbreviation	Manufacture
1	Amoxicillin	AX	Bioanalyse
2	Gentamicin	CN	Bioanalyse
3	Cefotaxime	CTX	Bioanalyse
4	Streptomycin	S	Mast diagnostics
5	Penicillin G	PG	Mast diagnostics
6	Vancomycin	VA	Mast diagnostics
7	Erythromycin	E	Mast diagnostics
8	Tetracycline	TE	Mast diagnostics
9	Methicillin	ME	Mast diagnostics
10	Amoxicillin/clavulanic acid	AMC	Mast diagnostics

2.1.3.2 Cellomics Multi-parameter Cytotoxicity 3 kit Composition used for Cytotoxicity Activity

Kit Contents	**Concentration**
Cytochrome C primary antibody	75 µl
DyLight™ 649 Conjugated Goat Anti-Mouse IgG	72 µl
Mitochondrial Membrane Potential Dye	1 ea
Permeability dye	25 µl
Hoechst dye	30 µl
Wash Buffer (10X Dulbecco's PBS)	100 ml
Permeabilization Buffer (10X Dulbecco's PBS with 1% Triton® X-100)	100 ml
Blocking Buffer (10X)	85 ml
Thin plate seal assembly	7/pack

2.2 Areas of the Study

A study area of this project has covered Garmian area –Kurdistan region-Iraq, an area about (4.0873 Km^2) which is about 0.94% of the total area of Iraq (434.934 Km^2). The area locates between (34^o .11$^-$ - 34^o.15$^-$ latitude and 44^o.30$^-$ 45^o.55$^-$ longitude), on the geographical coordinate (Azeez, 2013). This area is located at the south of Sulaymaniyah, north of Diyala, and west of Kirkuk Province, it also has border with Iran from the east; Kalar, Kefri and Khanqeen are considered as the main districts of Garmian area Figure (2.1 and 2.2).

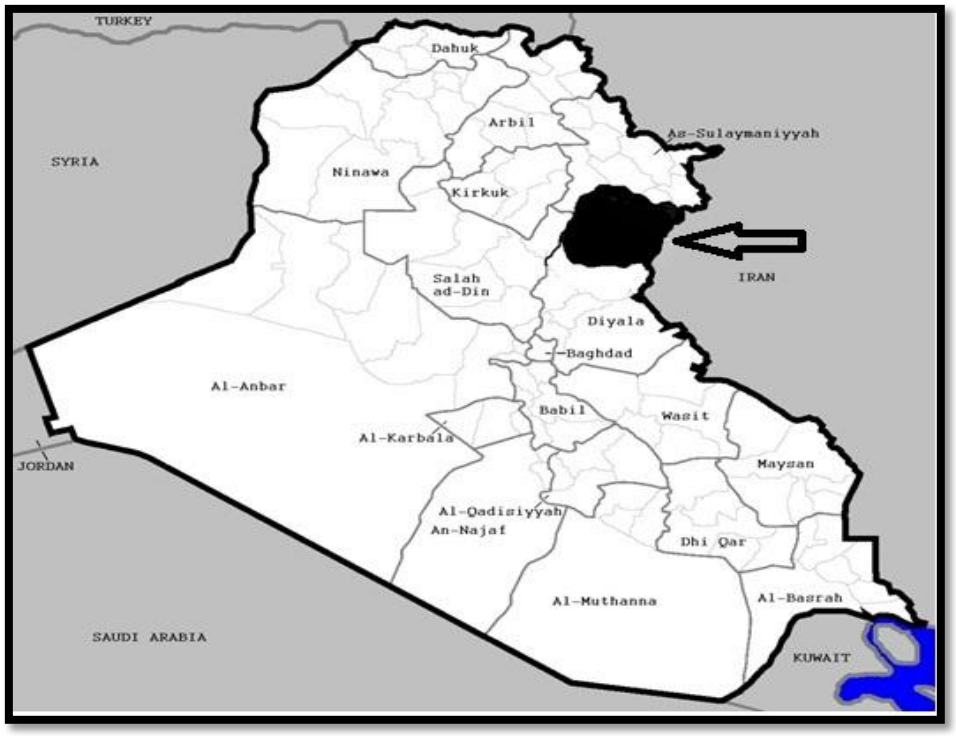

Figure 2.1: Location of Garmian area on the Iraqi map, which shows borders, highlighted black area represents Garmian (From Geographic Department, College of Education: Garmian University).

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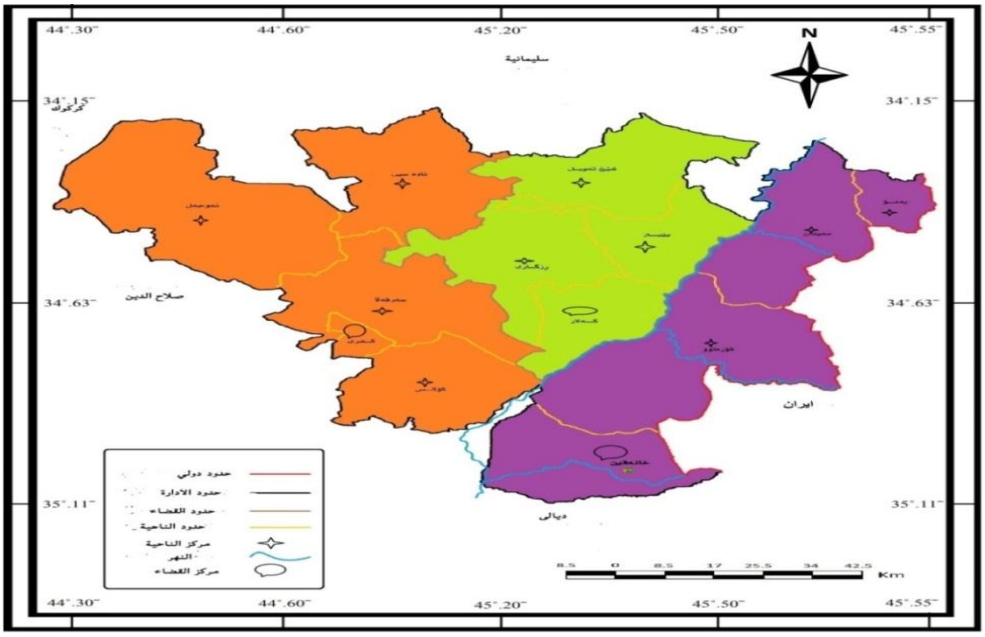
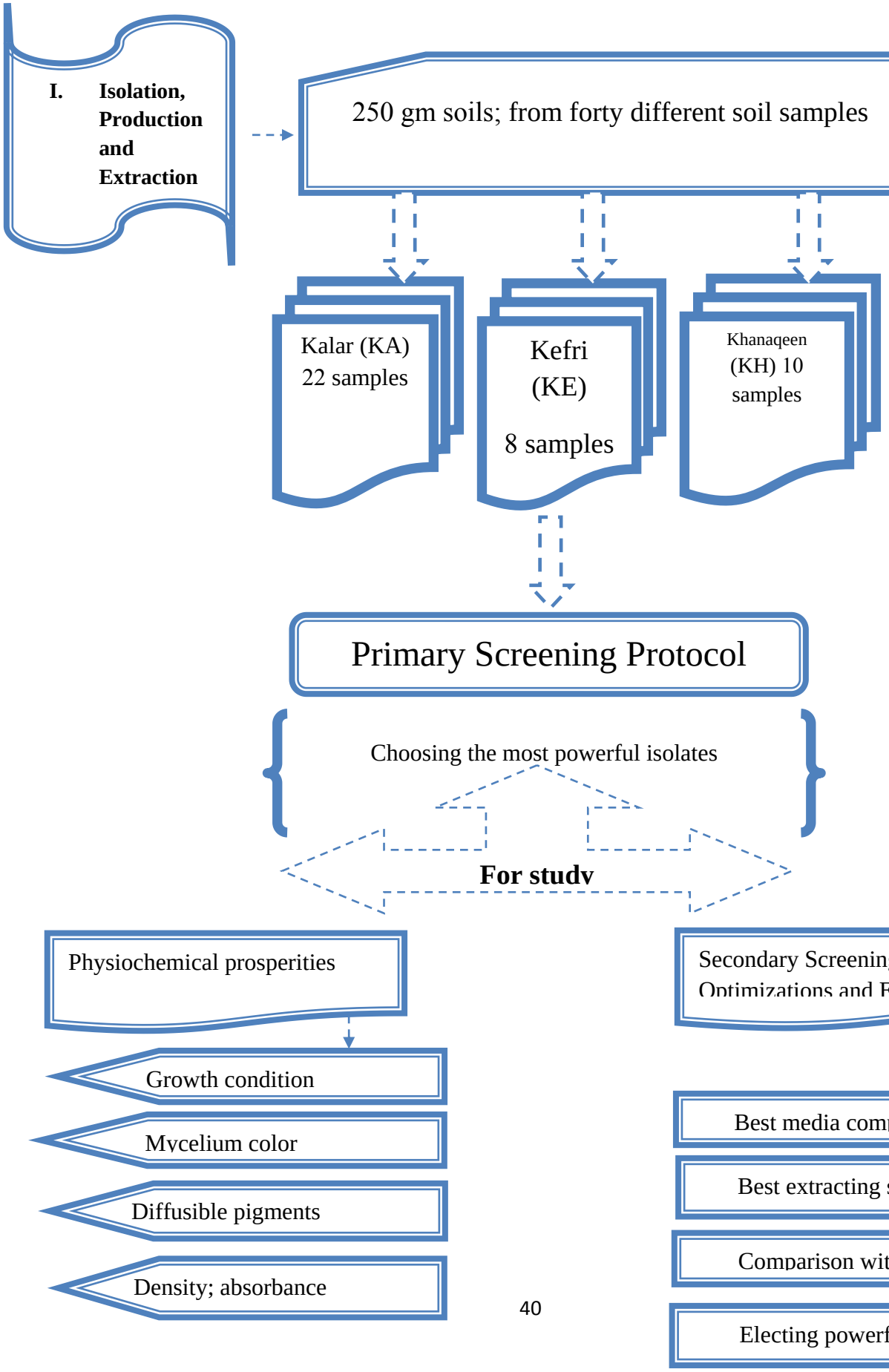
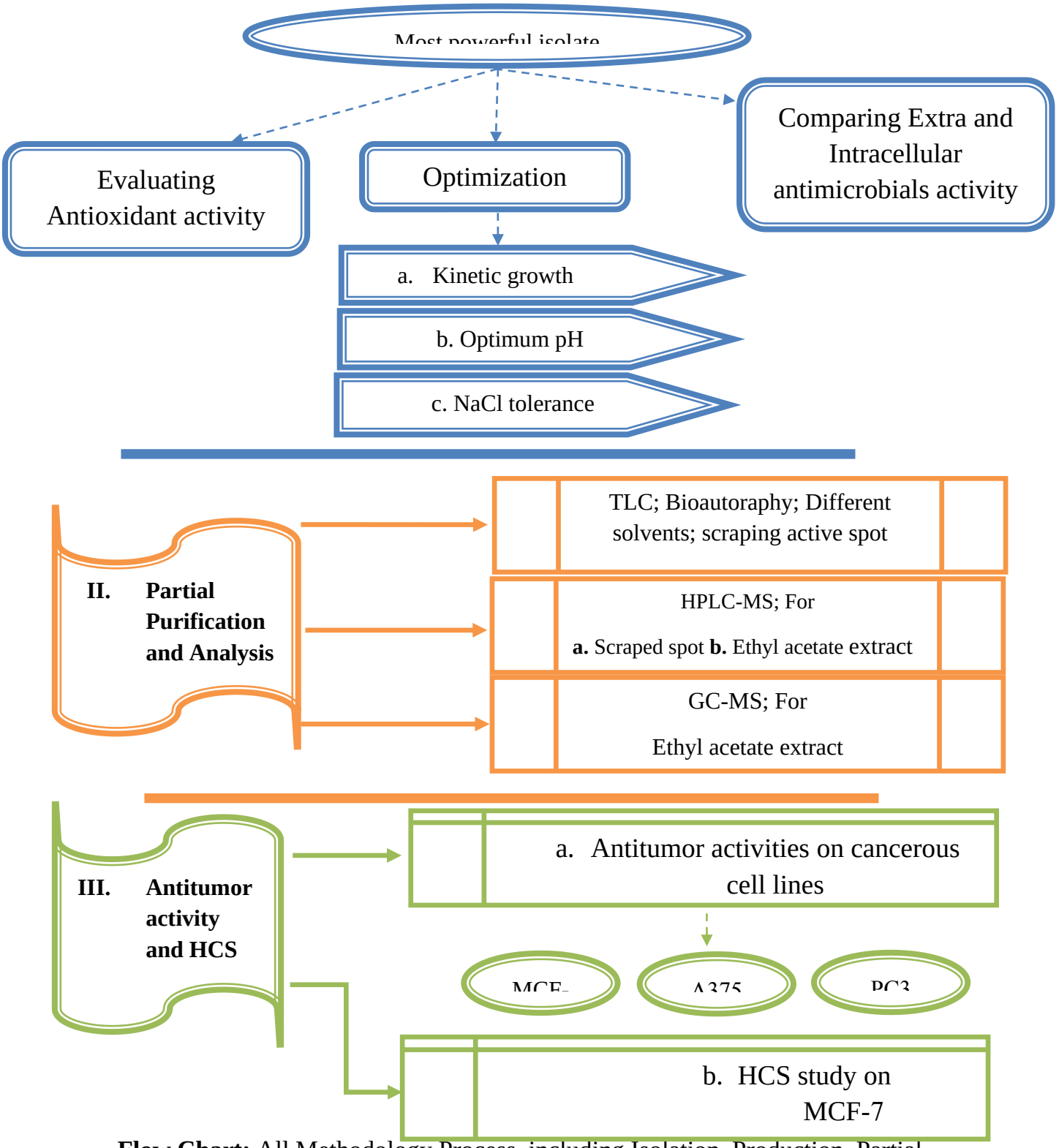


Figure 2.2: Map of the Garmian area that shows three main districts (Kalar, Kefri and Khanaqeen) with all locations of the study area (From Geographic Department, College of Education, Garmian University).

2.3 Methodologies

The current study was carried out in the laboratory of microbiology in Kalar Technical Institute, and Part of this work was carried out in the Research Lab. of the Biology Department in Faculty of Education, School of Scientific Education; University of Garmian. The overall procedure for the isolation, production, partial purification and studying both antitumorand cytotoxicity was summarized in the flow chart (1.1).


I. Isolation, Production and Extraction
250 gm soils; from forty different soil samples
Kalar (KA) 22 samples
Kefri (KE) 8 samples
Khanaqeen (KH) 10 samples
Primary Screening Protocol
Choosing the most powerful isolates
For studv
Physiochemical prosperities
Growth condition
Mycelium color
Diffusible pigments
Density; absorbance
Secondary Screening (Production, Optimizations and Extraction)
Best media composition
Best extracting solvents
Comparison with discs
Electing powerful one



Flow Chart: All Methodology Process, including Isolation, Production, Partial Purification, Antitumor Prosperities and High Content Screen

2.3.1 Samples Collection

All samples were collected randomly depending on farming and none farming soil from three main districts Kalar, Kefri and Khanaqeen summarized in Table (2.1, 2.2 and 2.3) during the period from 2nd Feb.to 15th Feb.2015. The total collected samples were 40 soil samples as shown in Figure (2.3).

Table 2.1: Site, Number and Soil conditions were collected for the isolation of locally actinomycetes in Kalar district.

No.	Site	Sample no.	Abbreviated	Soil condition
1	Kalar	1	KA 1	Farming
2	Kalar	2	KA 2	Farming
3	Kalar	3	KA 3	None farming
4	Kalar	4	KA 4	None farming
5	Kalar	9	KA 9	Farming
6	Kalar	10	KA 10	None farming
7	Kalar	17	KA 17	Farming
8	Kalar	18	KA 18	None farming
9	Kalar	19	KA 19	None farming
10	Kalar	21	KA 21	None farming
11	Kalar	22	KA 22	None farming
12	Kalar	23	KA 23	None farming
13	Kalar	24	KA 24	None farming
14	Kalar	25	KA 25	None farming
15	Kalar	26	KA 26	Farming
16	Kalar	27	KA 27	Farming
17	Kalar	35	KA 35	Farming
18	Kalar	36	KA 36	None farming
19	Kalar	37	KA 37	None farming
20	Kalar	38	KA 38	Farming
21	Kalar	39	KA 39	Farming
22	Kalar	40	KA 40	None farming

Table 2.2: Site, Number and Soil conditions were collected for the isolation of locally actinomycetes in Kefri district

No.	Site	Sample no.	Abbreviated	Soil condition
23	Kefri	20	KE 20	Farming
24	Kefri	28	KE 28	None farming
25	Kefri	29	KE 29	None farming
25	Kefri	30	KE 30	None farming
27	Kefri	31	KE 31	Farming
28	Kefri	32	KE 32	Farming
29	Kefri	33	KE 33	Farming
30	Kefri	34	KE 34	None farming

Table 2.3: Site, Number and Soil conditions were collected for the isolation of locally actinomycetes in Khanaqeen district

No.	Site	Sample no.	Abbreviated	Soil condition
31	Khanaqeen	5	KH 5	Farming
32	Khanaqeen	6	KH 6	Farming
33	Khanaqeen	7	KH 7	None farming
34	Khanaqeen	8	KH 8	Farming
35	Khanaqeen	11	KH 11	None farming
36	Khanaqeen	12	KH 12	Farming
37	Khanaqeen	13	KH 13	None farming
38	Khanaqeen	14	KH 14	None farming
39	Khanaqeen	15	KH 15	Farming
40	Khanaqeen	16	KH 16	None farming

Figure 2.3: Forty different soil samples were collected from (Kalar "KA", Khanaqeen "KH" and Kefri "KE") for isolating locally actinomycetes.

2.3.2 Treatment of the Collected Samples

Two hundred fifty grams of soil samples was collected from the regions mentioned previously at a depth ranged from 5 to 15 cm, and they were kept in polyethylene bags 20x 40 cm. The soil samples were exposed to the air for a week. Soil samples pretreated with CaCO3 at a ratio of 10:1 soil: CaCO3 and kept at ambient temperature for a week to enrich actinomycetes which usually prefer alkaline conditions and also to reduce the contamination with molds and yeast, as described by (El-Nakeeb and Lechevalier, 1963 and Abdulhameed, 2013).

2.3.3 Isolation and Identification of Actinomycetes from Soil

One gram of dried and treated soil samples was used to make suspension, by adding it to 99 ml of sterile distilled water (stock suspension), The samples were shacking in a shaker at 120 rpm for 30 minutes at room temperature. Serial dilutions from 10^1 to 10^3 were made from the stock suspension and left for 10 minutes. After shaking, 0.1 ml of each dilution was pipetted and put on supplemented Glycerol yeast extract agar (GYEA) with Cyclohexamide 50 ug/ml and Streptomycin 50 ug/ml, then spread by a sterile swab to make a uniform distribution of the suspension on the surface of the media. The inoculated plates were incubated at 28°C for 7 to 14 days. Based on cultural characteristics, suspected colonies of actinomycetes were selected for being characterized as small, white, and pin-point, rough, chalky and a clear zone of inhibition around them. The suspected colonies was subjected their identification by types of Gram's stain, aerial and substrate mycelium color, pigment production and pigment color.The colonies were transferred from the mixed culture into separate agar plates and incubated at 28±1°C for 7 days. In order to obtain a pure growth of actinomycetes species, the last steps were repeated several times. The pure culture was kept at 4°C until a further study, (Abussaud and Saadoun, 1991 and Oskay *et al.*, 2004).

2.3.4 Microbial Pathogens used for Antimicrobial Activities

The pathogenic microorganisms were used as reference strains for testing the antimicrobial activities are listed in the table below,

Reference strain	**Source**
Staphylococcus aureus ATCC 25923	Irbil medical center
Escherichia coli ATCC 25922	Sulaimani University- Biology Dept.
Candida albicans ATCC 10231	Irbil medical center

2.3.5 Primary Screening for Antimicrobial Activities of Suspected Actinomycetes

Initial screening (primary screening) for antimicrobial activities was done by the cross-streak method, in which the isolated actinomycetes were used against the three different microbial pathogens. The suspected actinomycetes were streaked as across lines in the center of plates poured with Malt extract yeast extract agar (ISP2) and inoculated plates were incubated at 28°C for 7 days. After the actinomycetes were completely cultivated, the tested bacterial pathogens were streaked perpendicular to the actinomycetes, and then plates were re-incubated at 37°C for 24 hours. The anatgonistics was observed by the naked eye in which the reference strains failed to grow near the actinimycetes line, (Oskay, 2009 and Kumar *et al.*, 2012).

2.3.6 Secondary Screening Processes (Fermentation)

The positive results were obtained from the primary screening protocol. Besides, it was ready to use the batch culture fermentation as well as agar well diffusion assay was used to determining the antimicrobial activities of the isolated actinomycetes. (Pallavi *et al.*, 2013).

2.3.6.1 Stock cultures Suspension Preparation

The general inoculums suspensions were prepared by the procedure described by Shirling and Göttlieb (1966). The full growth plates of isolated actinomycetes 7 days age at 28°C were used in making bacterial stock culture suspensions. One loopfull of spores with their mycelial growth was transferred to a conical flask containing 250 ml of sterilized ISP2 , then incubated at 28°C for 7days. The prepared stock solution was used as a stock culture to inoculate all protocols used, and was kept at 4°C until next uses.

2.3.6.2 Fermentation Condition

Secondary screening for the production of antimicrobial metabolites was done by the inoculation of 1.5ml of prepared stock suspension cultures into 150 ml of production media and incubated at 29±1°C, 150rpm for 7days in a shaking incubator. The process was carried out in the Biology department, Faculty of Education, Garmian University, (Khan and Patel, 2011 and Pallavi *et al.*, 2013).

2.3.6.2.1 The Best Media Composition for Antimicrobial Production

Different media were used for isolation and identification of actinomycetes spp. Furthermore, to achieve the best types of media (composition) for the production of antimicrobial metabolites, different media with different compositions were used as summarized in Table (2.4). After 7days of incubation, antimicrobial metabolites extractions were carried out, (Shirling and Göttlieb, 1966 and Khan and Patel, 2011).

Table 2.4: Different media with different compositions were used to achieve the best media composition for production of antimicrobial metabolites.

No.	Medial name	Abbreviation
1	Tryptone-yeast extract broth	ISP1
2	Yeast extract-malt extract broth	ISP2
3	Inorganic salts-starch broth	ISP4
4	Glycerol-asparagine broth	ISP5
5	Glycerol yeast extract broth	GYE

2.3.6.2.2 Extraction of Extracellular Antimicrobial Metabolites

Extracellular crude extracts were carried out from fermented broth by taking 5ml of the fermented broth from each active isolated actinomycete in the primary screening program, after 7days of incubation and transferring it into a test tube, then it was spun at 6000rpm for 5 minutes. Supernatants were filtered through Whatman No.1, and treated as extracellular crude extract. Agar well diffusion procedures were followed to determine the antimicrobial activity. Besides, the tested microbial pathogens were spread on Mueller Hinton agar 20ml media per plate. Via using a sterilized pasture pipette 6mm diameter, wells were dug on the seeded plates. The wells were loaded with 60 ±5 ul of separated supernatants (extracellular crude extracts), then the inoculated plates with the microbial pathogens and extracellular crude extract were incubated overnight at 37°C. After finishing the incubation period, the plates were observed for a zone of inhibition by the naked eyes. The zones of inhibition were determined by using a metric ruler, (Khan and Patel, 2011 and Pallavi *et al.*, 2013).

2.3.6.2.3 Solvent Extraction of Extracellular Antimicrobial Metabolites

The supernatant obtained from fermented broth, after filtering it via WhatmanNo.1 filter paper, was used to extract antimicrobial metabolites by using different solvents methanol, ethyl acetate, chloroform, n-butanol and n-hexane. In order to achieve the maximum ability of solvents to dissolve the antimicrobial metabolites and determine their antimicrobial activities by agar well diffusion protocol. 1ml of extracellular crude extract (supernatant) is to be added after filtering through Whatman No. 1 into a test tube and mixed with 1ml of the above mentioned solvents, gently shaked in a shaking plate for 60minutes, and then spun at 10000rpm for 10minutes. After centrifugation, the mixture was separated into two phases. The upper phase containing the dissolved

antimicrobial metabolite was collected by micropipette from test tubes into the sterilized petridish and the plates were kept in hot air oven at 45± 2°C.

After the solvents completely vaporized the remaining residues was re-suspended in 500 ul of the sterilized distal water. This mixture was treated as a solvent extracellular extract for detecting antimicrobial activity, via agar well diffusion procedure mentioned previously against the reference strains, (Gurung*et al.*, 2009 and Hemashenpagam, 2011).

2.3.6.2.4 Intracellular Extraction of Antimicrobial Metabolites

Intracellular extracts were achieved after the separation of the extracellular crude extracts by centrifugation. The residual in the tube contained a bacterial cells pellet with its intracellular antimicrobial metabolites. These components were used to determine the intracellular antimicrobial activity by agar well diffusion as follows: the pelleted cells were re-suspended in the test tube containing lysis bufer 1ml TE buffer "Tris 200ml and 50ml EDTA, 60 ul of 10% SDS and 6 ul of proteinase K, with a gentl shaking, the mixture were incubated at 37°C for 60 minutes. That caused bacterial cell walls disruption as well as intracellular metabolites liberation.

Six hundred µl of the intracellular crude metabolites was taken and mixed with 600 µl of methanol. The mixture was gently mixed and left for 60minutes. Then the tubes were spun at 1000rpm for 10 minutes at room temperature. The mixture was separated into two phases, the upper phase methanolic phase containing dissolved metabolites, was collected and transferred to the sterilized petridish, then kept in a hot air oven 45°C for 24 hrs to dry the dissolved intracellular crude extract.

Finally, the dried intracellular crude extracts were dissolved in double volume of sterilized distal water 1200ul. Agar well diffusion procedures as

mentioned previously were used to determine the antimicrobial activity against tested microbial pathogens, (Khan and Patel, 2011).

2.3.7 Comparisons for Antimicrobials Activities between Synthetic Antibiotic Discs with Extracellular Crude Extract

Antimicrobial susceptibility test was performed for all reference strains by the Kirby-Bauer's disk-diffusion method. Mueller-Hinton agar plates were used for the assay of 10 different synthetic antibiotics as described in Table (2.5). Then, the antimicrobial activities of extracellular crude extract metabolites were compared with synthetic antibiotic against the same strains based on the inhibition zone, as mentioned by (Eucast, 2000).

Table 2.5: Antibiotic sensitivity discs abbreviations; concentration and manufactured company were used as standard antimicrobial activities.

No.	Antibiotic name	Abbreviation	Concentration	Manufacture
1	Amoxicillin	AX	25 ug	Bioanalyse
2	Gentamicin	CN	10 ug	Bioanalyse
3	Cefotaxime	CTX	30 ug	Bioanalyse
4	Streptomycin	ST	10 ug	Mast diagnostics
5	Azithromycin	AZM	15ug	Mast diagnostics
6	Vancomycin	VA	10 ug	Mast diagnostics
7	Erythromycin	E	15 ug	Mast diagnostics
8	Tetracycline	TE	30 ug	Mast diagnostics
9	Methicillin	ME	10 ug	Mast diagnostics
10	Amoxicillin/clavulanic acid	AMC	30 ug	Mast diagnostics

2.3.8 Growth (Kinetic) Curve Study for Maximum Antimicrobial Production

The stationary phase in growth curve gives the maximum yields of secondary metabolites, in order to achieve growth patterns of locally isolated actinomycetes. Furthermore, to determine the accurate days for the isolate to reach the stationary phase, 50ml of ISP2 was prepared in conical flask and 30ml of the same media ISP2 in other conical flasks. The latter was treated as a negative control (blank).

Fifty ml prepared broth of ISP2 was inoculated with 1ml of stock culture suspension. Both broth media were incubated at 28°C with a soft shaking twice a day. Every 24 hours of incubation, the absorbance of both inoculated and none inoculated broths were read at 600nm.This process continued until reaching a maximum optical density (OD). So it was considered as a stationary phase, (Khan and Patel, 2011).

2.3.9 Determination the Optimum pH for Fermentation Process

Locally isolated actinomycetes were grown on ISP2 was supplemented with different pH 4, 5, 6, 7, 8, 9 and 10 in order to obtain optimum pH for the isolates. Screw cap tube 50ml occupied with 30ml of ISP2 in duplicates, one with 0.5ml stock culture suspension and other as a negative control (blank) without culture suspension, then incubated at 28°Cfor 4 days and the growth was checked by reading their absorbance at 600nm, (Khan and Patel, 2011).

2.3.10 KH14 Isolate Salt-Tolerance

Sodium chloride tolerance of locally isolated actinomycetes was evaluated by growing them in a ISP2 medium was supplemented with graded doses of sodium chloride 1, 2, 3, 4, 5, 6, 7, 8, 9 % w/v, with the negative control without inoculating it with the isolate actinomycetes. Finally, after 4 days of incubation

at 28°C, the results were observed by reading their absorbance at 600nm. The strain which survived in the highest (maximum) NaCl concentration was considered and remained viable, as the strain salt-tolerance, (Tresner *et al.*, 1968).

2.3.11Characterization of Isolated Actinomycetes

2.3.11.1Morpholoical Characterization of Actinomycetes

The actinomycetes isolates were inoculated on ISP2 broth and agar and incubated at 28±1°C for 7 days, then made a dry preparation from the colonies. Furthermore, the smear stained with a Gram's stain. Morphological characteristics of actinomycetes strains were observed such of Gram's stain patterns, aerial and substrate mycelium color, pigment production and pigment color, (Vimal *et al.*, 2009 and Reddy *et al.*, 2011).

2.3.11.2 Physical and Chemical Parameters Change during Fermentation

During the entire fermentation processes for the production of antimicrobial metabolites, several parameters have been changed through the reaction between the actinomycetes and medium consumed such as broth color, pH of the medium and the absorbance of the broth. After seven days of incubation at 28±1°C, they were compared with initial time of inoculation (blank).

2.3.12 Minimum Inhibitory Concentrations (MICs) Determination

The minimum inhibitory concentrations (MICs) of the locally isolate actinomycetes were determined by a serial dilution technique. MICs were defined as the lowest concentration of an antimicrobial that inhibits the noticeable growth of a microorganism after their incubation for overnight. To determine MIC, 1ml of nutrient broth was taken into15 test tubes numbered from 1 to 15, tube no. 14 as a negative control only broth plus extract and tube no. 15 as a positive control broth plus microbial pathogens. Then antibacterial

compound extracellular crude extract was added into the first test tube and made a serial dilution to obtain a final concentration 1, 2, 4, 8, 16, 32, 64, 125, 250, 500, 600, 700, 800 ul /ml, 50µl of the tested microbial pathogens was added into each test tube except negative control and incubated at 37°Cfor overnight. After incubation, the last tube which does not show the growth by the naked eye and this was consided as the MIC values, (Reiner, 1982 and Andrews, 2001).

2.3.13 Bacteriostatic and Bactericidal Properties of Extracellular Crude Extract

Bactericidal and bacteriostaticare define as "one that exclusively kills bacteria and another that only inhibits growth", and the *in vitro* antimicrobial determination of whether an antibacterial agent is bactericidal or bacteriostatic may be influenced by growth conditions, test duration, bacterial density and extent of the reduction in bacterial numbers. To determine if bacteria are only in stasis or dead, a sample from the area of the zone inhibition around the well was taken by the means of the sterile swab then inoculated into nutrient agar. If bacteria grow in the nutrient agar, the action of the extracellular crude extract was probably to be bacteriostatic, while it would be bactericidal if no growth seen, after 24 hours of incubation at 37 °C, (Pankey and Sabath, 2004).

2.3.14 Antioxidant activity of KH14 Extracellular Crude Extract

2.3.14.1 Preparation of Standard Solution

The standard solution was prepared by using 50 mg of ascorbic acid and dissolving it in 50ml 100% of ethanol. From the stock solutions, different concentrations of ascorbic acid ranging from 1, 0.5, 0.25, 0.125, 0.064 and 0.032 mg/ml were prepared, mixed with 2, 2- diphenyl 1-picralhydrazyl (DPPH) solution 0.002mg/100ml, and then kept for 30 minutes at room temperature in a

dark container. Absorbance was read after 30minutes of reaction by spectrophotometer at 517nm, to obtain a standard curve for calculating DPPH radical scavenging activity and calculating IC_{50} (the dose was required to cause a 50% inhibition of free radical formed). All tested concentrations were analyzed in duplicates, (Khalaf *et al.*, 2008).

2.3.14.2 Preparation of Test Solution and Protocol Assay

The antioxidant properties DPPH radical scavenging assay was carried out to locally isolated actinomycetes. A 0.002gm DPPH dissolved in 100ml ethanol and kept in a dark container. An extracellular crude extract concentration was mixed with ethanol to obtain the final dilution concentration as 0.1, 0.5, 1.0, 3.0, 5.0, 10.0, 20.0 and 30.0 mg/ml, exactly 2.5ml of DPPH solution and 0.5ml of crude extract was mixed at various concentrations. The mixture was shaken by hands for two minutes, and then kept at room temperature in a dark place for 30 minutes.

De-colorization of DPPH was determined by measuring the absorbance at 517 nm using a visible spectrophotometer. Absorbance was read at 517nm after 30min of reaction. The antioxidant activity inhibition of DPPH activity, or free radical scavenging activity was calculated, and was expressed as IC_{50} (mg/ml), the dose required to cause a 50% inhibition. In addition, ascorbic acid was used as a standard for making a standard curve, and for calculating the IC_{50} powerful. The ability to scavenging the DPPH radical was calculated as follows:

$$\textit{DPPH radical scavenging activity\%} = (A_0 - A1) / A_0 * 100$$

Where A_0 is the absorbance of the control at 30 minutes, and A1 is the absorbance of the sample at 30 minutes. All samples were analyzed in duplicates, (Khalaf *et al.*, 2008 and Sunil *et al.*, 2012).

2.3.15 Purification of Extracellular Crude Extract

2.3.15.1 Partial Purification by Thin Layer Chromatography (TLC)

Partial purification of extracellular crude extract was carried out by a TLC .The extracellular crude extract was dissolved in ethyl acetate and spotted by means of capillary tubes on TLC plates (TLC 20x20 cm, silica gel 60F $_{254}$, Merk co, USA) using different mobile phase solvents such as Ethyl acetate 100%, methanol 100%, chloroform100%, water 100%, n-hexane 100%, toluene 100%, acetone 100%, ethanol 100%, n-butanol 100%, acetic acid 100%, ethyl acetate: methanol: water (10:1.5:1), toluene: ethyl acetate (93:7), methanol : water (6:4), Ethyl acetate : Chloroform : Water (95:5:5), Methanol : Chloroform (6:4), Ethyl acetate: Toluene: Methanol :Chloroform: Water (1:1:1:1:1), in order to determine the best solvent system for separates the bioactive compounds, as well as determining the retardation factor R_f values of the bioactive compounds, and their ability for dissolving the extracellular crude extract.

After running the spots, the plates were dried and chromatograms TLC plates were observed visually by eyes under UV light, to detect the spots position. The retardation factor R_f, is defined as "the distance traveled by the compound divided by the distance traveled by the solvent" and as follows, (Attimarad *et al.*, 2012)

R_f= distance traveled by the compound/ distance traveled by solvent

2.3.15.2 Detecting the Antimicrobial Metabolites Position by Bioautography

Bioautography assay follows the thin layer chromatography to determine the active antimicrobial metabolites position, after running the TLC plates. TLC strips were placed on the surface of a Mueller Hinton agar plate seeded with the microbial pathogens, then, incubated at 37°C for 24 hrs. After incubation, the

inhibition zones around the active spots of antimicrobial metabolites were observed by the naked eyes. The clear zone on the media indicated the presence of active antimicrobial compounds which inhibited the growth of the tested microbial pathogens, (Hozzein *et al.*, 2011).

The active spot was visualized by ultraviolet light, then scraped from the TLC plates by a sterilized scrapple and collected in a clean disposable test tube, and used for studying and analyzing it by the High Performance Liquid Chromatography (HPLC).

2.3.15.3 High-Performance Liquid Chromatography – Mass Chromatogram (HPLC-MS) Analysis and Partial Purification of the KH14 Extract

Both the ethyl acetate extract and scraped active spot from the TLC plates were analyzed by a high-performance liquid chromatography (HPLC) for purity, molecular weight detection, and UV absorbance of the content. The solution for HPLC was prepared by using 100 mg of ethyl acetate and active spots separately were dissolved in 1ml of methanol and shacked well, then filtered by passing them through a millipore micro filter (0.2 µm pores) by using a disposable syringe.

The separation was carried out with a HPLC-MS (Waters 2545-USA) quaternary gradient module equipped with a system fluidics organizer (waters-SFO) coupled with SQ detector and operated in a positive ionization mode at range from m/z = 200-900 with 2 scan/min, combined with photodiodes array detector (Waters 2998) with sampling rate 2 points/sec (Lambda range 190-800 nm). A C18/4.5 x 155 mm RP column (X Bridge) was used for separation with a solvent system consisting of A: deionized water, B: methanol, each contain 0.1% formic acid. The following gradient was applied: 0-2 minutes 30% A, and 70%

B, 2-10 minutes A linear from 30 to 95%, B linear from 70% to 5%. Ten microliter of the extract was injected using an auto-sampler (Waters 2767), and run with a flow rate of 1 ml/min using an auto-sampler (Waters 2767). The process was carried out in Koya University, College of Health and Sciences, Advance Research Center, (Boudjelal *et al.,* 2011).

2.3.15.4 Gas Chromatography –Mass Spectrometry (GC-MS) Analysis of KH14Ehtyl Acetate Extract

Ethyl acetate extract of KH14 was analyzed by GC-MS for detecting the volatile compounds presented in the ethyl acetate extract, as well as, their quantity and quality ratios. The sample was reconstituted using 1ml Ethyl acetate, and then one hundred microliters of the extract was transferred into auto sampler glass vials. GC-MS analysis was performed on GC-MS QP-2010 (Shimadzu Instruments, Japan); an inert cap pure wax capillary column was used 30m x 0.25mm x 0.25µm film thickness with Helium as the carrier gas at a flow rate of 1.51 ml/minutes.

The source was operated in positive ionization mode electron impact energy: 70eV and the detection were performed in a full-scan mode scan range: 35.00 – 700.00 m/z for a total run time of 27 minutes. The inlet and the transfer line temperatures were maintained at 240°C, while the ion source was kept at 200°C. Samples were injected in a split-less mode and separated by using a temperature gradient program as follows: 100°Cfor 3minutes, to 150°C at 10°C/min and then maintained at 150°C for 2minutes; then to 240°C at 10°C/min and maintained at 240°Cfor further 8 minutes. GC-MS spectra were evaluated by posting the software and searched in the National Institute of Standards and Technology (NIST), MS Search V2.0 browsers. The Sample was

analyzed at Al-Mustansiriyah University, College of Science; Department of Chemistry, (Ara *et al.*, 2012 and Rajesh *et al.*, 2013).

2.3.16 Antitumor and High Content Screening Properties of KH14 Extracellular Crude Extract

2.3.16.1 Cancer Cell Lines

The cell lines used in the study for screening the antitumor activity of KH14 extracellular crude extract were Breast cancer cell (MCF-7), (Human Prostate cancer cell (PC3) and Human malignant melanoma, skin cancer (A375). These cell lines were supplied by Pharmacology Department/Medicine College/Malayia University.

2.3.16.2 *In vitro* Evaluating Antitumor Activity of KH14 Extracellular Crude Extract

Antitumor activities were determined by MTT assay for KH14 extracellular crude extract. The process was carried out at the Pharmacology Department/Medicine College/Malayia University, against different cell lines used such as Breast cancer cells (MCF-7), Human Prostate cancer cells (PC3) and Human malignant melanoma, skin cancer (A375). Cell lines were seeded in 96-well tissue culture plate's 5000 cells/well. Besides, from lyophilized extracellular crude extract stock solutions were prepared in sterile distilled water and diluted to the required concentrations by serial dilutions 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.562, 0.781, 0.390, and finally 0.195 ug/mL. They were done via the cell culture medium Eagles Minimum Essential Medium (EMEM) containing 10% fetal bovine serum (FBS). Then, the EMEM were mixed with cancerous cell lines treated as a control, however, all treated and none treated sample were done in triplicate trials.

The cells were incubated at 37°C with 5% CO2 and 95% air in 100% relative humidity in Thermo Forma Series II Water Jacketed CO_2 incubator,

After 72 hrs of incubation; the solution in the medium was removed. An aliquot amount of 100 µl of medium containing 1 mg/ml of 3-(4, 5-dimethylthiazol- 2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) was loaded to the plates. The cells were cultured for 4 hrs in a dark chamber for the conversion of MTT to formazan, and then the solution in the medium was removed. The well was loaded with an aliquot amount of 100 µl of dimethyl sulphamide (DMSO) being added to the plates, and then shaken was done until the formed crystals were dissolved.

The cytotoxicity against cancer cells line was determined by measuring the absorbance of the converted dye at 570 nm in a (Hidex Chameleon plate reader). Cytotoxicity of each concentration was expressed as % inhibition values, and the equation used for calculating the inhibition rate of the cells on the basics that the MTT assay was reduced. Their absorbance in metabolically active cells to yield an insoluble purple formazan product was done as an end product, by calculating the optical density between control well and the treated well with an extracellular crude extract and as follow. (Mosmann, 1983 and Saravana Kumar *et al.*, 2014)

Cell viability % = (mean OD control x mean OD treated)/ (mean OD control)*100%

2.3.17 High Content Screening Multi-parametric analysis (HCS) of KH14 Extracellular Crude Extracts on MCF-7 Cancer Cell Line

A High content screening (HCS) was carried out for KH14 extracellular crude extract on MCF-7 by using cellomics multi-parameter cytotoxicity 3 kit protocol. The procedure was carried out at the Pharmacology Department/Medicine College/Malayia University. The procedure was carried as follows;, according to the procedure described by (Abraham *et al.*, 2008).

2.3.17.1 Cell Line Preparation

Eagles Minimum Essential Medium (EMEM) medium used with the following supplements 10% fetal bovine serum, 1 mM sodium pyruvate, 1X non-essential amino acids, 100 units/ml of penicillin and 100 µg/ml of streptomycin. When the MCF7 cells were split and reached 90% confluence at a dilution of1:4, as well as, the MCF-7 cells were harvested by trypsinization, then they were diluted into EMEM complete medium and cell density was determined. Cells were diluted to 7.5×10^4 cells/ml in EMEM complete medium.

After completing the dilution, the cell suspension 100 µl was added to each well of a 96-well micro plate to achieve 7,500cells/well as recommended by the manufacturing instructions (recommended plating density). Finally, the prepared cells were incubated overnight at 37°C in 5% CO_2.

2.3.17.2 High Content Screening Procedure Assay

Twenty five µl of different concentrations of KH14 extracellular extracts 25, 50,100 and 200 µg/ml was added to the prepared cell lines manifested as treated samples, and 25 µl of 5.0 µM paclitaxel was used as a positive control, while unloading wells were considered as a negative control (vehicle). The cells were incubated at 37°C for 24 hours, then 50µl of live cell staining solution was added to each well, and the staining cells was incubated at 37°C for 30 minutes. The medium with the staining solution were gently aspirated and 100 µl/well of fixation solution was added, plate was incubated for 20 minutes at room temperature.

The fixing solution was gently aspirated and 100 µl/well of 1X wash buffer was added. Wash buffer was removed and 100 µl/well of 1X permeabilization buffer was added. The plate was incubated for 10 minutes at

room temperature and protected from light. Permeabilization buffer was aspirated and plate was washed twice with 100 µl/well of 1X wash buffer. Wash buffer was aspirated and 100 µl of 1X blocking buffer was added and the plate was incubated for 15 minutes at room temperature.

Blocking buffer was aspirated and 50 µl/well of primary antibody solution was added. Plate was incubated for 60 minutes and protected from light at room temperature. The primary antibody solution was aspirated and the plates were washed three times with 100 µl/well of 1X wash buffer. Wash buffer was aspirated and 50 µl/well of the secondary antibody/staining solution was added. Plate was protected from light and incubated for 60 minutes at room temperature.

Secondary antibody staining solution were aspirated and the plate was washed three times with 100 µl/well of 1X wash buffer, and 100 µl/well of 1X wash buffer was added, finally the plate was sealed and evaluated on the array scan HCS reader.

2.3.17.3 Microscope Information

Cells prepared and labeled according to the kit protocol were used and analyzed by fluorescence microscopes using the appropriate filter set(s) or confocal microscopy. Optimization was required when using slides, cover slips or multi-well chamber slides, and the image-processing used software to quantify the targets. The approximate absorption/emission maxima of the fluorescent dyes are as follows: DyLight 649 Conjugates = 646/674 nm Hoechst Dye = 350/461 nm.

2.3.17.4 Statistical Analysis

Statistical analysis and significances of high content screening (HCS) were determined using one-way analysis of variance (ANOVA) followed by a post hoc testing using Dunnett's test. Duplicated data for each concentration of extracellular crude extract KH14 were compared with the vehicle controls (Untreated sample), for all comparisons. The results were considered as significant if $p \leq 0.05$, (Breier *et al.,* 2008).

Chapter Three: Results and Discussion

3.1 Isolation of Actinomycetes from Soils

The serial dilution technique was used to isolate actinomycetes from forty different soil sources after inoculating the plates with soil suspension on glycerol yeast extract agar and incubating the inoculated plates for 7- 14 days with a dilution ranged from 10^1 to 10^3. The data presented in Table (3.1) summarize all suspected actinomycetes obtained from the above soil sources on the basis of forming pinpoint colonies with inhibitory or clear zone of inhibition around them: small white, rough and chalky as recommended by Oskay *et al.*, (2004).

Out of forty soil sources, only 26 soil sources 65% were suspected to contain actinomycetes with a total of 55 colonies with different morphotypes from all soil sources were obtained. The color of selected suspect colonies ranged from white, creamy to gray. They were grown on a glycerol yeast extract agar, and this was in agreement with that described by Saadoun *et al.* (2015).

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. Distinguishing between the repeated colonies wasnot carried out because of high difficultly to separate anyone of them among all the soil sources and dilution, i.e., some of these 55 suspected colonies may be repeated within the different soil sources.

Stackebrandt *et al.* (1991) and Ramazani *et al.* (2013) described actinomycetes colonies being slow growing, glabrous or chalky, aerobic, piled, as well as with different color of aerial and substrate mycelium. In addition, all isolated colonies possess an earthy odor.

Fifty-five colonies were obtained from the isolation processes, sub-cultured on ISP2, in order to obtain pure colonies culture of locally isolated strains and the process of sub-culturing repeated more than one time. Morphologically, on the basis of aerial mycelium colorthere are many similarities among a large number of them, especially when purified on plates. However, all 55 purified colonies were kept at 4°C in a refrigerator for further study primary screening programs.

The results was in agreement with the finding of both Zhou *et al.* (2007) and Portillo *et al.* (2009) concerning the isolation process that each plate was often contained one or few colony types ranging from two to four colonies, and from similar habitats the actinomycetes diversity exhibited few different colony types.

Kariminik and Baniasadi (2010), mentioned that because of their stringent aerobic metabolisms, actinomycetes ,especially *Streptomyces,* exist in different types of soil and they are abundant on the surface layer of soils as well as they favor the alkaline soils, compost, river's mud and riverbeds. Nonoh *et al.* (2010) described the physical properties, organic matter content, pH, moisture, soil reactions and soil texture which were considered as the main factors that the concentration (distribution) of *Streptomyces* depended on.

Table 3.1: Total suspected actinomycetes colonies appear on glycerol yeast extract agar

Soil sources	Dilution (no. of colonies)			Total suspected colonies per soil source
	10^1	10^2	10^3	
KA 3	1	1	2	4
KA 4	-	1	-	1
KH 5	-	1	1	2
KH 6	2	2	3	7
KH 7	-	1	-	1
KH 13	-	-	1	1
KH 14	1	1	-	2
KH 16	-	1	-	1
KA 18	1	1	-	2
KA 19	1	1	1	3
KE 20	1	-	-	1
KA 21	-	1	-	1
KA 22	1	-	-	1
KA 23	1	1	-	2
KA 24	-	1	-	1
KA 25	-	1	-	1
KA 27	-	1	-	1
KE 29	1	2	-	3
KE 30	-	1	1	2
KE 32	1	2	1	4
KE 33	1	1	1	3
KE 34	1	1	2	4
KA 36	1	1	-	2
KA 37	-	1	1	2
KA 38	1	-	-	1
KA 39	1	1	-	2
Total				**55 colonies**

3.2 Primary Screening for Antagonistic Activity

A total of fifty five locally isolated actinomycetes that obtained from forty soil sources were tested for the antimicrobial activities against human microbial pathogens (bacteria and yeasts) by the cross streak technique, such as *Staphylococcus aureus* ATCC 25923 was for gram positive bacteria model, *Escherichia coli* ATCC 25922 for gram-negative bacteria model and Candida *albicans* ATCC 10231 as a model of yeasts.

The results presented in Figure (3.1) summarize the process of primary screening program against human microbial pathogens. Furthermore, the activity of each isolated actinomycetes was done against each microbial pathogen and the strains which were selected for completing the secondary antimicrobial screening (fermentation) were highlighted in Table (3.2). The data presented in Figure (3.1) were applied to conclude the antimicrobial activity. To achieve this potential, all isolates were streaked as a straight line on nutrient agar media separately, and incubated at 28 °C for 7 days. After completing their growth as shown in plate A Figure (3.1), different strains of microbial pathogens were streaked at right angle, without touching each other, in addition to being incubated at 37 °C for 24 hours.

Rana and Salam (2014) summarize the primary process, if the microbial pathogens were susceptible to the antimicrobial compound produced by the isolated actinomycetes; it would not allow their growth near the actinomycetes as presented in Plate B Figure (3.1). The process was done for all 55 locally isolated actinomycetes, instead, most of the isolates had a power against the tested pathogens, but our designed protocol concentrated on choosing the most active and broad spectrum activity. For this , our results presented in Table (3.2) by (+ve and -ve) indicated capability or incapability to prevent the growth of the tested microbial pathogens respectively, so that most of the isolates were

ignored because of their incapability to prevents the growth of microbial pathogens. Finally, the highlighted isolates achieved a high activity to produce a large amount of antimicrobial compounds as secondary metabolites which prevent the growth of tested microbial pathogens.

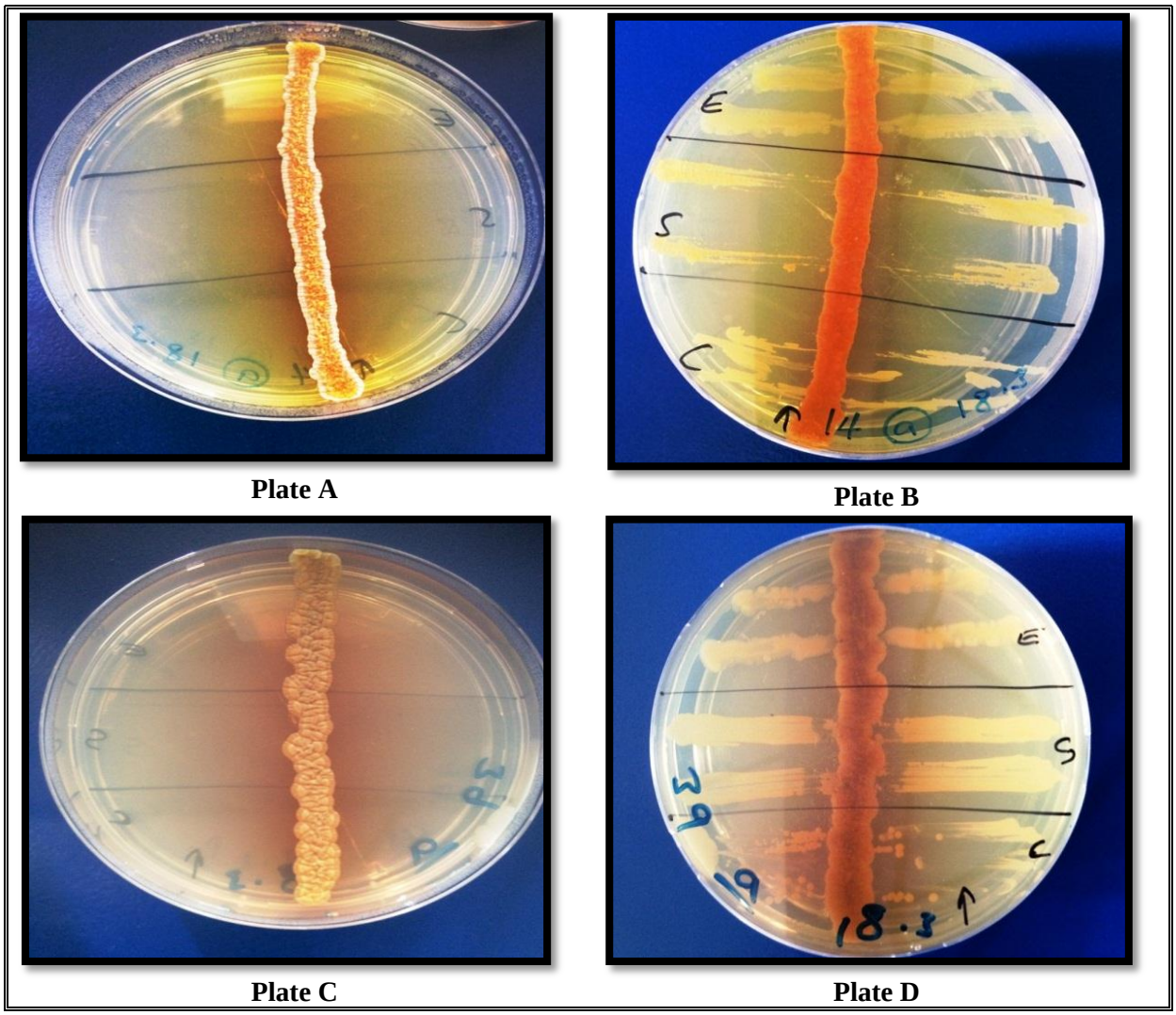


Figure 3.1: Primary screening program for locally isolated actinomcetes, plate A and B for isolate KH14, while Plate C and D for isolate KA39.

Table 3.2: Primary screening outcome obtained from locally isolated actinomycetes from soils.

Isolate No.	Tested pathogens			Notes
	***Staphylococcus aureus* ATCC 25923**	***Escherichia coli* ATCC25922**	***Candida albicans* ATCC 10231**	
KA 3 A,B,C,D	-ve	-ve	-ve	Ignored
KA 4	-ve	-ve	-ve	Ignored
KH 5 A,B	-ve	-ve	-ve	Ignored
KH 6A,B,C,D,E,F,G	All –ve	-ve	-ve	Ignored
KH 7	-ve	-ve	-ve	Ignored
KH 13	-ve	-ve	-ve	Ignored
KH 14 A,B	A +ve	A +ve	both -ve	KH 14A selected
KH 16	both +ve	both +ve	both -ve	KH 16 selected
KA 18 A,B	+ve	-ve	+ve	KH 18 A Selected
KA 19 A,B,C	+ve	-ve	+ve	KA 19 B selected
KE 20	-ve	-ve	-ve	Ignored
KA 21	-ve	-ve	-ve	Ignored
KA 22	-ve	-ve	-ve	Ignored
KA 23 A,B	+ve	-ve	-ve	Ignored
KA 24	-ve	-ve	-ve	Ignored
KA 25	-ve	-ve	-ve	Ignored
KA 27	-ve	-ve	-ve	Ignored
KE 29 A,B,C	-ve	-ve	-ve	all Ignored
KE 30 A,B	+ve	-ve	-ve	Ignored
KE 32 A,B,C,D	+ve	-ve	-ve	All Ignored
KE 33 A,B,C	-ve	-ve	-ve	All Ignored
KE 34 A,B,C,D	-ve	-ve	-ve	All Ignored
KA 36 A,B	-ve	-ve	-ve	Ignored
KA 37 A,B	-ve	-ve	-ve	Ignored
KA 38	-ve	-ve	+ve	Selected
KA 39 A,B	A +ve	A +ve	-ve	KA 39 A selected

The data presented in (Table 3.3) summarize the details of primary screening results of the locally isolated actinomycetes activities against the tested microbial pathogens, which were selected to complete the secondary screening program (fermentation process). Additionally, Table (3.3) also represented the

rename of the isolated strains. The studies of Suthindhiran and Kannabiran (2009) and Ravi *et al.* (2015) investigated that screening for microorganisms belonging to the Actinomycetaceae family, revealed from the soil sources, can serve as an important source for obtaining novel antimicrobial compounds and for finding new chemicals used for achieving new therapeutic agents.

The differences in results between primary and secondary screening among suspected actinomycetes colonies may be due to appearances of actinomycetes as fragmenting mycelia and filamentous mycelia when growing in liquid media and solid media respectively, in addition to showing the difference in the morphology of actinomycetes as described by Bushell (1993). Moreover, Porter (1971) ; Maruyama et *al.* (1975); Bushell (1988); Zgurskaya and Nikaido (2000); Dehnad *et al.* (2010) and Ramazani *et al.* (2013) also investigated that some actinomycetes species were producing antimicrobial metabolites on agar media (plating) and primary screening but the field in liquid media and secondary screening meant that flask shaking methods obtained the fewest amount of antimicrobial because of the significantly breakage of the hyphal component of certain isolated actinomycetes occurring in case of liquid culture rather than on agar media in plates.

The selected isolates were chosen to complete the fermentation process grown on ISP2 as shown in Figure (3.2) in a pure culture form. Furthermore, the morphological characters such Growth state of (mycelium), aerial mycelium color, substrate (reverse) color, soluble pigment and Gram's reaction were determined after growing them for 7-14 days at 28±1°C. This is summarized in Table (3.4).

Table 3.3: Renaming the selected isolates with detailed results against tested pathogens

Isolate No.	Tested pathogen			Notes	Renaming isolated strains
	***Staphylococcus aureus*ATCC 25923**	***Escherichia coli* ATCC25922**	***Candida albicans* ATCC 10231**		
KH 14 A,B	A +ve B –ve	A +ve B –ve	both –ve	KH 14A selected	**KH 14**
KH 16 A,B	both +ve	both +ve	both –ve	KH 16 A selected	**KH 16**
KA 18 A,B	both +ve	both -Ve	A +ve B –ve	KH 18 A Selected	**KH 18**
KA 19 A,B,C	all +ve	B +Ve A and C –ve	B +ve A and C –ve	KA 19 B selected	**KA 19**
KA 38	+ve	–ve	+ve	Selected	**KA 38**
KA 39 A,B	A +ve B –ve	both –ve	A +ve B –ve	KA 39 A selected	**KA 39**

The results presented in Table (3.4) summarize the morphological observation of all six isolated actinomycetes growing on ISP2, for 14 days at 28±1°C. They are in agreement with those obtained by Ravel et *al.* (2000) and Hemashenpagam (2011) who considered that all isolated actinomycetes found to bce Gram positive bacteria and similar to fungal characters in possessing branched mycelium in their cell morphology. Moreover, the results agree with those described by Rana and Salam (2014) for isolating actinomycetes when incubated for 10 days and the colonies were detected with white ,orange, grey, yellow, cream, pale brown, pink and red surfaces with firm and difficult to scrap textures which are considered as the main characteristics of actinomycetes; some of them produced salty and brownish pigmentation, and various appearance such greyish-white, yellowish, milky and brownish on the aerial mycelia and with pale yellow, white and brownish pigmentation on the reverse of the colonies.

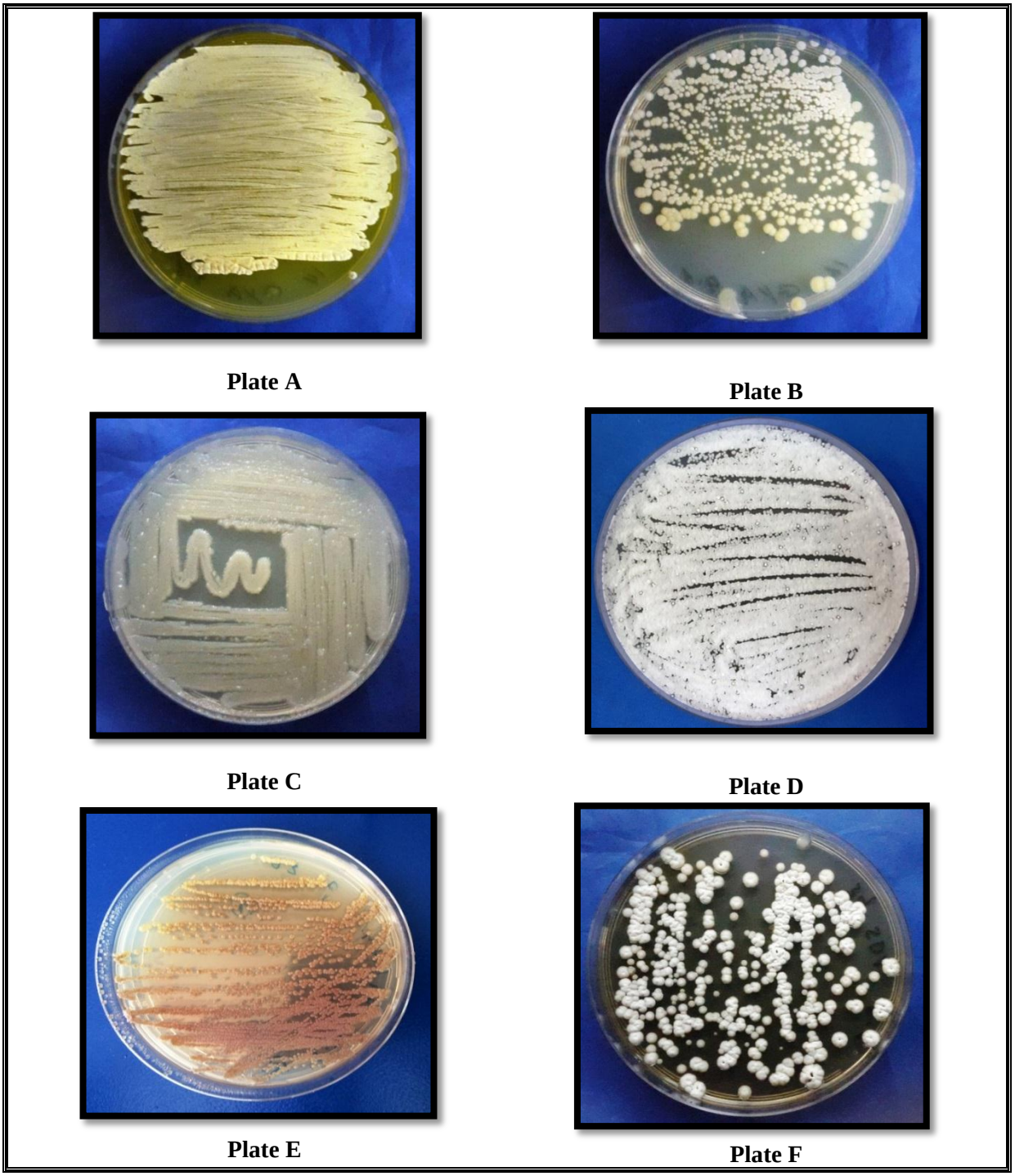


Figure 3.2: Pure isolated culture of locally isolated actinomycetes growing on ISP2, after 7-14 days at 28±1°C, **Plate A** showing the isolate KH14, **plate B** isolate KH16, plate **C** isolate KH18, **Plate D** isolate KA19 ,**plate E** isolate KA38 and **plate F** isolate KA39.

Table3.4: Morphological and cultural characteristics of locally isolated actinomycetres, growing on ISP2, after 7-14 days at 28±1°C.

Isolate no.	Growth state (mycelium)	Aerial mycelium color	Substrate (reverse) color	Soluble pigments	
				Presence	Color
KH 14	Very good	White	Yellow	+	Orange
KH 16	Moderate	Yellow	Creamy	-	-
KH 18	Good	Creamy	Creamy	-	
KA 19	Good	White	Yellow	-	-
KA 38	Slightly	White	brown	+	Brown
KA 39	Moderate	White	Gray	+	Brown

(+) = presence; (-) = absence

3.3 Secondary Screening Methods

Locally isolated actinomycetes successfully prevented or inhibited the growth of tested microbial pathogens by primary screening programs KH14, KH16, KH18, KA19, KA38 and KA39 as presented in Table (3.3). They were selected to perform the secondary screening programs in order to evaluate several parameters best media composition for obtaining maximum antimicrobial compounds, best organic solvents for extracting secondary metabolites , extraction of intra and extracellular antimicrobial compounds, comparison between synthetic antibiotic discs with extracellular crude extract and finally physical and chemical changes throughout the entire fermentation process.

3.3.1 Best Medium Composition to Achieve Maximum Antimicrobial Activities (Extracellular crude alone)

The data presented in Table (3.5; 3.6; 3.7; 3.8 and 3.9) represented the results for ISP1, ISP2, ISP4, ISP5 and Glycerol yeast extract broth, respectively.

The details procedures were presented in Figure (3.3) for some of the locally isolated actinomycetes activities, as well as, for some of medium cultures and extraction of antimicrobials extraction with organic solvents. According to Khan and Tripathi (2011) and Song *et al.* (2012), the secondary bioactive metabolites of actinomycetes were influenced by many factors for their growth and production. Such parameters include temperature, fermentation time and initial pH that effect the production of bioactive secondary metabolites. The medial composition also influenced the metabolites production and growth rate.

The obtained results recorded the best zone of inhibition on gram positive bacteria *Staphylococcus aureus* from that of Abdulhameed (2013) who obtained the highest inhibition zone about 14 mm against *Staphylococcus aureus* and also than Gurung *et al.* (2009) 20mm; Boudjelal *et al.* (2011) 25mm inhibition; Hozzein *et al.* (2011) 27mm; Attimarad *et al.* (2012) 15mm; Naine *et al.* (2015) 24 mm and Abdul Wahab et *al.* (2015) 24mm. These results were compared with our obtained results especially those obtained by KH14 and KA19 about 30 and 33mm inhibition respectively when growing on ISP2 as presented in (Table 3.6).

Table 3.5: Antimicrobial activities of locally isolated actinomycetes (extracellular crude alone), fermented on ISP1

Isolate No.	Zone of inhibition (mm)		
	***Staphylococcus aureus*ATCC25923**	***Escherichia coli* ATCC25922**	***Candida albicans* ATCC 10231**
KH 14	23	0	0
KH 16	0	0	25
KH 18	0	9	0
KA 19	17	0	15
KA 38	0	0	0
KA 39	0	0	13

0= no inhibition

Table3.6: Antimicrobial activities of locally isolated actinomycetes (extracellular crude alone), fermented on ISP2.

Isolate No.	Zone of inhibition (mm)		
	***Staphylococcus aureus*ATCC25923**	***Escherichia coli* ATCC25922**	***Candida albicans* ATCC 10231**
KH 14	30	13	12
KH 16	16	13	11
KH 18	13	0	0
KA 19	33	0	21
KA 38	0	0	23
KA 39	16	0	24

0= no inhibition

Among all used media for the production of antimicrobial metabolites, the ISP2 performed high activities by KH14 and KH16 against *Escherichia coli* recording only 13mm for both isolate as presented in Table (3.6). The rest of other used media moderately showed the activities of all isolated actinomycetes against gram negative bacteria *Escherichia coli* as presented in Table (3.5; 3.7; 3.8 and 3.9) especially the glycerol yeast extract broth which didn't show any activity for all isolated actinomycetes against *Escherichia coli* as presented in Table (3.9).

Comparing the obtained results from all the used media types for all isolated actinomycetes against gram negative bacteria, *Escherichia coli* were less than that obtained by Gurung *et al.* (2009) who obtained 17mm zone of inhibition Hozzein *et al.* (2011), from isolate D 332, obtained 25mm inhibition and Attimarad *et al.* (2012) from isolate ACT-A2 , obtained the inhibition zone 15mm, Abdul Wahab *et al.* (2015), recorded 23mm against gram negative bacteria *Escherichia coli* from isolate GZO23.

Chawawisit *et al. (*2015) described that most of the bioactive compounds extracted from actinomycetes were performed against Gram-positive bacteria with a strong activity, while against Gram-negative bacteria and yeast with a moderate activities. In another study, Ramazani *et al.* (2013) stated that Gram positive bacteria are naturally less resistant to antimicrobials compounds than Gram negative bacteria and this has been attributed to the collective exclusion of antimicrobial compounds by a barrier of double membranes and trans-membrane efflux present in this group of bacteria (gram negative). Moreover, Ilic *et al.* (2007) and Ramazani *et al.* (2013) explained that the results of primary and secondary screening programs showed the most active isolates exhibiting their activities against gram positive bacteria rather than gram negative bacteria.

Table 3.7: Antimicrobial activities of locally isolated actinomycetes (extracellular crude alone), fermented on ISP4.

Isolate No.	Zone of inhibition (mm)		
	***Staphylococcus aureus*ATCC25923**	***Escherichia coli* ATCC25922**	***Candida albicans* ATCC 10231**
KH 14	23	0	0
KH 16	0	0	0
KH 18	0	13	9
KA 19	0	0	0
KA 38	0	0	13
KA 39	18	12	13

0= no inhibition

Table 3.8: Antimicrobial activity of locally isolated actinomycetes (extracellular crude alone), fermented on ISP5.

Isolate No.	Zone of inhibition (mm)		
	***Staphylococcus aureus*ATCC25923**	***Escherichia coli* ATCC25922**	***Candida albicans* ATCC 10231**
KH 14	15	0	0
KH 16	0	0	26
KH 18	0	8	0
KA 19	23	0	0
KA 38	0	0	0
KA 39	12	8	18

0= no inhibition

The best results for inhibiting *Candida albicans* were obtained by isolate KH16 26mm; 25mm on ISP5 and ISP1 respectively, as presented in Table (3.5 and 3.8) ,while the rest of other isolates and media types showed moderate activities. Our results was inagreement with finding of Abdul Wahab *et al.* (2015) who obtained 15mm zone of inhibition , and than the isolate strain of

actinomycetes by Attimarad et *al.* (2012) which do not show any activity against *Candida albicans* , while Hozzein *et al.* (2011) obtained better results than our results from the isolated strain D 332 which recorded 30mm zone of inhibition against *Candida albicans* .

Hacène *et al.* (2000) described that the secondary metabolites and antimicrobial products of isolated actinomycetes species are almost extracellular products and they appear as extracellular products in nature exhibiting the powerful antimicrobial metabolites activities.

Table 3.9: Antimicrobial activities of locally isolated actinomycetes (extracellular crude alone), fermented on GYEB.

Isolate No.	Zone of inhibition (mm)		
	***Staphylococcus aureus*ATCC25923**	***Escherichia coli* ATCC25922**	***Candida albicans* ATCC 10231**
KH 14	0	0	0
KH 16	0	0	0
KH 18	12	0	9
KA 19	25	0	15
KA 38	0	0	0
KA 39	0	0	0

0= no inhibition

The best media composition for the production of extracellular crude extract antimicrobial metabolites are taken from media types ISP1, ISP2, ISP4, ISP5 and GYEB against tested human microbial pathogens as presented previously. The ISP2 showed the best media composition for the production of antimicrobial metabolites where 66.6% of which gave the inhibition zone against all tested microbial pathogen. However, other media composition, ISP5, ISP4,

ISP1 and GYEB exhibited 38.8%, 33.3%, 33.3% and 11% respectively, through revealing the antimicrobial activities

The results for achieving the best media for the production of antimicrobial metabolites from locally isolated actinomycetes were in agreement with those found by Maataoui *et al.* (2014), who showed that ISP2 was the best media formulation for obtaining sufficient antimicrobial metabolites.

Kumar (2010) and Gopi and Ramakrishna (2011) reported that manipulating the nutritional, physical and chemical parameters of the culturing conditions will be augmented in the growth of actinomycetes. Besides, media composition plays a significant role in the economics and productivity of the vital process. Mahalaxmi *et al.* (2010) also stated that substrate composition plays an energetic role in the production of the antibiotics.

Badji *et al.* (2005) results indicated that both ISP1 and GYEA media allowed satisfactory production in comparison to ISP2 and nutrient agar, whereas Cheraiti and Gacemikirane (2012) exhibited that the activity of actinomycetes strain was very important on GYEA medium as compared to ISP2 and ISP1.

Sharon *et al.* (2014) also summarized the best medium composition for the production of the maximum production of antibiotics by actinomycetes species which experimentally optimized several suitable factors including medium ISP2 with, temperature 28^0C, incubation period 7days, carbon source dextrose, agitation 200rpm, nitrogen source yeast extract, sodium chloride 2.5%and inoculum concentration 2%.

Waksman *et al.* (2010) explained that the ability to inhibiting microorganisms by the actinomycetes species was highly specific, so this was selective, and not only depending on the strain and species of the isolated actinomycetes, but also the medium composition was used for growing it and the

condition of the fermentation remained the powerful point for production of inhibitory substances.

Among all locally used isolated actinomycetes KH14, KH16, KH18, KA19, KA38 and KA39 were used to complete the fermentation programs against human microbial pathogens Gram positive, negative and yeast via using different media composition and within the same fermentation condition. The isolates KH14 and KH16 showed broad antimicrobial activities against all tested microbial pathogens, as presented in Table (3.6). The isolate KH14 on the other hand showed more activity than KH16 in the ability of inhibition. However, the rest of other isolates showed varied activities against human microbial pathogens.

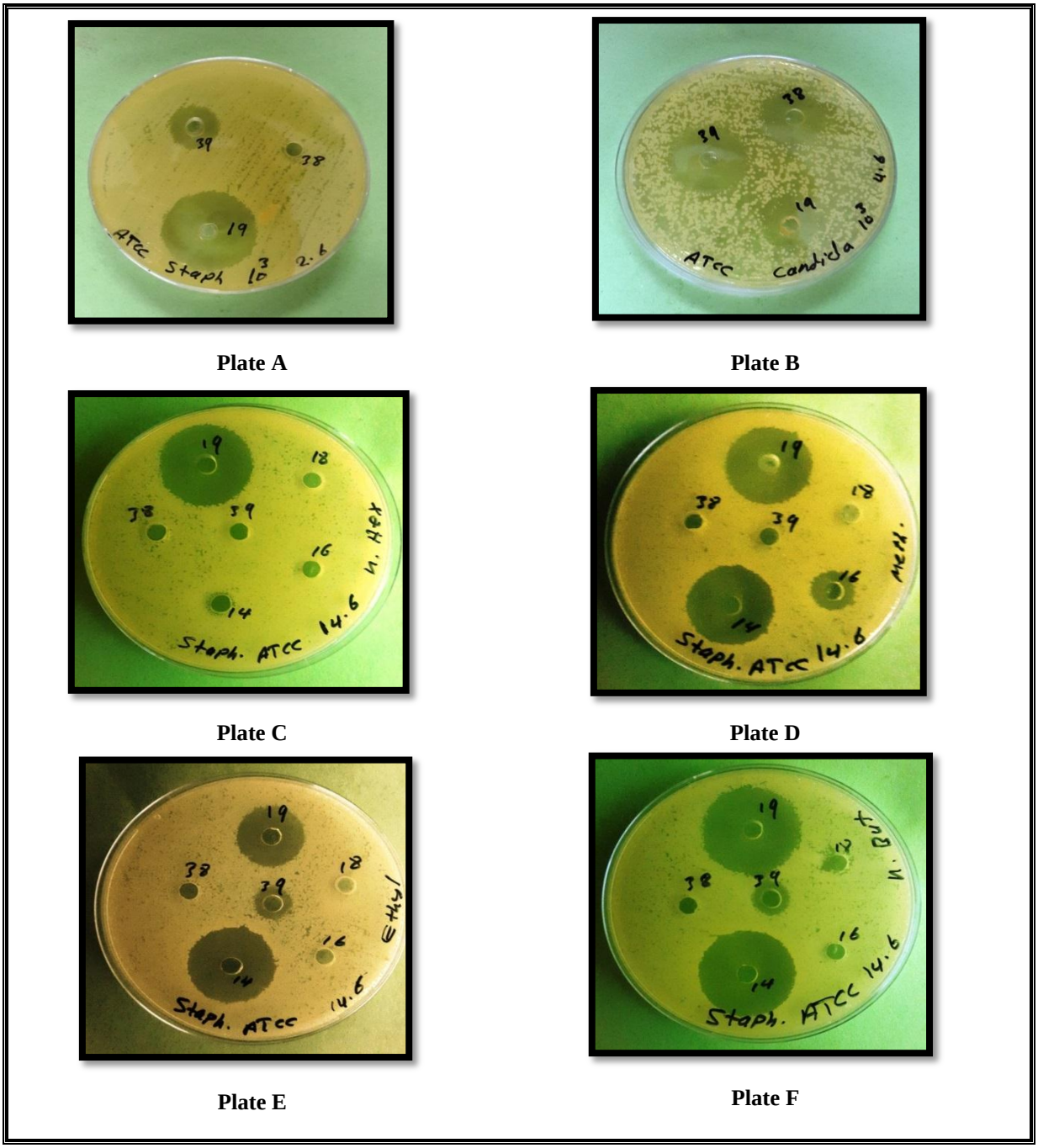


Figure 3.3: Secondary screening program for locally isolated actinomycetes against tested pathogens, **plate A**, extracellular crude extract activities against *Staphylococcus aureu*, **Plate B**, extracellular crude activities against *Candida albicans*. **Plate C** and **D** represent n-Hexane and methanol crude extract, while **plate E** and **F** represent ethyl acetate and n- butanol crude extract.

3.3.2 Best Organic Solvents for Extracting Extracellular Antimicrobial Metabolites

Depending on the results presented in Table (3.6) ISP2 was considered as the best media composition for obtaining the highest extracellular antimicrobial metabolites against all tested microbial pathogens in comparison to other media used from the fermentation program. Among the best organic solvents that extract the highest amount of antimicrobial metabolites, five different organic solvents methanol, ethyl acetate, chloroform, n-butanol and n-hexane were used to achieve the best extraction of antimicrobial metabolites. The results presented in Table (3.10, 3.11, 3.12, 3.13, and 3.14) representes the antimicrobial activities against tested microbial pathogens for methanol, ethyl acetate, chloroform, n-butanol and n- hexane extraction respectively.

The data presented in Table (3.10) summarized the extraction of extracellular antimicrobial metabolites with methanol against the tested microbial pathogens as shown in Figure (3.3) plate D. The isolates KH14, KH16 and KA19 performed antimicrobial activities against *Staphylococcus aureus*ATCC 25923 about 27, 14 and 24 mm zone of inhibition respectively, while KH18, KA38 and KA39 didn't show any activities against gram positive bacteria *Staphylococcus aureus* ATCC 25923. Extracting with methanol for all isolates didn't show any antimicrobial activities against *Escherichia coli* ATCC25922. Furthermore, in the case of *Candida albicans* ATCC 10231, only the isolates KA38 and KA39 showed antimicrobial activities about 14 and 20 mm, respectively.

Table 3.10: Antimicrobial activities of locally isolated actinomycetes (methanolic extract), fermented on ISP2.

Isolate No.	Zone of inhibition (mm)		
	***Staphylococcus aureus*ATCC 25923**	***Escherichia coli* ATCC25922**	***Candida albicans* ATCC 10231**
KH 14	27	0	0
KH 16	14	0	0
KH 18	0	0	0
KA 19	24	0	14
KA 38	0	0	20
KA 39	0	0	0

0= no inhibition

Table3.11: Antimicrobial activities of locally isolated actinomycetes (ethyl acetate extract), fermented on ISP2.

Isolate No.	Zone of inhibition (mm)		
	***Staphylococcus aureus*ATCC 25923**	***Escherichia coli* ATCC25922**	***Candida albicans* ATCC 10231**
KH 14	25	0	0
KH 16	0	0	0
KH 18	0	0	0
KA 19	20	0	0
KA 38	0	0	0
KA 39	11	0	0

0= no inhibition

The data presented in Table (3.11) summarized the extraction of extracellular antimicrobial metabolites with ethyl acetate against the tested microbial pathogens as shown in (Figure 3.3) plate E. The isolates KH14, KA19 and KA39 perform antimicrobial activities were intended for *Staphylococcus aureus* exactly 25, 20 and 11 mm zone of inhibition respectively, while for gram

negative bacteria *Escherichia coli* and yeast *Candida albicans* none of them showed antimicrobial activities. The data summarized in Table (3.12) demonstrated the extraction of extracellular antimicrobials metabolites with chloroform against tested microbial pathogens, which assessed no any antimicrobial activities against the tested pathogens for all isolates except isolate KH16 that achieved an antimicrobial activity against *Escherichia coli* ATCC25922 about 10mm.

Khan and Patel (2011) extracted metabolites with chloroform about 12 mm and 13 mm inhibition for *Staphylococcus aureus* and *Escherichia coli* respectively, but they did not record any activities for the extraction with ethyl acetate. Results for the extraction of antimicrobial metabolites with organic solvents were showed better than those of Lertcanawanichakul *et al.*, (2015) from *Streptomyces lydicus* A2. They obtained antimicrobial activities, extracted by ethyl acetate and methanolic about 16mm and 17 mm zone of inhibition against *Staphylococcus aureus* respectively, while there was not any inhibition against *Escherichia coli*. Extraction with n-hexane also did not show any antimicrobial activity.

Bode *et al.* (2002) explained that the antimicrobial compounds secreted to the culture medium (fermented broth) can be easily extracted when using one of the most suitable organic solvents. Selvameenal et *al.* (2009) stated that the extracellular crude extract from fermented cultures was prepared for secondary screening protocol and when extracting their bioactive antimicrobial metabolites, different organic solvents were used on the basis of their differentiation in their polarity to organic solvents against antimicrobial metabolites presented in actinomycetes.

Suthindhiran and Kannabiran (2010) explained that the bioactive compounds (secondary metabolites) after filtration can be recovered with a suitable organic solvent as an extraction method, by treating it with an equal volume of appropriate solvents for about 1 to 2 hours such methanol, n-butanol and ethyl acetate. Vijakumar *et al.* (2010) evaluated the extraction with organic solvents for bioactive compounds from isolated actinomycetes and the results indicated that the extraction with ethyl acetate gave the maximum antimicrobial activity against the tested microorganisms, while extracting with other solvents showed a moderate activity or no activity as antimicrobial compounds.

Table 3.12: Antimicrobial activities of locally isolated actinomycetes (Chloroform extract), fermented on ISP2.

Isolate No.	Zone of inhibition (mm)		
	***Staphylococcus aureus*ATCC 25923**	***Escherichia coli* ATCC25922**	***Candida albicans* ATCC 10231**
KH 14	0	0	0
KH 16	0	10	0
KH 18	0	0	0
KA 19	0	0	0
KA 38	0	0	0
KA 39	0	0	0

0= no inhibition

The data presented in Table (3.13) summarized the extraction of extracellular antimicrobial metabolites with n-butanol against tested microbial pathogens as shown in Figure (3.3) plate F. The isolates KH14, KA19 and KA39 performed antimicrobial activities against gram positive bacteria *Staphylococcus aureus* ATCC 25923 about 27, 28 and 11 mm respectively, while KH16, KH18 and KA38 didn't show any an antimicrobial activities, while the isolate KH16 showed antimicrobial activity about 9mm zone of inhibition, on the other hand,

for *Candida albicans* ATCC 10231 all isolates showed antimicrobial activities except isolates KH16 and KH18 as presented in Table (3.13).

As a final point, the data presented in Table (3.14) summarize the extraction of extracellular antimicrobial metabolites with n-hexane against the tested microbial pathogens as shown in Figure (3.3) plate C. All isolates failed to show any antimicrobial activities against all tested microbial pathogens except isolate KA19 that performed antimicrobial activities 27 and 11 mm for *Staphylococcus aureus* and *Candida albicans* respectively.

The results indicated that all the isolated actinomycetes had higher powerful activities against *Staphylococcus aureus* than *Escherichia coli*. Similar results were earlier obtained by Anansiriwattana *et al.* (2006); Charoensopharat *et al.* (2008) Hozzein *et al.* (2011); Al-Hulu *et al.* (2011); Kekuda *et al.* (2012) and Lertcanawanichakul *et al.* (2015).

Table 3.13: Antimicrobial activities of locally isolated actinomycetes (n-butanol extract), fermented on ISP2.

Isolate No.	Zone of inhibition (mm)		
	***Staphylococcus aureus*ATCC 25923**	***Escherichia coli* ATCC25922**	***Candida albicans* ATCC 10231**
KH 14	27	0	10
KH 16	0	9	0
KH 18	0	0	0
KA 19	28	0	11
KA 38	0	0	13
KA 39	11	0	20

0= no inhibition

Table 3.14: Antimicrobial activities of locally isolated actinomycetes (n-Hexane extract), fermented on ISP2.

Isolated no.	Zone of inhibition (mm)		
	***Staphylococcus aureus*ATCC 25923**	***Escherichia coli* ATCC25922**	***Candida albicans* ATCC 10231**
KH 14	0	0	0
KH 16	0	0	0
KH 18	0	0	0
KA 19	27	0	11
KA 38	0	0	0
KA 39	0	0	0

0= no inhibition

The results of the extraction of antimicrobial metabolites with organic solvents were summarized being related to their ability to extract extracellular antimicrobial metabolites. They showed that n- butanol, methanol, ethyl acetate, n- hexane and chloroform had activities against the tested microbial pathogens by producing the zones of inhibition as follows 44.4%, 27.7%, and 16.6%, 11.1% and 5.55% respectively. In addition, these obtained results were in a disagreement with those obtained by Abdul Wahab *et al.* (2015), who documented that ethyl acetate provided the best solvent for the extraction of antimicrobial metabolites followed by chloroform, while both ethanol and methanol solvents were unable to extract the antimicrobial metabolites. Furthermore, within the obtained results, ethyl acetates followed by methanol showed the best solvents for the extraction of antimicrobial metabolites especially against *Staphylococcus aureaus*, but chloroform seems to fail to extract the main extracellular antimicrobial metabolites except isolate KH16 against *Escherichia coli.* This was in disagreement with the result obtained by Khan and Patel (2011) who recorded that chloroform was the best solvent for

extracting the extracellular metabolites, while, the organic solvent such n-hexane ranged in between for their activity among all used organic solvents.

3.3.3 Extraction of Intracellular Antimicrobial Compounds (Metabolites)

The data presented in Table (3.15) summarize the antimicrobial activities of the intracellular extract against reference microbial strains *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*.

All isolates showed antimicrobial activities against *Staphylococcus aureus* ATCC25923, in which the isolate KA19 recorded the greatest antimicrobial activity exactly 40mm zone of inhibition. On the other hand, both KH16 and KA39 recorded the lowest activities, exactly 19mm; while for *Escherichia coli* ATCC25922 only isolate KH18 showed an antimicrobial activity 30mm but the rest of other isolates did not show any activities. Moreover, only isolates KH16 and KH18 did not show any antimicrobial activities against *Candida albicans* ATCC 10231, and the rest of other isolates performing antimicrobial activities KH14, KA19, KA38 and KA39 about 20mm, 11mm, 15mm, and 25mm zone of inhibition respectively as presented in Table (3.15).

The results for performing antimicrobial metabolites activities from intracellular metabolites exhibited that most of the isolates showed activities against both *Staphylococcus aureus* and *Candida albicans* while only one isolate showed activity against *Escherichia coli*. In comparison to the extracellular crude extract, a powerful result achieved in Table (3.6) with intracellular crude extract results as in Table (3.15). In general, the extracellular crude extracts exhibited somewhat better activity than intracellular crude extracts at the basis of their recording positive cases against tested microbial pathogens, in which recording 12 and 11 cases for extracellular cellular extract alone and intracellular extract alone activities, respectively.

Saravana Kumar *et al.* (2012) studied the antagonistic activity of intracellular extract for isolated actinomycetes species against gram positive and negative bacteria. The inhibition zone against *Staphylococcus aureus* was 21mm while against *Escherichia coli* was 20mm. The study of Khan and Patel (2011) also documented intracellular extract activities extracted with methanol against *Staphylococcus aureus* about 24mm zone of inhibition and 25mm against Escherichia *coli*.

Table 3.15: Antimicrobial activities of locally isolated actinomycetes (intracellular metabolites extract), fermented on ISP2.

Isolate No.	**Zone of inhibition (mm)**		
	***Staphylococcus aureus*ATCC 25923**	***Escherichia coli* ATCC25922**	***Candida albicans* ATCC 10231**
KH 14	23	0	20
KH 16	19	0	0
KH 18	17	30	0
KA 19	40	0	11
KA 38	20	0	15
KA 39	19	0	25

0= no inhibition

3.3.4 Comparison between Synthetic Antibiotic Discs Activities and Extracellular Crude Extract

The results presented in Table (3.16) showed that the highest activity against *Staphylococcus aureus*ATCC 25923 was recorded by Cefotaxime 30 ug exactly 35mm zone of inhibition, while the lowest result was recorded by Tetracycline 30 ug 12mm zone of inhibition against *Staphylococcus aureus*ATCC 25923. On the other hand , the highest results for *Escherichia coli* ATCC25922 were documented by Gentamicin 10 ug exactly (30mm zone of inhibition) , but the lowest results failed were recorded by Amoxicillin 25 ug, Erythromycin 15 ug, Tetracycline 30 ug, Methicillin 10ug, Amoxicillin/clavulanic acid 25 ug in which none of them showed any activities against *Escherichia coli* ATCC25922. Unfortunately, none of the synthetic antibacterial discs used showed antimicrobial activities against *Candida albicans* ATCC 10231.

Table 3.16: Antimicrobial activities of antibiotic discs on tested microbial pathogens.

No.	Antibiotics name	Abbreviation	Zone of inhibition (mm)		
			***Staphylococcus aureus*ATCC 25923**	***Escherichia coli* ATCC25922**	***Candida albicans* ATCC 10231**
1	Amoxicillin 25 ug	AX	24	0	0
2	Gentamicin 10 ug	CN	26	30	0
3	Cefotaxime 30 ug	CTX	35	27	0
4	Streptomycin 10 ug	ST	16	16	0
5	Pencillin G 10nuit	PG	21	20	0
6	Vancomycin 10 ug	VA	30	24	0
7	Erythromycin 15 ug	E	23	0	0
8	Tetracycline 30 ug	TE	12	0	0
9	Methicillin 10 ug	ME	30	0	0
10	Amoxicillin/clavulanic25	AMC	18	0	0

0= no inhibition

The comparison between synthetic antibiotic discs and locally isolated actinomycetes activities against tested microbial pathogens were presented in Table (3.16) and the results presented previously in Table (3.6), which represented the antimicrobial activities of locally isolated actinomycetes extracellular crude extract fermented on ISP2. The result presented in Figure (3.4, 3.5 and 3.6) shows the comparison between synthetic antibiotic discs and locally isolated actinomycetes activities extracellular crude extract against *Escherichia coli* ATCC25922, *Staphylococcus aureus*ATCC 25923 and *Candida albicans* ATCC 10231 respectively.

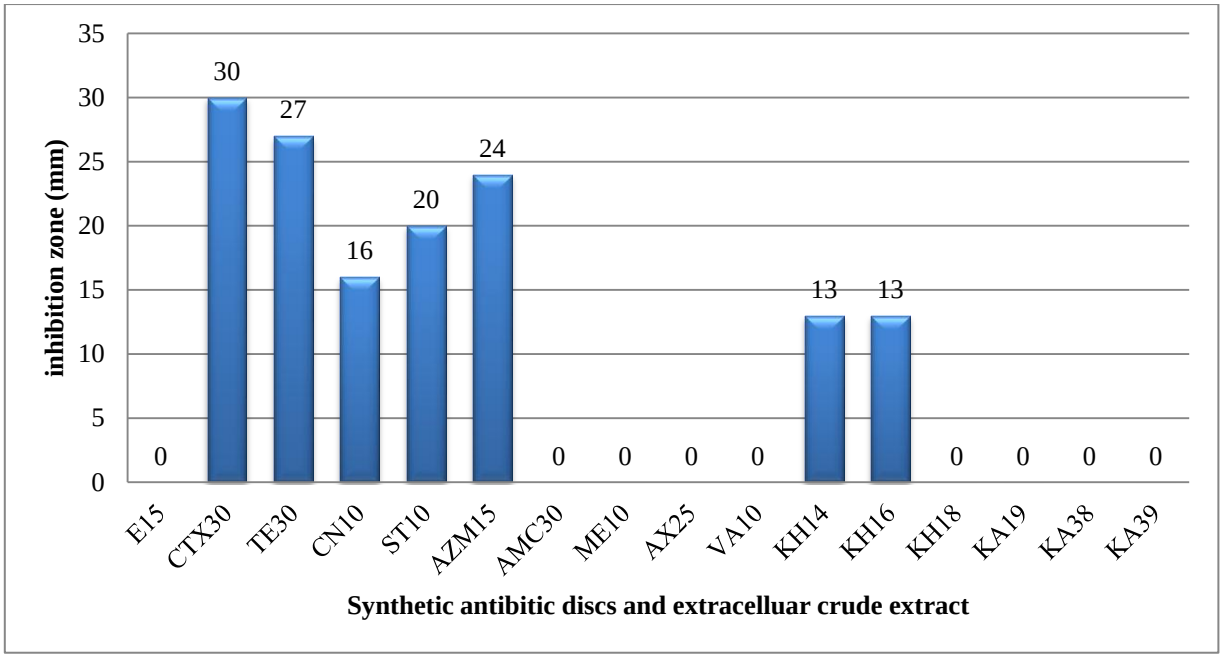


Figure 3.4: Comparisons between different synthetic antibiotic discs with antimicrobial activities of locally isolated actinomycetes (extracellular crude extract) on *Escherichia coli* ATCC25922

The data presented in Figure (3.4) summarize the comparisons between different synthetic antibiotic discs with antimicrobial activities of locally isolated actinomycetes extracellular crude extract on *Escherichia coli* ATCC25922, only two of locally isolates showed antimicrobial activities KH14 and KH16 exactly 13mm zone of inhibition, while the others failed to perform any activities against gram negative bacteria *Escherichia coli.* On the other hand, many of the synthetic antibiotic discs also failed to perform antimicrobial activities such Amoxicillin, Erythromycin, Tetracycline, Methicillin and Amoxicillin/clavulanic acid. The most antimicrobial activity against *Escherichia coli* was obtained by Cefotaxime 30 ug exactly 30mm zone of inhibition.

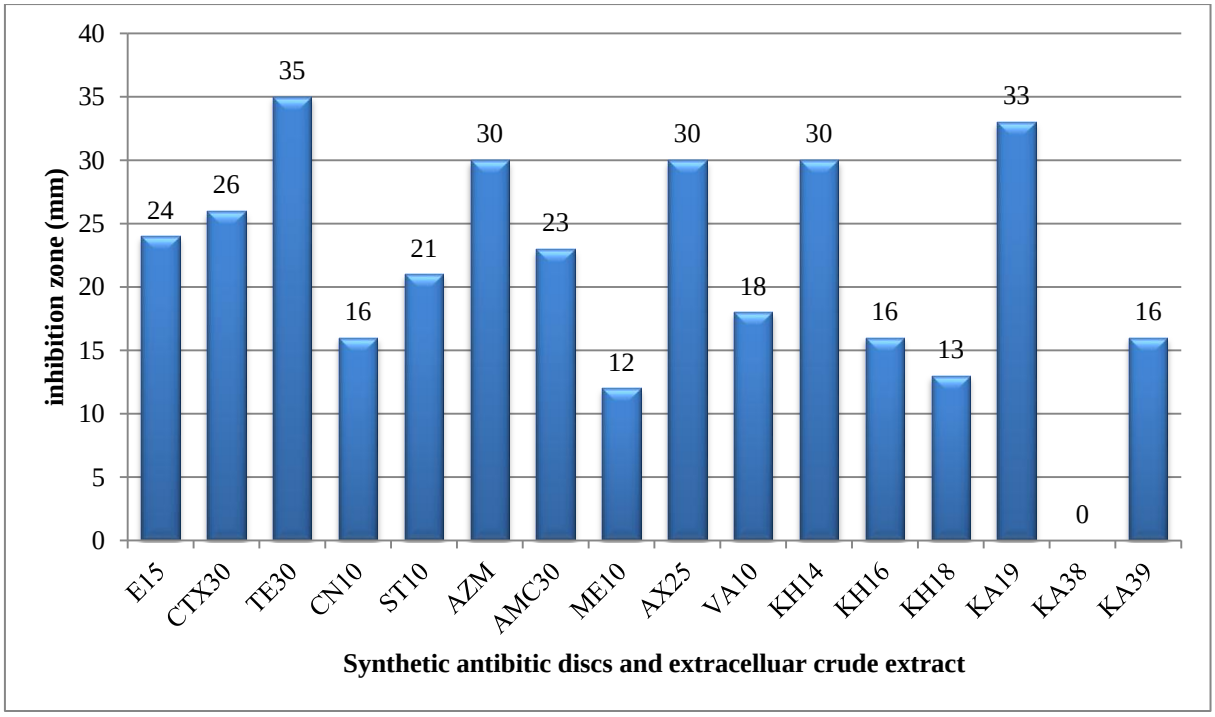


Figure 3.5: Comparisons between different synthetic antibiotic discs with antimicrobial activities of locally isolated actinomycetes (extracellular crude extract) on *Staphylococcus aureus*ATCC25923.

The comparison between different synthetic antibiotic discs with antimicrobial activities of locally isolated actinomycetes extracellular crude extract for gram positive bacteria *Staphylococcus aureus* was presented in Figure (3.5), which summarized that all used antibiotic discs having antimicrobial activities ranged between 12 to 35mm zone of inhibition ,as well as, all locally isolates actinomycetes also having antimicrobial activities except isolate KA38 which failed to inhibit the growth of *Staphylococcus aureus*.

The highest antimicrobial activities for synthetic antibiotic discs were recorded by Tetracycline 30 ug 35mm zone of inhibition followed by Azithromycin 15 ug and Amoxicillin 25 ug each with 30mm inhibition, on the other hand, the locally isolate actinomycetes KA19 exhibited the highest activity against gram positive bacteria *Staphylococcus aureus* exactly 33mm zone of inhibition which indicated that its antimicrobial activity greater than all used

synthetic antibiotic discs except tetracycline 30 ug which record 35mm inhibition. The remaining isolates activities against *Staphylococcus aureus* ranged from 30, 16, 16 and 13 mm zone of inhibition for KH14, KH16, KA39 and KH18 respectively.

Figure (3.6) summarize the comparisons between different synthetic antibiotic discs with antimicrobial activities of locally isolated actinomycetes extracellular crude extract on *Candida albicans* ATCC 10231. The results showed that none of the synthetic antibiotic discs were used exhibiting antimicrobial activities against *Candida albicans* ATCC 10231, because all used antibiotic discs were characterized as antibacterial activities, in contrast, all isolated actinomycetes performed the antimicrobial activities against *Candida albicans* ATCC 10231, except isolate KH18 did not perform any activity. The maximum antimicrobial activities were recorded by KA39 exactly 24mm, while other isolates KA38, KA19, KH14 and KH16 recorded 23, 21, 12 and 11 mm respectively as presented in (Figure 3.6).

The results obtained from locally isolated actinomycetes against tested microbial pathogens inhibition, were in agreement with those obtained by Al-Hulu *et al*. (2011) and Jaralla and Noor (2014) from locally isolated actinomycetes through having effective and significantly activities when comparing to synthetically antibiotic discs against several human microbial pathogens.

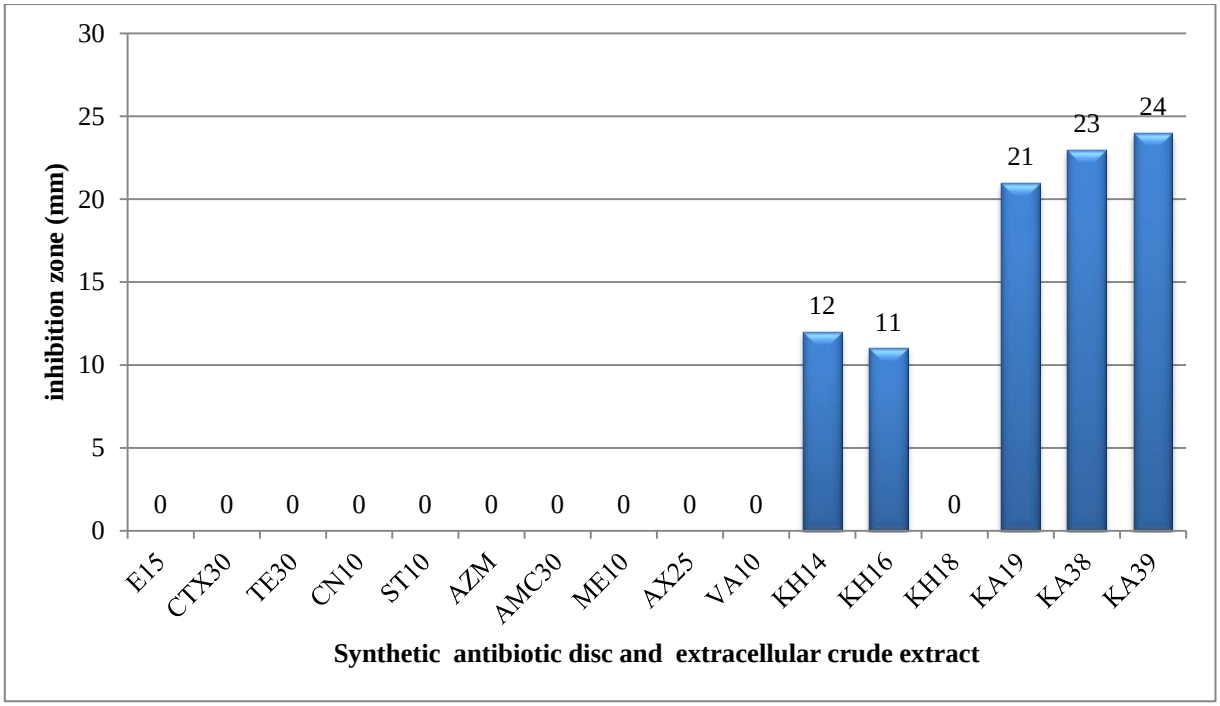


Figure 3.6: Comparisons between different synthetic antibiotic discs with antimicrobial activities of locally isolated actinomycetes (extracellular crude extract) on *Candida albicans* ATCC 10231.

3.3.5 Physio-chemical and Biological Properties Changes throughout Fermentation Process

Interaction between growing isolated actinomycetes isolates with culture medium (broth) during the entire fermentation process caused several changes in physiochemical properties such as broth color, absorbance (O.D) of the growth state and pH of the medium's broth after completing the fermentation process 7 days of incubation at 28±1°C, the results are summarized in Table (3.17) and Figure(3.7).

Table (3.17) shows the physio-chemical properties changed throughout the entire fermentation process, the general properties of the broth before inoculated with isolates were golden, 0.020 and 7.3 for broth color, optical density at 600nm and pH of broth culture respectively. However, after completing the

fermentation process the color of the broth became widely different to the initial time of inoculation as presented in Table (3.17) and Figure (3.7).

The results were in agreement with Dietz (1994) who described that studying the actinomycetes species at the level of microscopic and macroscopic levels showed a high diversity and distinction for determining their characterization patterns such as color, appearance and physio-chemical properties.

The growth condition was studied by determining their optical density values (growth state with the highest optical density (O.D) having high amount growth rate and vice versa) of the broth absorbance at 600nm, after 7 days of incubation at 28±1°C for all isolates. The isolates KA38 and KA39 recorded the highest level of O.D which gave 0.932 and 0.922 respectively, while the rest of all other isolates O.D (growth) ranged from 0.850 to 0.580 as summarized in Table (3.17).

Table 3.17: Comparison of physiochemical properties of fermented broth throughout fermentation process of locally isolated actinomycets growing on ISP2.

Isolate No.	Broth color		O.D at 600 nm		pH	
	Initial time	After 7days fermentation	Initial Blank	After 7days fermentation	Initial time	After 7days fermentation
KH 14	Golden	Orange	0.020	0.591	7.3	8.1
KH 16	Golden	Milky	0.020	0.687	7.3	6.5
KH 18	Golden	Milky	0.020	0.841	7.3	7.9
KA 19	Golden	Creamy	0.020	0.607	7.3	8.7
KA 38	Golden	Brownish gray	0.020	0.936	7.3	9.1
KA 39	Golden	Brownish gray	0.020	0.922	7.3	8.6

Valli *et al.* (2012) and Gebreyohannes *et al.* (2013) described the morphologically characteristics of the isolated actinomycetes, and our results were in agreement with their results in case of aerial mycelium, substrate mycelium growth, color and pigmentation depending on the culture media, inoculation amount and incubation periods showed a distinct variation. Colonies also appeared as leathery, white powdery, creamy, pinpoint and powder colonies of actinomycetes.

Also summarize the changes occurred in the pH value at the zero time (initial). After seven days fermentation, all isolated actinomycetes strains increased their pH value to become more alkaline except KH16 which was decreased under the initial time of inoculation pH 7.3. However, the highest pH value was recorded by KA38 exactly 9.1 and the other isolates recorded 8.1, 6.5, 7.9, 8.7 and 8.6 for KH14, KH16, KH18, KA19 and KA39, respectively.

The results also conformed to those described by Desai and Phatak (2010) who explained that the growth of actinomycetes species at neutral pH was decayed and its growth would be increased when cultivated in a higher pH range as well as increased their growth rate. In general the actinomycetes species preferred alkaline condition, On the other hand, both Augustine *et al.,* (2005) and Muiru *et al.* (2007) revealed that the stability of antimicrobial metabolites (antibiotics) was highly affected by the amount of pH values.

Figure 3.7: Physiochemical properties comparison during the entire fermentation process for locally isolated actinomycetes, growing on ISP2, N.C = negative control.

3.4 Electing the Most Active Isolated Actinomycetes to Complete the Study

According to the obtained results, the isolate KH14 showed the higher sntimicrobisl metabolities production, and it was selected for the following steps and completing the Amis of the study.

3.5 Evaluating Cultural Growth Requirement of KH14 Isolate

The production of antimicrobial metabolites is considered as the one of the main products of the secondary metabolites, which they are not straightly concerned in the normal growth, development, or reproduction of an organism (Shomurat *et al.*, 1979), this means that the microorganisms reached to the stationary phase. However, high microorganisms' population as well as high amount, production of secondary metabolites, will achieve this truth. Our project

will rely on several parameters that lead to optimize the isolated production, such as growth curve studying (growth kinetic), optimum pH value and salt tolerance (NaCl) value.

3.5.1 Evaluating Growth Curve (Growth Kinetics) of KH14 Isolate

Stationary phase of the normal growth curve for bacterial growth was considered as a phase that gives the highest amount of secondary metabolites as mentioned by Khan and Patel (2011). For determining the stationary phase, the isolated KH14 was growing on malt extract yeast extract broth ISP2, through recording the growth state by measuring the absorbance of the growth and obtaining the optical density (OD) at 600nm. The results presented in Figure (3.8) summarize the growth state activity of the isolate KH14 when their growth starts after 24 hours of incubation, with starting inoculums. OD was about 0.067in the first day and in second day it reached into 0.315 , which indicated that the isolate completed its lag and log phase in the growth curve only through two days ,because at the 3^{rd} day of incubation it was reached the maximum OD 0.619. This means that the isolate reached to the stationary phase, therefore, the isolate would be able to start the production of secondary metabolites after three days of incubation, while as presented in Figure (3.8) the curve would decrease down after 3^{rd} days of incubation.

The results also agreed with those obtained by Boudjelal *et al.* (2011) with regard to the maximum growth of 3 days incubation, Attimarad *et al.* (2012) maximum of 3 days incubation 72 hours and maintained at this level till 129 h; Chawawisit *et al.* (2015) maximum recorded of 3days. In contrast, in the study of Khan and Patel (2011) for isolate MJA 1105 in 4^{th} days the stationary phase was reached. Abdul Wahab *et al.* (2015) also obtained the same results in

the study of GZO24 when the stationary phase was reached in 4 days and ended in 6 days of incubation. When the isolate started producing antimicrobial metabolites at log phase, the concentrated activity was found at the stationary phase. Besides, Kojiri *et al.* (1992) recorded the maximum activity in 6 days; Bouras *et al.* (2013) started from 7 days and continued to 9 days, and, finally Maataoui *et al.* (2014) started at 7 days and remain till 10 days of incubation.

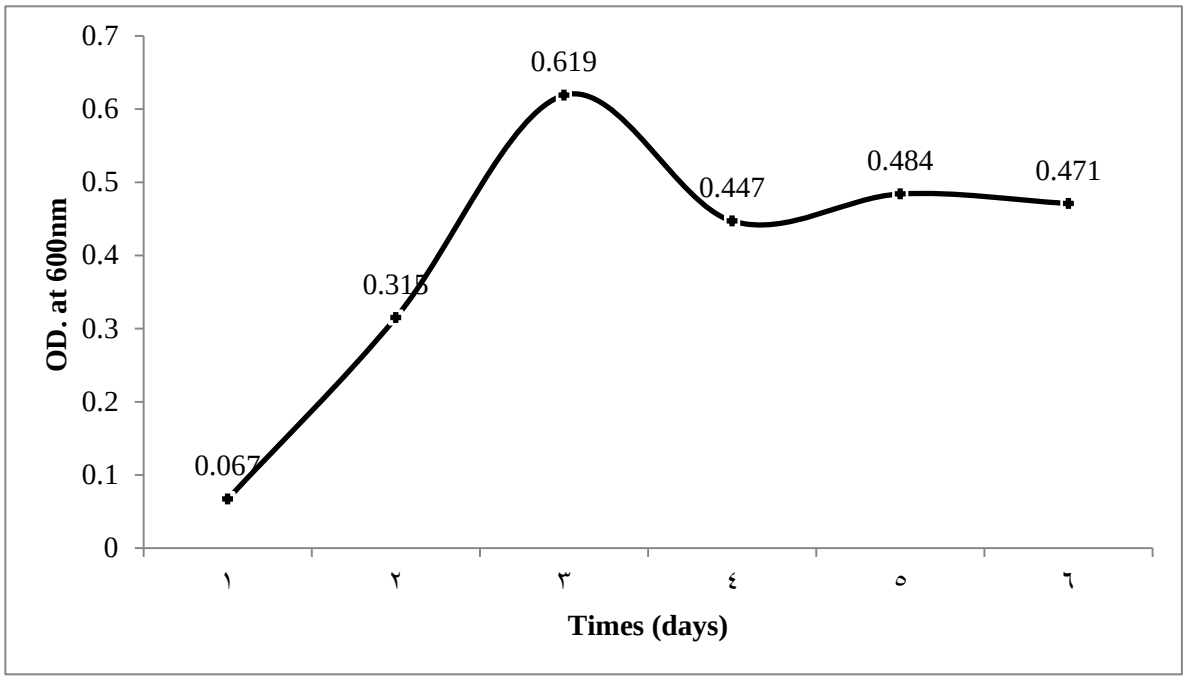


Figure 3.8: Growth kinetic curve of locally isolate actinomycetes KH14, growing ISP2 for 6 days.

3.5.2 Determination of Optimum pH Value of KH14 Isolate

Kutzner (1986) and Locci (1989) state that Streptomycetes are well-known species of actinomycetes which preferred neutral to alkaline environment pH when the optimal growth of them ranged 6.5 to 8. However, some actinomycetes (streptomycetes) have been found in acidophilic environment as described by Kontro et *al.* (2005). For determining optimum pH, the isolated actinomycete KH14 was growing on yeast extract malt extract broth with

different pH values ranging from 4, 5, 6, 7, 8, 9, and 10. Their growth rate was measured spectrophotometrically at 600 nm. The results presented in Figure (3.9) summarize the determination of the optimal pH value for isolate KH14. According to the presented data the highest amount of absorbance 0.409 was recorded at pH 8, which means the maximum amounts of growth was achieved after four days of growth of isolate KH14 with pH 8, while the remaining pH values gave high O.D values especially pH 9 and pH 7 about 0.204 and 0.211 respectively. This result indicated that the isolated had a wide range for growth in different pH values, especially at alkaline ranges.

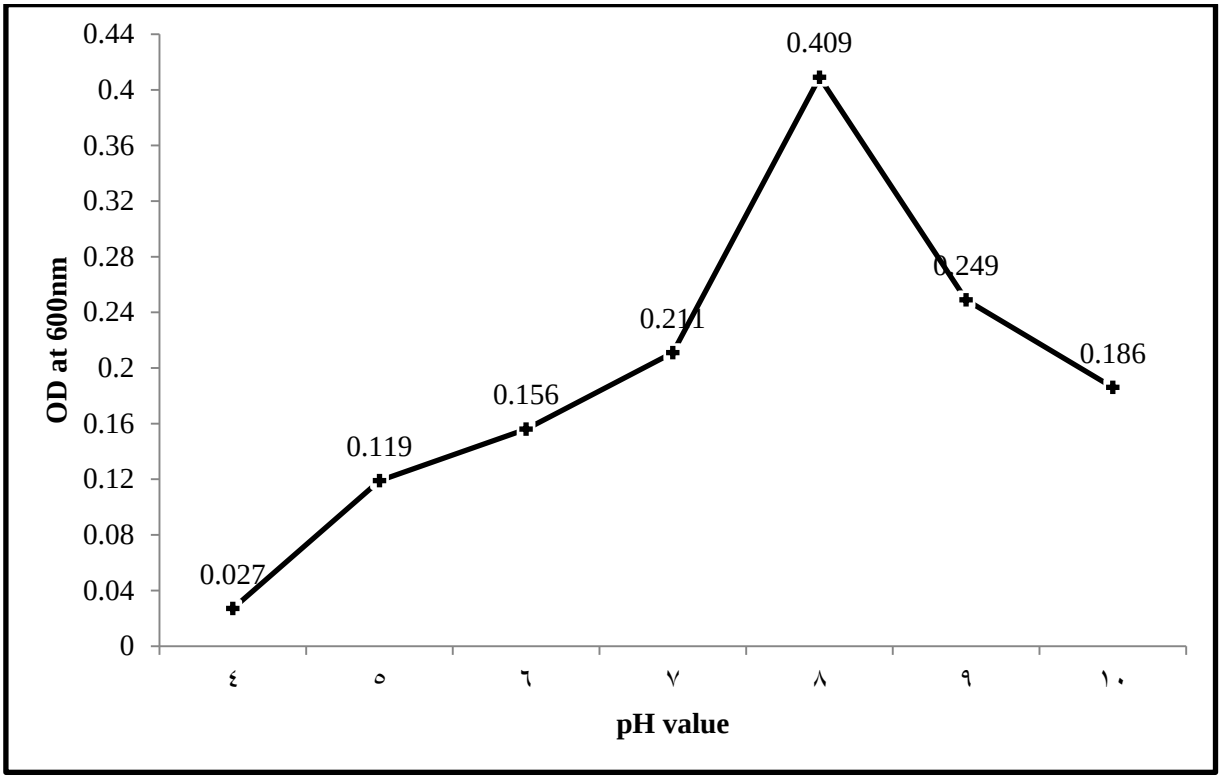


Figure 3.9: Evaluating optimal pH value of locally isolate actinomycetes KH14 growing on ISP2 after 4 days of incubation.

The optimum pH value for the locally isolated of actinomycete KH14 recorded the optimum value at pH 8. This result was in between with regard to the results obtained by the previously published research. Boudjelal *et al.* (2011) recorded that the pH varied between 7.2 and 8.6 during the incubation of

its isolated strain, while Khan and Patel (2011) documented the optimum pH for their isolated strain MJA 1105 exactly with pH 9. Finally, Attimarad *et al.* (2012) observed that the optimum pH was 7 for their isolated strain.

3.5.3 Determination of KH14 Isolate Salt-Tolerance NaCl

The ability of the isolate KH14 for growing ISP2 which supplemented with different NaCl salt concentrations ranging from 1 to 9% NaCl, which was determined at the basis of salt tolerance against growth rate state, and the growth rate was measured spectrophotometically through determining the amount of optical density at 600nm.

The data in Figure (3.10) summarize the salt tolerance of isolate KH14, which indicated that the isolate tolerated salt concentration till 5%, while above this concentration from 6% to 9% no growth was seen as shown in Figure (3.10), because the absorbance became below the blank value which means there was not any growth. In the study of Singh, *et al.* (2014) on *Streptomyces sannanensis* SU118, salt tolerance of NaCl was found about 3%. This results were also obtained by Khan and Patel (2011); Abdul Wahab *et al.* (2015). Furthermore, the results were in agreement with those obtained by Boudjelal et *al.* (2011) and Gebreyohannes *et al.* (2013) who isolated actinomycetes species that survive till 5% NaCl concentration.

Selvakumar *et al.* (2010) mentioned that the bioactive potential of *Streptomyces* it was revealed that many species are found in salty soil and marine so that they are considered highly tolerant to salinity.

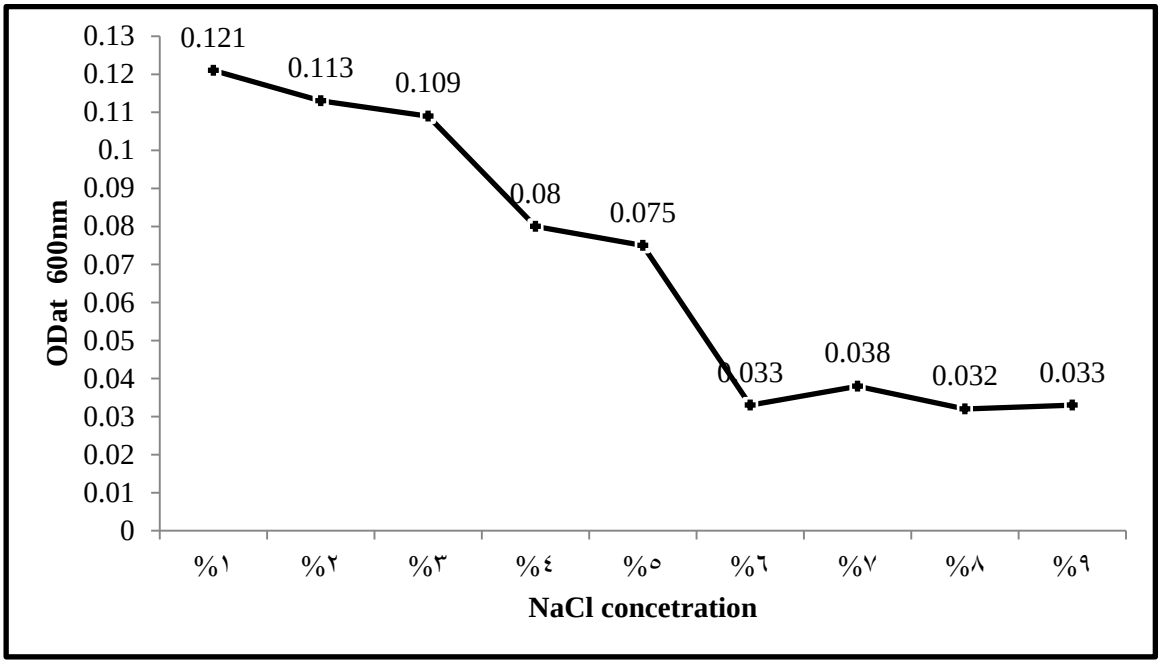


Figure 3.10: Evaluating NaCl salt tolerance of KH14 isolate growing on ISP2 supplemented with different NaCl concentration.

3.6 Determination of Minimum Inhibition Concentrations (MICs) of KH14 Isolate Extracellular Crude Extract

Extracellular crude extract of isolate KH14 was used to determine its MICs against references microbial strians. The concentrations were used ranged from 1, 2, 4, 8, 16, 32, 64, 125, 250, 500, 600, 700 and 800 ul /ml, and the nutrient broth was used as a culture medium broth.

The data presented in Table (3.18) summarize minimum inhibition concentrations against microbial pathogens. The MIC values for microbial strains *Escherichia coli* ATCC25922, *Staphylococcus aureus* ATCC 25923 and *Candida albicans* ATCC 10231 were recorded 500 ul /ml, 64 ul /ml and 500 ul /ml respectively. This indicated that the extracellular crude extract was very strong against *Staphylococcus aureus*, as well as it had a moderate activity against both *Escherichia coli* and *Candida albicans*. Our results were agreed with those described by Pandey *et al*. (2004); Mukai *et al*. (2006); Xie *et al*. (2007); Gurung

et al. (2009); Ababutain *et al.* (2012) and Gebreyohannes *et al.* (2013) which were intended for the activities of the bioactive metabolites against both gram positive and negative bacteria. Kumar *et al.* (2012) and Nanjwade *et al.* (2015) reported the activity of their isolated actinomycetes against only *Staphylococcus aureus* and *Candida albicans* without obtaining any activity against *Escherichia coli*.

Ababutain *et al.* (2012) studied the MIC of locally isolated actinomycetse in fine points and they obtained results after 24 hours of incubation on nutrient agar *Staphylococcus aureus* ATCC 6538P 31.25µg/ml; *E. coli* ATCC 7839 15.62µg/ml and *Candida albicans* ATCC 10231 31.25µg/ml**.**

The activity of extracellular crude extract of isolated KH14 against tested microbial pathogens cannot be achieved with Kirby-Bauer test because this can not determine their bacteriostatic and bactericidal activities. However, the results presented in Table (3.18), also summarize the activity and determination of both bacteriostatic and bactericidal. All the tested microbial pathogens after inhibiting them by the activity of the extracellular crude extract have the ability to re-grow on prepared nutrient agar. In conclusion, the isolate KH14 has bacteriostatic activities on the microbial pathogens without achieving any bactericidal activity.

Table 3.18: Estimating minimum inhibition concentrations, bacteriostatic and bactericidal activity of extracellular crude extract of isolate KH14.

Extracellular Crude concentration ul /ml	***Staphylococcus aureus*ATCC 25923**	***Escherichia coli* ATCC25922**	***Candida albicans* ATCC 10231**
1	+	+	+
2	+	+	+
4	+	+	+
8	+	+	+
16	+	+	+
32	+	+	+
64	-(MIC)	+	+
125	-	+	+
250	-	+	+
500	-	- (MIC)	-(MIC)
600	-	-	-
700	-	-	-
800	-	-	-
NC (broth +crude)	-	-	-
PC(broth +pathogen)	+	+	+
Bacteriostatic assay	**+**	**+**	**+**
Bactericidal assay	**-**	**-**	**-**

NC; Negative control, PC; positive control, +; growth, - ; No growth (inhibition)

3.7 Antioxidant Activity of the KH14 Isolate

The results presented in Figure (3.11) summarize the antioxidant activity of KH14 crude extract. A gradually increase of the extracellular crude extract concentration caused an increasing scavenging free radical ability until reaching the 20mg/ml of extract which scavenged 91% of the formed DPPH free radical. Our results were also supported by those found by Mbwambo *et al.* (2007) that DPPH radicals scavenging was in manner of dose-dependently increased. Koleva *et al.* (2001) stated that the DPPH assay when obtain one more electron; the absorbance would decrease when an antioxidant agent was present. The IC_{50} for our extracellular crude extract was 5.24 mg/ml that inhibits 50% of free radical

formed by DPPH, while the IC_{50} of the ascorbic acid as a standard was only 0.492 mg/ml.

Extracellular crude extract of KH14 was able to convert the violet color of DPHH free radical to yellow diphenyl picryl hydrazine after 30 minutes in a dark place at room temperature. According to the principle of DPHH assay described by Braca *et al.* (2001), the DPPH was considered as a good reagent for evaluating the free radical scavenging, with the ability of hydrogen donating as antioxidant.There fore, transferring the hydrogen atoms or electrons to formed DPPH radicals, which finally neutralized their radical activity. Furthermore, Asatian *et al.* (2007) described that each of nucleic acids, proteins, lipids or DNA, when oxidized with a free radical, the main characteristic of the antioxidant agents was to trap this formed free radicals and all the organisms possessing a highly specialized defense mechanism that attacks the free radical generated which is called "antioxidant system". The result for scavenging free radical activity showed less activity than those obtained by Saravana Kumar et *al.* (2014); Eva *et al.* (2014) and Naine *et al.* (2015) who obtained IC_{50} of their extracts from isolated actinomycetes with a concentration less than our concentration.

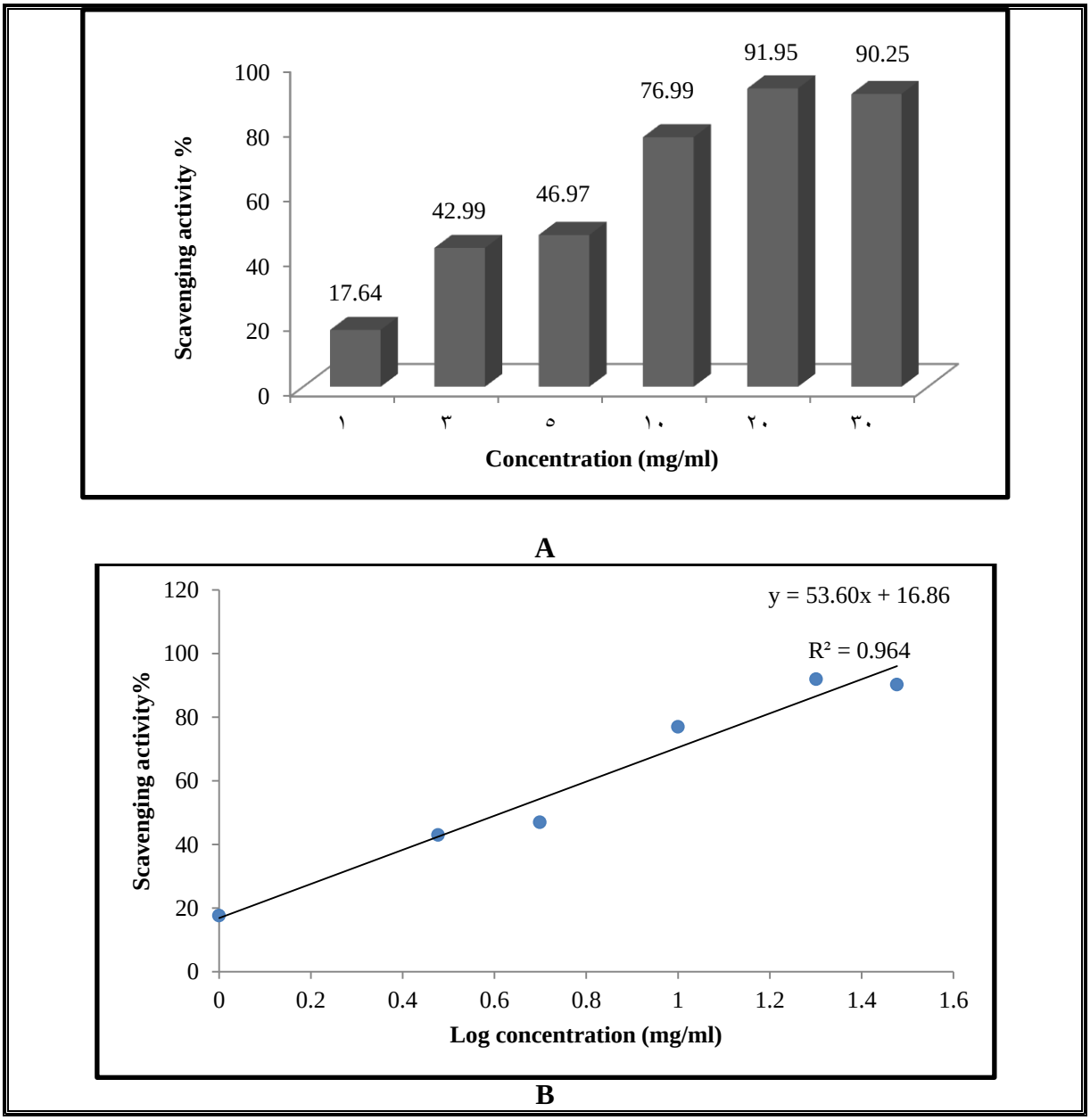


Figure 3.11: DPPH scavenging activity and total antioxidant activity of extracellular crude extract (KH14). (**Plate A**) percentage antioxidant scavenging activity of crude concentration; (**Plate B**) the results presented as Log concentration in contrast to the percent activity for inhibition.

The results presented in Figure (3.12) summarize the evaluation the antioxidant properties of ascorbic acid with different concentrations, used as a standard for measuring the ability of our isolate scavenging activity via making a

standard curve from the ascorbic acid, and calculating the IC_{50} scavenging activity.

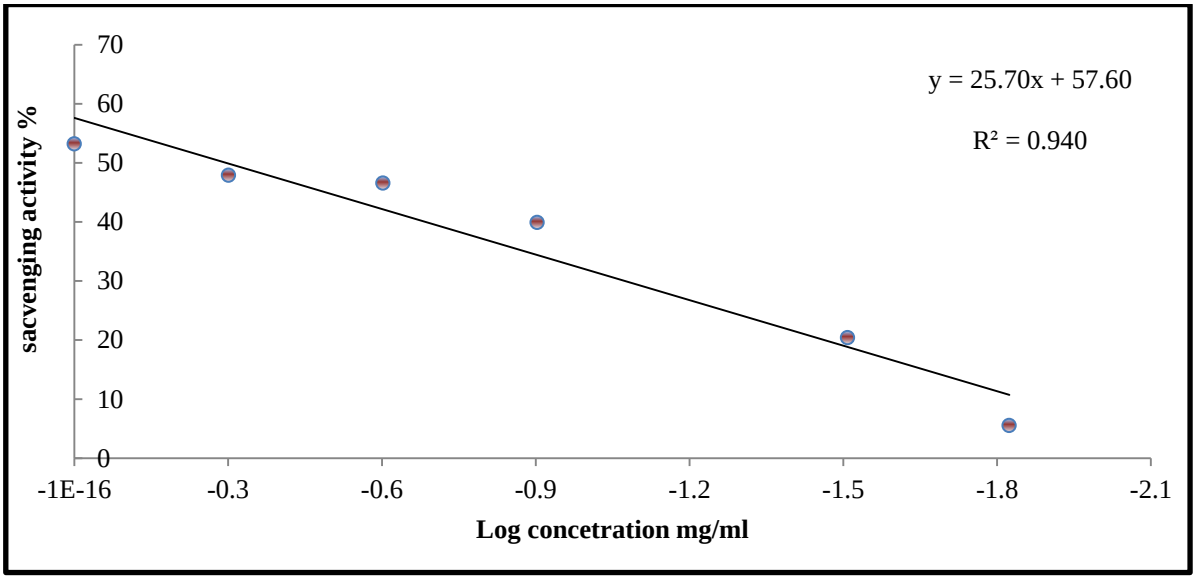


Figure 3.12: DPPH scavenging activity and total antioxidant activity of different concentrations of ascorbic acid.

3.8 Partial Purification of Extracellular Crude Extract of KH14 Isolate

Partial purification of extracellular crude extract was carried out by extracting the ethyl acetate and determining the bioactive compounds with a thin layer chromatography (TLC) through carrying out bioautography in the presences of tested microbial pathogens. However, the ethyl acetate extract and scraped spots of TLC were further studied by analyzing the bioactive compounds using HPLC-MS and GC-MS.

3.8.1 Partial Purification by Thin Layer Chromatography (TLC) and Bioautography

The extracellular crude extract was subjected to TLC in order to separate and identify the bioactive compounds. When the extracellular crude extract was spotted on pre-coated silica gel sheet, different mobile phases (solvents) were

used to achieve the best separation through determining the R_f values of the separated spots. The overall procedure and results for carrying out thin layer chromatography and bioautography were presented in Figure (3.13**)**. Starting with testing different mobile phases, determining different spot place, determining the bioactive compound by bioautography and finally testing isolated spot separately against tested microbial pathogens were presented in Figure (3.13 A, B, C and D respectively). Furthermore, the data in Figure (3.14) including the plate (E, F, G and H) exhibited a series of spots placed on TLC plates and run at the same time to obtain large amounts of bioactive compounds and dry separated spots in order to be scraped for collecting bioactive compounds and finally collecting scraped spots of bioactive compounds, respectively.

The extracellular crude extract showed four different spots on TLC plate as shown in Figure (3.14 B), but only one of them exhibited an antimicrobial activity when testing via bioautography, as presented in Figure (3.14C). Considinet and Mallette (1965) and Awais *et al.* (2007), stated that thin layer chromatography and bioautography are regularly used for primary separation and characterization of antimicrobial compounds from producing microorganisms especially determining the position of the hot spot on the TLC plate. According to the above mentioned procedure and displayed results, only one spot exhibited an antimicrobial activity against the tested microbial pathogens when evaluating its activity through bioautography as presented in Figure (3.13 Plate C). In order to determining the R_f of the bioactive spot and its solubility with different mobile phase tested. The results presented in Table (3.19) summarize all R_f values for each solvents used with its ability to dissolve the spots that carry the bioactive compounds.

The results agreed with those obtained by El-Naggar *et al.* (2006); Boudjelal *et al.* (2011) and Rana and Salam (2014). All such researchers obtained a single active spot in their isolated actinomycetes with different R_f values which were using different organic solvents in mobile phase, after applying the direct bioautography assay for determining their position, Maataoui *et al.* (2014) obtained three spots on TLC plate and only one of them showed an antimicrobial activity.

Among the solvents used as mobile phases, 100% ethanol exhibited the highest Rf about 0.92 and the lowest R_f was obtained by 100% water about 0.3, while both solvents toluene 100% and n-Hexan 100% didn not show any actions to separate the extracellular crude extract. On the other hand, in the case of solubility of extracellular crude extract , ethyl acetate 100% exhibited the highest solubility(+++), while toluene and n- Hexane were not capable of dissolving the extracellular crude extract. Furthermore, in case of using mixing solvents (mixture) as a mobile phase, the highest R_f value was (0.69) obtained by Ethyl acetate :Methanol: Water (10:1.5:1) , as well as performing the highest solubility activity(+++), but (Methanol : Chloroform 6:4) recorded the lowest R_f value 0.38, and Toluene: Ethyl acetate (93:7) did not record any R_f value as presented in Table (3.19).

These results agreed with those obtained by El-Tayeb *et al.* (2004); Atta (2010) and Ababutain *et al.* (2012) who stated that the bioactive antimicrobial metabolites were insoluble in n-Hexane while soluble in methanol, ethyl acetate, chloroform, n-butanol, acetone with different R_f values ranged from 0.92 cm to 0.3 cm.

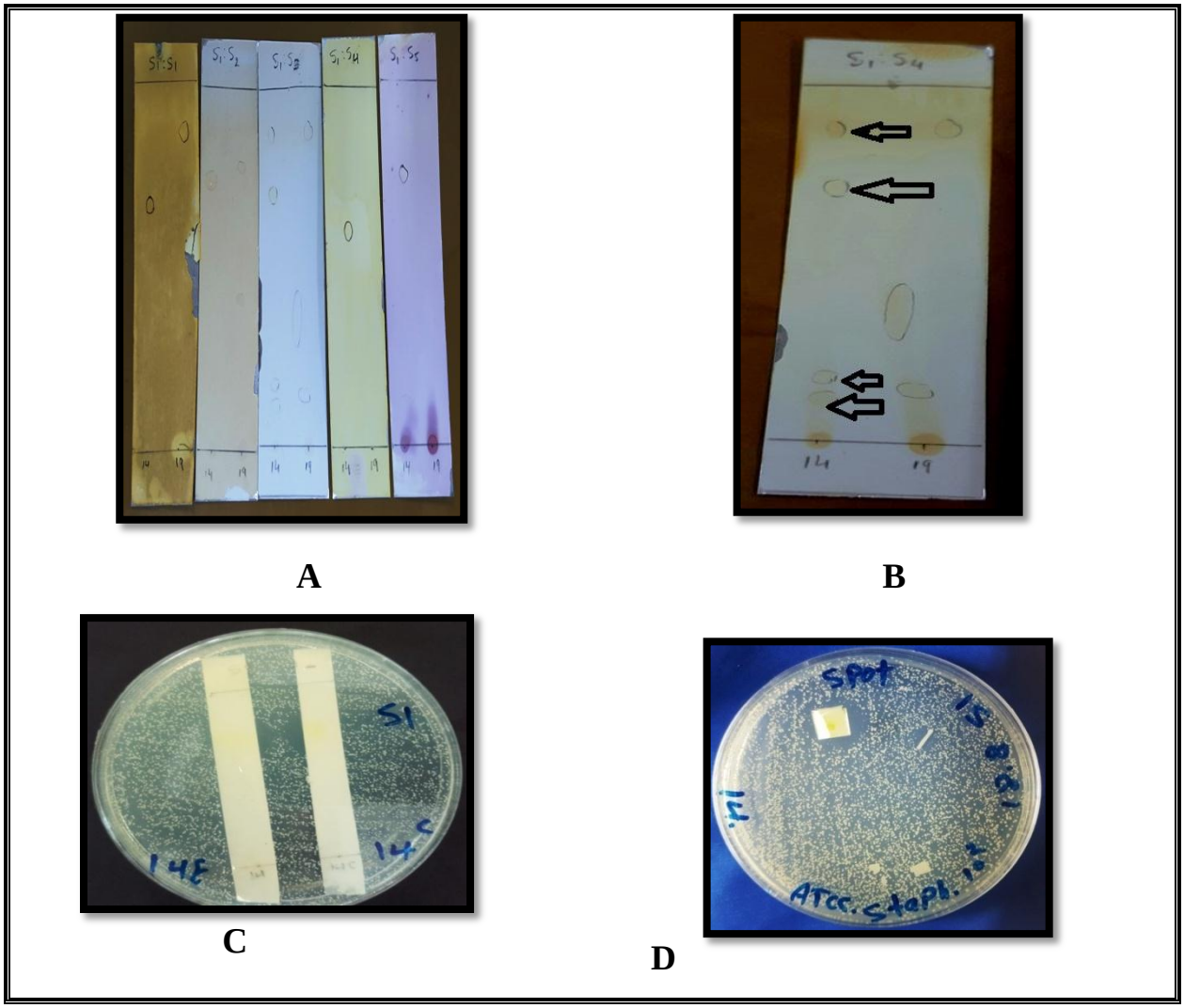


Figure 3.13: Overall procedure and results of TLC and bioautography of extracellular crude extract of KH14. **Plate A** different solvents were used, **Plate B** four clearly spots were separated with different R_f values, **plate C** showed bioautography only one spot was capably for inhibition microbial pathogens, **Plate D** showing the spot that containing bioactive compounds cropped by scissor and tested.

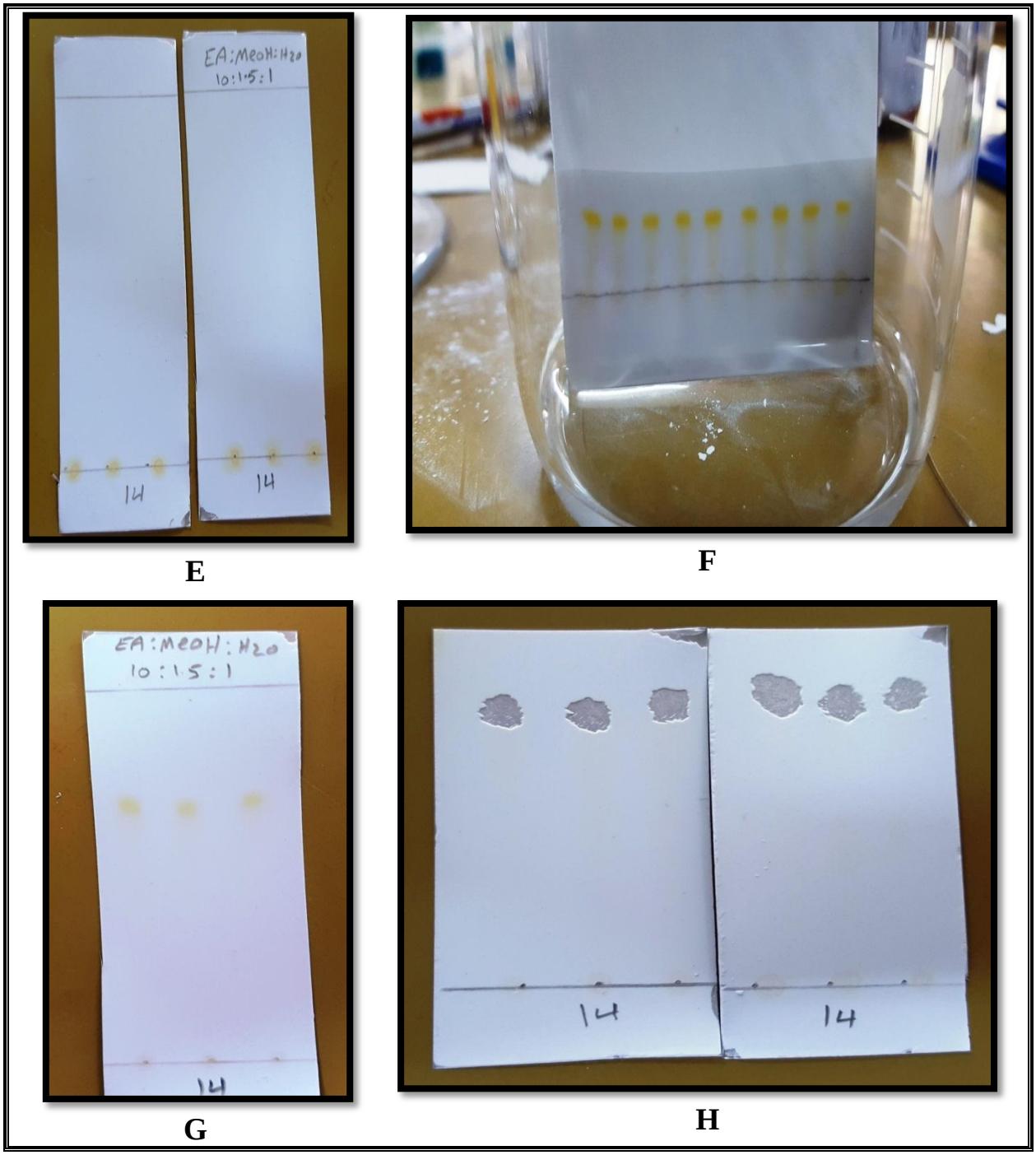


Figure 3.14: Overall procedure and results of TLC and bioautography of extracellular crude extract of KH14, **Plate E** and **F** showed a series of extracellular crude extract placed on silica gel plates, **Plate F** the plate was in state of running, **Plate G** separated spots were dried at room temperature, **Plate H** the spots after separating were scraped and collected in dry tube.

Table 3.19: R_f values and solubility of KH14 extracellular crude extract

Solvents (mobile phase)	R_f value (cm)	Solubility
Ethyl acetate 100%	0.81	+++
Methanol 100%	0.84	++
Chloroform100%	0.14	+
Water 100%	0.3	++
n-Hexan 100%	0.0	-
Toluene 100%	0.0	-
Acetone 100%	0.9	++
Ethanol 100%	0.92	++
n-Butanol 100%	0.6	+
Acetic acid 100%	0.87	+
Ethyl acetate :Methanol: Water (10:1.5:1)	0.69	+++
Toluene: Ethyl acetate (93:7)	0.0	-
Methanol : water (6:4)	0.65	++
Ethyl acetate : Chloroform : Water (95:5:5)	0.40	+
Methanol : Chloroform (6:4)	0.38	+
Ethyl acetate : Toluene :Methanol :Chloroform :Water (1:1:1:1:1)	0.59	+

(+++; very good solubility: ++ Good solubility: + Moderate solubility: - ; not dissolved)

3.8.2 Analyzing the Bioactive Compounds by High Performance Liquid Chromatography Mass Spectrometer (HPLC-MS)

Both ethyl acetate extract and scraped spots from TLC were subjected to (HPLC-MS) in order to analyze the bioactive compounds (antimicrobials). Separation and analysis were carried out with a HPLC-MS (Waters 2545-USA), quaternary gradient module operated in positive ionization mode at a range from

(mass-to-charge ratio) m/z = 200-900, combined with photodiodes array detector (Waters 2998) with a sampling rate of 2 point/sec (Lambda range 190-800 nm). Different analyzing states of both ethyl acetate extract and scraped spot from TLC were obtained in the form of Mass chromatogram, UV chromatogram, Mass spectra and finally UV absorbance.

The results presented in Figure (3.15) displayed the mass chromatograms, in which Hites and Biemann (1970) defined the chromatogram as "a mass chromatogram is a representation of mass spectrometry data as a chromatogram, in which x-axis represented the times, while the y-axis represented the signal intensity". However, Figure (3.15) showed different peaks on the chromatogram. The figure represents the different components in the sample mixture for ethyl acetate extract.

The results presented in Figure (3.15) represent the mass chromatogram of ethyl acetate extract. Clearly, there are more peaks appeared when comparing with the scraped spot extract. This is means that our scraped spot contains only bioactive compounds that exhibited antimicrobial activities. It also indicats that our separation protocol by TLC procedure was done in the correct manner. The highest peak signal intensity was obtained between retention times 1.73 to 2.30 minutes.

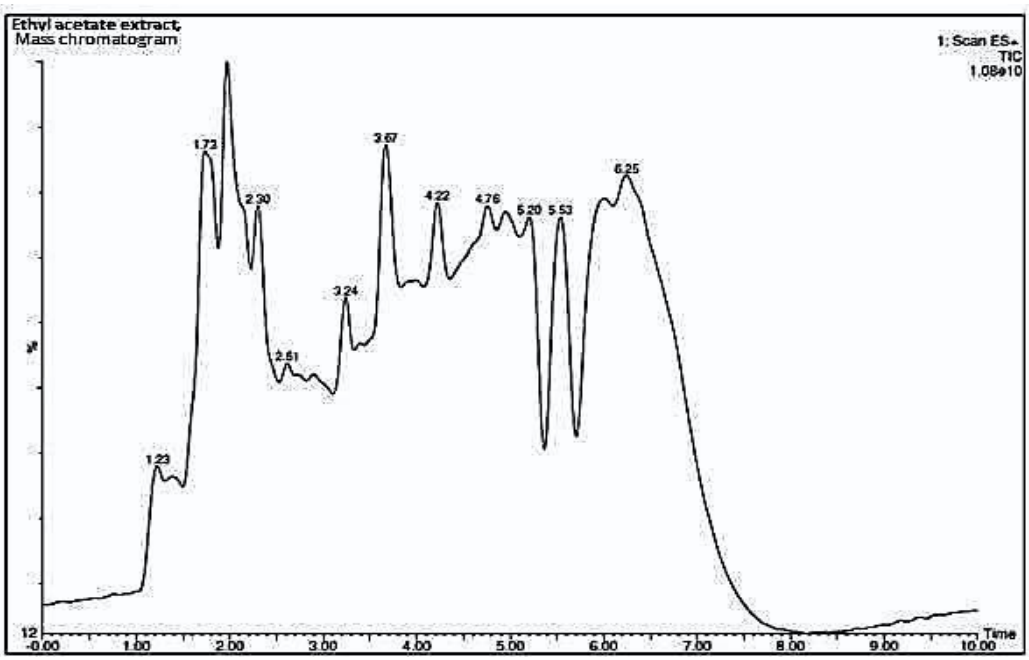


Figure3.15: Mass chromatogram of ethyl acetate extract was done by HPLC-MS; all separating time was 10 minutes.

High Performance Liquid Chromatography-Mass spectra with its Photodiodes array was used to detect and identify compounds in the samples for both ethyl acetate extract and scraped spot extract, in which a HPLC- UV detector used light to analyze the samples through measuring the sample's absorption of UV light at different retention time by scheming a Figure (relation) between X-axis which denoted to the times (retention time) against Y- axis which denoted to the absorption units (AU).

The results presented in Figure (3.16) summarize the UV chromatogram of ethyl acetate extract, there were three clearly sharp peaks that appeared at 1.68, 5.38 and 5.72 minutes which indicated a very well separated mixture at the above mentioned time with a clear absorption unit detected by UV detector.

In addition to identifying the exact compounds, they were founded at this peak, clicking on them in available software within HPLC-MS instrument; help us determine the molecular mass of the existing compounds at this retention time.

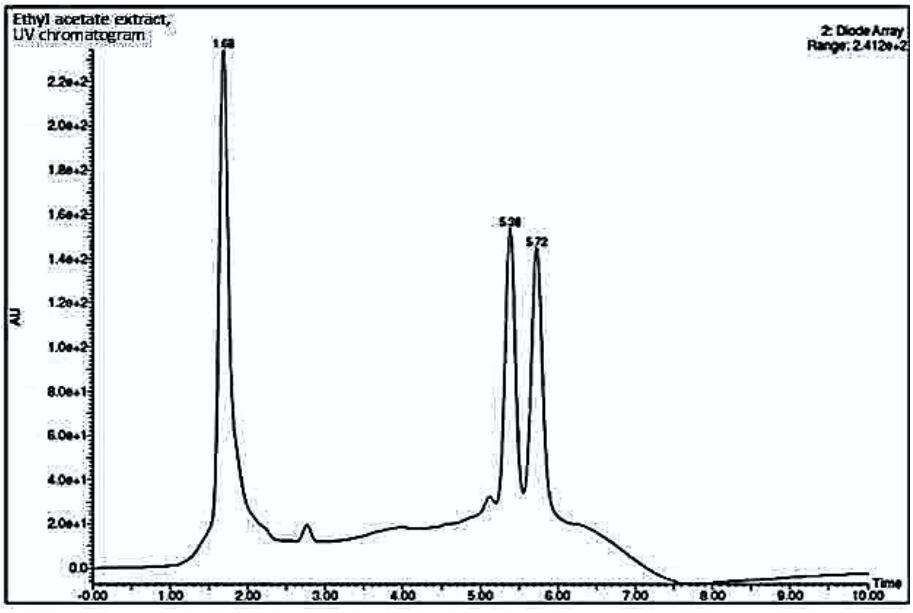


Figure 3.16: UV chromatogram of ethyl acetate extract was done by HPLC-MS; all separating time was 10 minutes.

The results presented in Figure (3.17) summarize all presented compounds (or fragments of the compound) for both ethyl acetate extract and scraped spot extract with their molecular mass separately. However, there was a huge aggregate of compounds presented in this area. Unfortunately, all these compounds may be formed as a result of captured compounds molecule during the process of extraction, preparation and/ or through preparing the solvent for HPLC analysis. Through consuming the results listed in Figure3.17 (A and B) between ethyl acetate extract and scraped spot extract, we are encouraged to find similarity between compounds or fragments of compound in both mass spectrums. The data presented in Table (3.20) summarize all similar molecular masses presented in both mass spectrums of ethyl acetate extract and scraped spot extract with some means of probability.

Table 3.20: Similar molecular mass presented in both ethyl acetate extract and scraped spot extract at peak (5.47) in mass spectra analysis by HPLC-MS

No.	Ethyl acetate extract mass spectrum (molecular mass at peak 5.47)	Scraped spot extract mass spectrum (molecular mass at peak 5.47)
1	281.07	282.20
2	329.20	329.26
3	443.19	443.38
4	344.20	344.32
5	413.37	413.31
6	441.35	441.28
7	455.15	455.33
8	657.34	657.84

Hamedi *et al.* (2015) obtained five compounds, which may be not previously isolated from microorganisms. So, they can made with five new probable bioactive metabolites with molecular mass of 274.2, 390.3, 415.3, 598.4 and 772.5, based on their UV absorption spectra and molecular weight values which did not match and verified with the natural compounds in the accepted product database. On the basis of this foundation, the obtained results may be new natural compounds that need a further study.

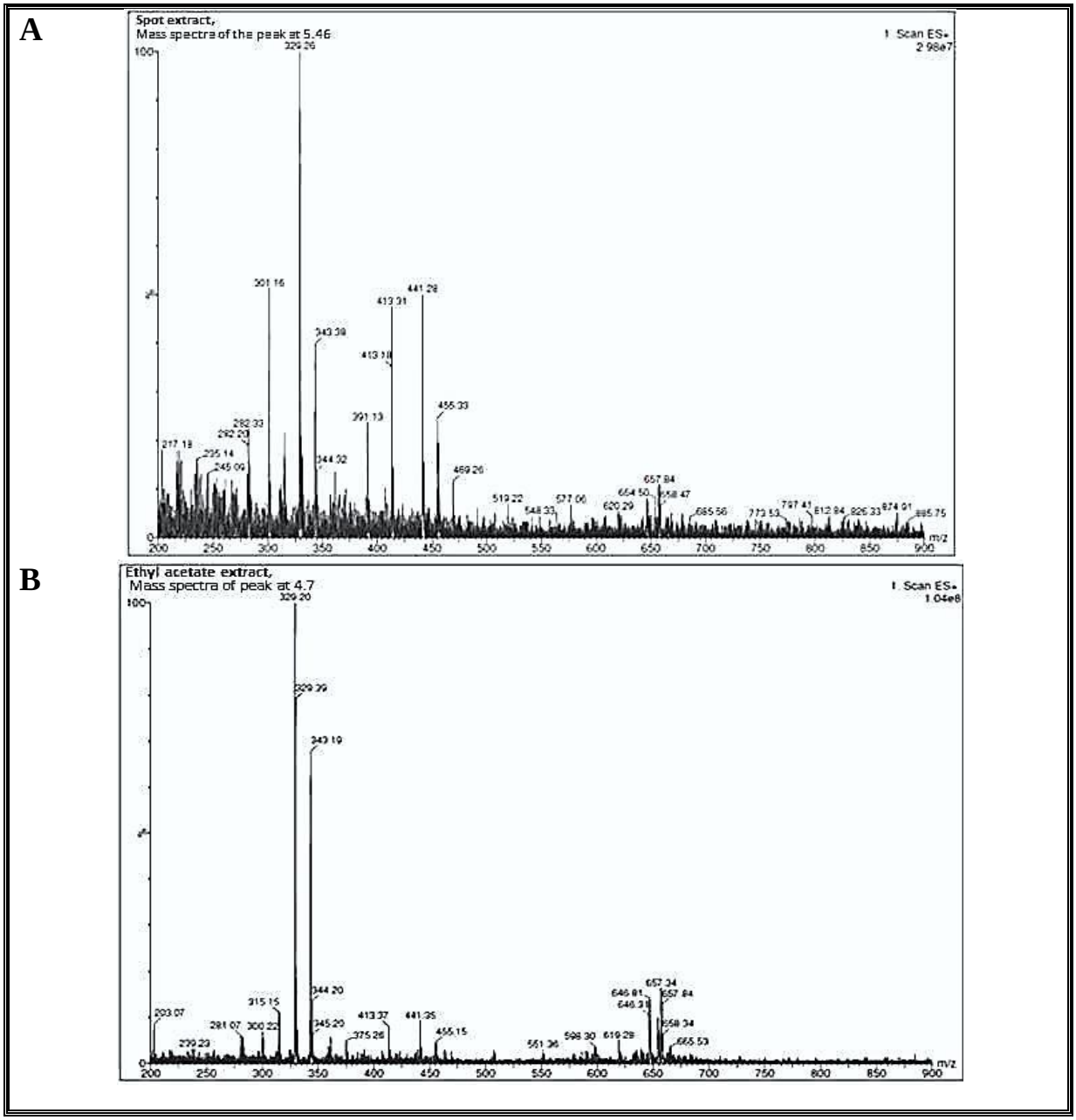


Figure 3.17: Mass spectra analysis of peak (5.47) for both ethyl acetate extract and scraped spot extract, Plate (**A**) represented scraped spot extract mass spectrum analysis while, plate (**B**) represented ethyl acetate extract analysis.

Besides to the results obtained in Figure (3.15), mass chromatogram for both ethyl acetate extract and scraped spot extract, UV absorbance, were carried out for determining the compounds or fragments of a compound found. Moreover, the potential UV absorbance carried out especially at peak 5.25 for both scraped spot extract and ethyl acetate extract. The results presented in

Figure (3.18) summarize all compounds that have a potential UV absorbance at the peaks were previsouly mentioned. In the case of scraped spot extract there were two sharp peaks that appeared to have UV absorbance at 221.85nm and 264.85nm, while in the case of ethyl acetate extract, there were three clearly peaks that appeared to have a potential UV absorbance at 222.85, 264.85 and 442.85 nm, furthermore, the similarity between the two extracts on the basis of UV absorbance was achieved by the second peak of both extracts which are 264.85nm indicating that this compound or fragment of a compounds played an essential role in antimicrobial activities. Our results agreed with those obtained by Pervez *et al.* (2015) that the bioactive compound of the isolated strain with ethyl acetate extract exhibited a maximum UV absorption at 217–221 nm, exactly for the first peak of the scraped spot.

Kurusu and Ohba (1987); Motta et *al.* (2006) and Sudha *et al.* (2013) reported that the maximum UV absorbance for most of the peptide antibiotics exhibited at 210-230 nm and 270-280 nm. Ababutain *et al.* (2012) also described the maximum absorption UV as a spectroscopic characteristic for the bioactive compound recorded at 269 nm.

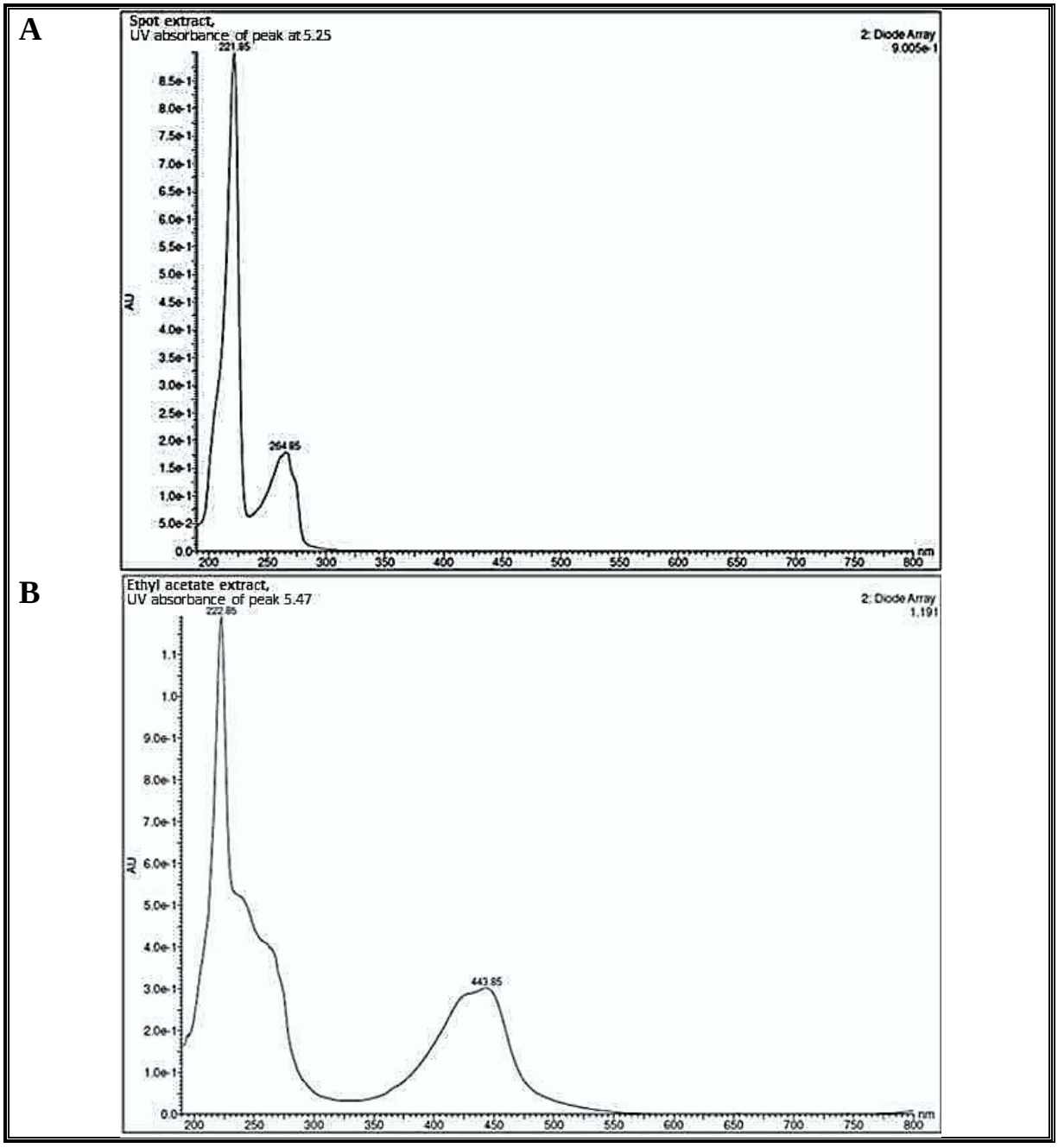


Figure 3.18: UV absorbance analysis of both ethyl acetate extract and scraped spot extract at (peak 5.25), Plate (**A**) represented scraped spot extract mass spectrum analysis while, plate (**B**) represented ethyl acetate extract analysis.

Saadoun *et al.* (1999) obtained a maximum absorbance of their bioactive compounds at 210 -260 nm. Finally Iii *et al.* (2005) recorded a maximum absorbance at 217 and 221 nm for their bioactive compounds extracted from their isolate.

3.8.3 Analyzing Ethyl Acetate Extract of KH14 by Gas Chromatography Mass Spectrometer (GC-MS).

The results presented in Figure (3.19) represent the GC-MS chromatogram of the ethyl acetate extract with all its separated 12 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 area percentage, retention time, the molecular formula and the molecular weight of all 12 compounds were identified as summarized in Table (3.21).

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 KH14 with GC-MS were found through analyzing all 12 peaks with some of their chemical info (chemical structure, mass peak, abundances and companies other names) as presented in Appendices.

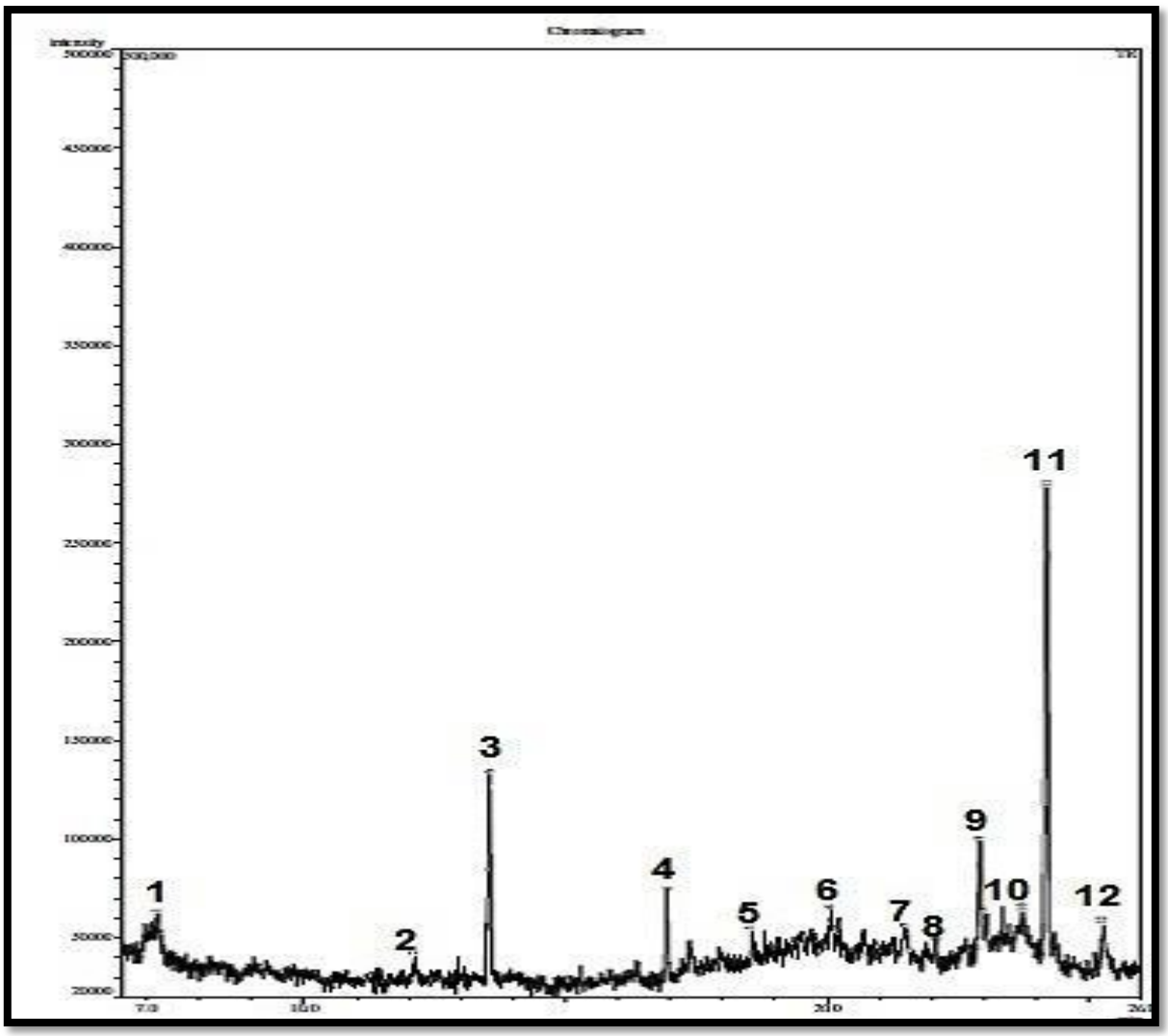


Figure 3.19: GC-MS chromatogram of the ethyl acetate extract of KH14 analyzed by GC-MS, with MS start time: 3.5 minutes and end time: 27 minutes.

The results of the present study agreed, on the basis of existing compounds or their fragments, with those obtained by Schöller *et al.* (2002) who studied the volatile compounds existent in actinomycetes ,they reported that among the most frequently produced compounds were acetone,isoprene,2-methyl-1-propanol, 1-butanol, 2-methyl-1-butanol, 3-methyl-3-buten-1-ol, 3-methyl-1-butanol, cyclopentanone, dimethyl disulfide, 2-phenylethanol, dimethyl trisulfide, and geosmin.

Table 3.21: GC-MS analysis profiles were applied for ethyl acetate extract of KH14 isolate

Peaks	R. time	Area%	Height%	Name	Molecular Formula	Molecular weight
1	7.22	10.69	3.85	2-Butanone, 3-hydroxy-	C4H8O2	88
2	12.12	2.39	2.32	DL-3-Methyl-2-butanol, acetate	C7H14O2	130
3	13.55	16.31	18.21	3-Heptanol, 4-methyl-	C8H18O	130
4	16.93	4.22	7.96	Hexadecanoic acid, methyl ester	C17H34O2	270
5	18.56	1.49	2.72	n-Pentadecanol	C15H32O	228
6	20.08	3.40	3.54	Piperazine-2,5-dione, 1,4-dimethyl-3,3'-bis-	C12H18N4 O4	282
7	21.51	3.00	2.21	1,2-Benzenedicarboxylic acid, diisooctyl ester	C24H38O4	390
8	22.08	1.25	1.93	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	C16H22O4	278
9	22.92	7.85	9.29	Pentadecanoic acid	C15H30O2	242
10	23.73	8.41	3.41	.alpha.-Tocopherol-.beta.-D-mannoside	C35H60O7	592
11	24.20	35.63	40.83	Hexanedioic acid, bis(2-ethylhexyl) ester	C22H42O4	370
12	25.28	5.35	3.73	n-Hexadecanoic acid	C16H32O2	256
		100.00	100.00			

Peak 8 with retention time 22.08 and molecular weight 278 Daltons represented 1, 2-Benzenedicarboxylic acid, bis (2-methylpropyl) ester. This

compound exhibited an antimicrobial activity in the study of Sastry and Rao (1995) against a number of bacteria. Furthermore, this compound with another company called (Diisobutyl phthalate) also accomplished another biological activity like eliminating tumor cells in the bone marrow. This was also documented by Nguyen, *et al.* (2007) who studied the effect of diisobutyl phthalate on the melanogenesis inhibition.

Peak 12 represented n-Hexadecanoic at retention time 25.28 minutes. This compound was also found by both Saravana Kumar *et al.* (2014) and Jalaluldeen *et al.* (2015) in their profiles analysis of GC-MS from their isolated strains of actinomycetes extracted with ethyl acetate.

3.9 Antitumor Activity of KH14 Extracellular Crude Extract

Determining the antitumor activities of extracellular crude extract of KH14 was carried out using MTT assay against different cancerous cell lines, like MCF7, PC3 and A375, through using different concentrations 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.562, 0.781, 0.390, and 0.195ug/mL. After incubating the cell lines with the mentioned concentrations for 72 hrs, at 37^0C the optical densities were recorded at 570nm in triplicates trial.

The results presented in Table (3.22) summarize the mean percent cell viability with its standard deviation for all cell lines used.

Table 3.22: Antitumor activity of KH14 extracellular crude extract on A375, PC3 and MCF-7 cancer cell lines.

Concentration ug/mL	Cell viability%		
	A375	PC3	MCF-7
	Mean± SD	Mean± SD	Mean± SD
400	46.870±1.808	69.1757±2.754	24.1576±14.636
200	77.796±1.225	74.9958±0.536	67.5356±10.018
100	89.246±5.068	86.7365±7.205	78.1615±8.540
50	94.329±3.536	93.8782±2.400	79.7513±8.734
25	95.559±3.792	94.9036±3.129	83.7888±7.449
12.5	97.967±3.298	96.3146±2.051	86.2923±6.175
6.25	99.465±3.383	97.7117±2.679	89.3378±3.444
3.125	100.107±3.034	98.3766±3.034	92.1626±2.339
1.5625	101.070±2.414	100.4862±2.794	93.8223±2.921
0.78125	101.498±3.012	102.0592±0.755	95.5351±2.579
0.390625	102.568±3.161	103.1014±0.356	96.6806±3.948
0.195313	105.564±8.140	104.1771±0.975	98.0274±3.930

The results listed in Table (3.22) only highlighted the concentrations 400, 200, 100, 50, 25, 12.5 ug/mL that gave variable results, while the rest of other results remained somewhat constant values. For all cell lines 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 .

The results presented in Figure (3.20) summarize the effects of different extracellular crude extract concentrations on PC3, the concentration 400 ug/mL exhibited the highest result 30.84% of the PC3 cells inhibited which means that can not form the final formazan product. However, 69.18 of the cells were remains viable during the entire incubation process. On the other hand, the lowest results was recorded by 12.5 ug/mL that means only 3.69 of the PC3 cell line were inhibited during the incubation process.

Results shown in Figure (3.21) represent the antitumor activities of extracellular crude extract of KH14 on the MCF7. The highest result (cell inhibition) was recorded by 400 ug/mL exactly 75.84% of MCF-7 were inhibited after 72hr incubation which means only 24.16% of MCF-7 were able to forminsoluble formazan products which remained as alive cells. On the other hand, the 12.5 ug/mL concentration recorded the lowest result only inhibits 13.71% of the breast cancer cells) as presented in Figure (3.23).

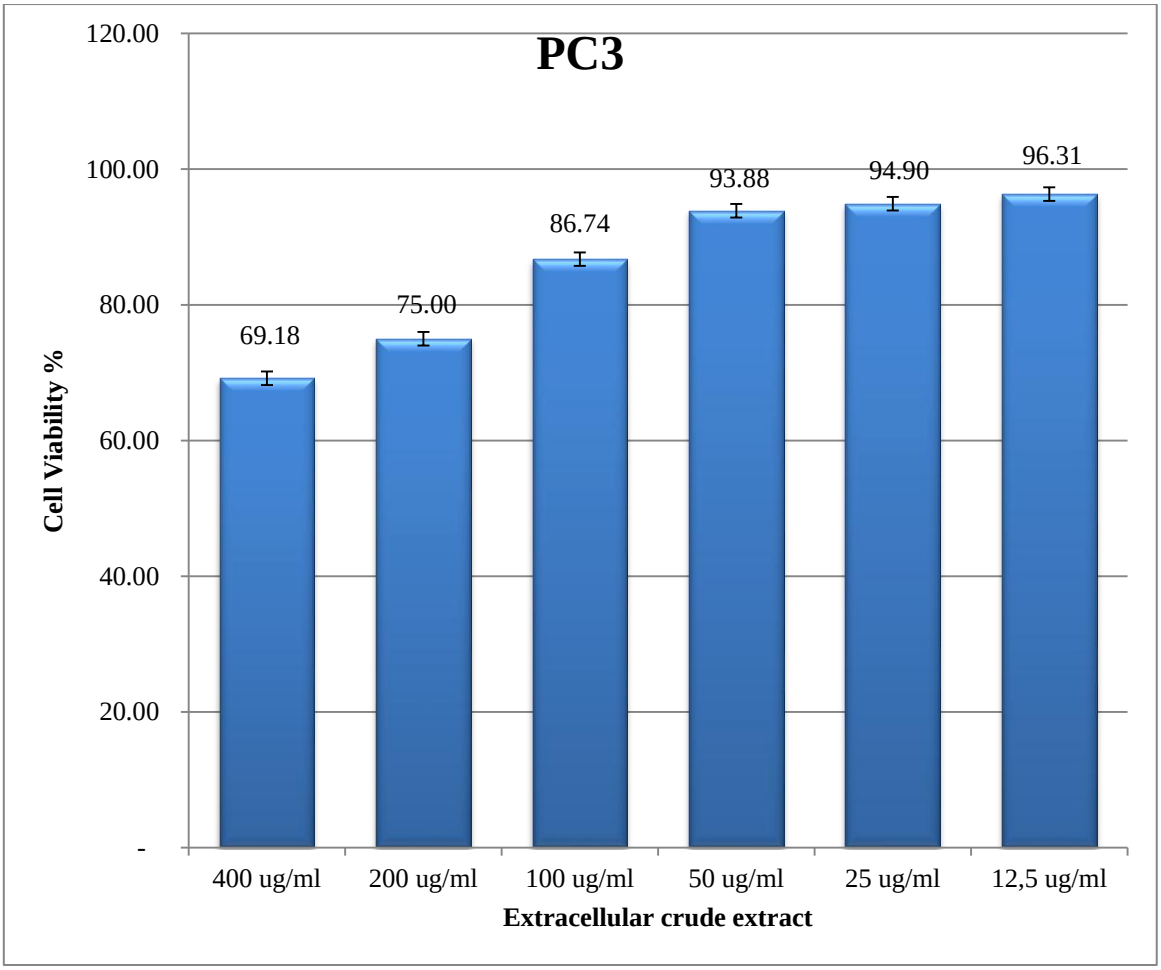


Figure 3.20: Antitumor activities of KH14 extracellular crude extract on PC3 cancer cell line

These results were higher than those of Abd-Elnaby *et al.* (2016) who obtained 56% inhibition of MCF-7 cell line by the activity of their isolated strain of actinomycetes EI-4 after 72 hours of incubation via using MTT assay. our extract inhibit 75.84% of MCF-7 cell line after 72 hours of incubation, and also than Huyen *et al.* (2014) who reported that the actinomycetes strain HP411 only inhibited 39.8% of MCF-7 cell line.

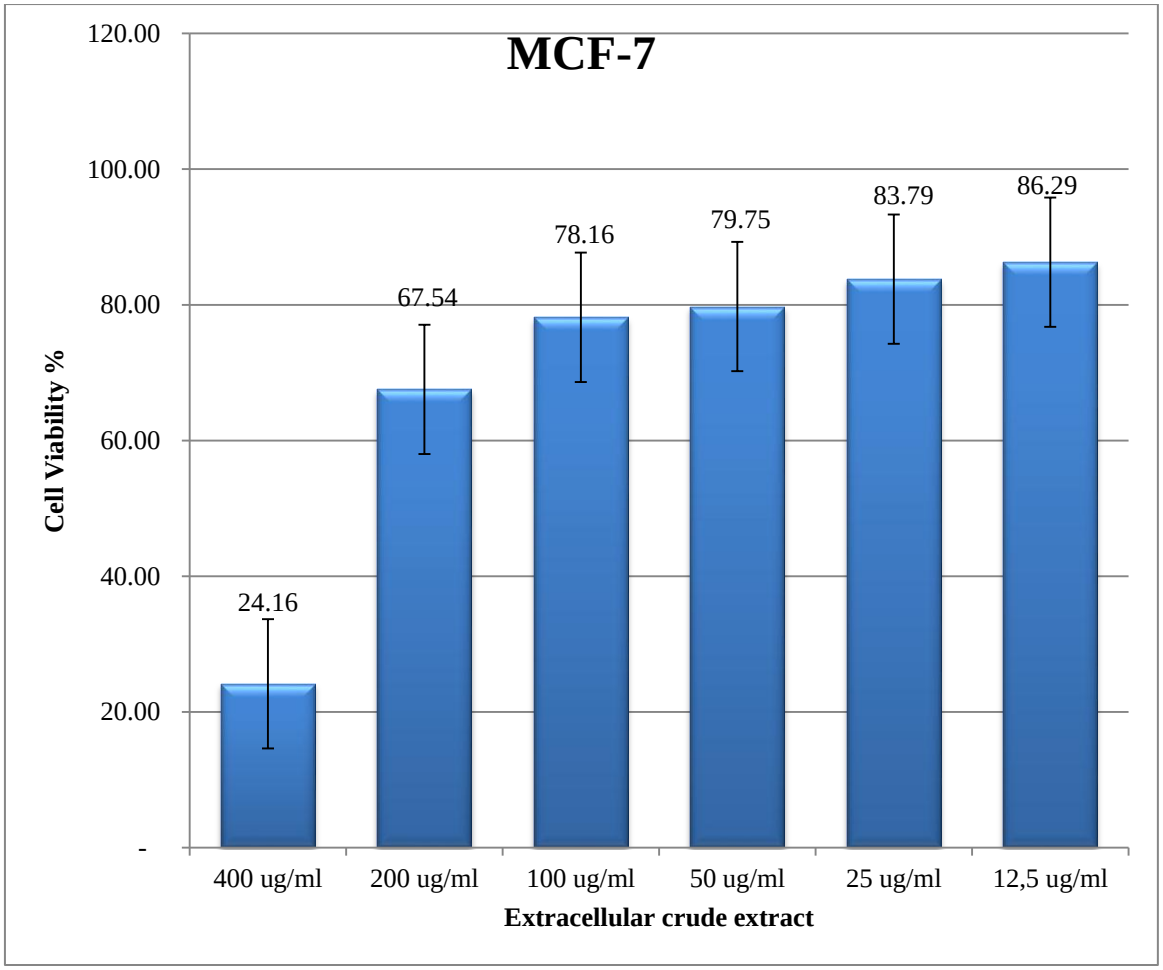


Figure 3.21: Antitumor activities of KH14 extracellular crude extract on MCF-7 cancer cell line

The results in Figure (3.22) summarize the antitumor activities of extracellular crude extract KH14 on the A375. The highest result for cell inhibition was recorded by 400 ug/mL, exactly 53.1% after 72hr of incubation, meaning only 46.9% of A375 were able to form insoluble formazan products which remain as live cells. On the other hand, 100, 50, 25 and 12.5 ug/mL concentrations recorded the percentage of cell viability as 77.8%, 89.2%, 94.3%, 95.6% and 98.0% respectively. Besides, the lowest results which were recorded by 12.5 ug/ml, only inhibit 2% as presented in Figure (3.23). These results were in agreement with those obtained by Saravana Kumar *et al.* (2014). The effect of

extracellular crude extract was achieved with a concentration-dependent manner (dose dependent).

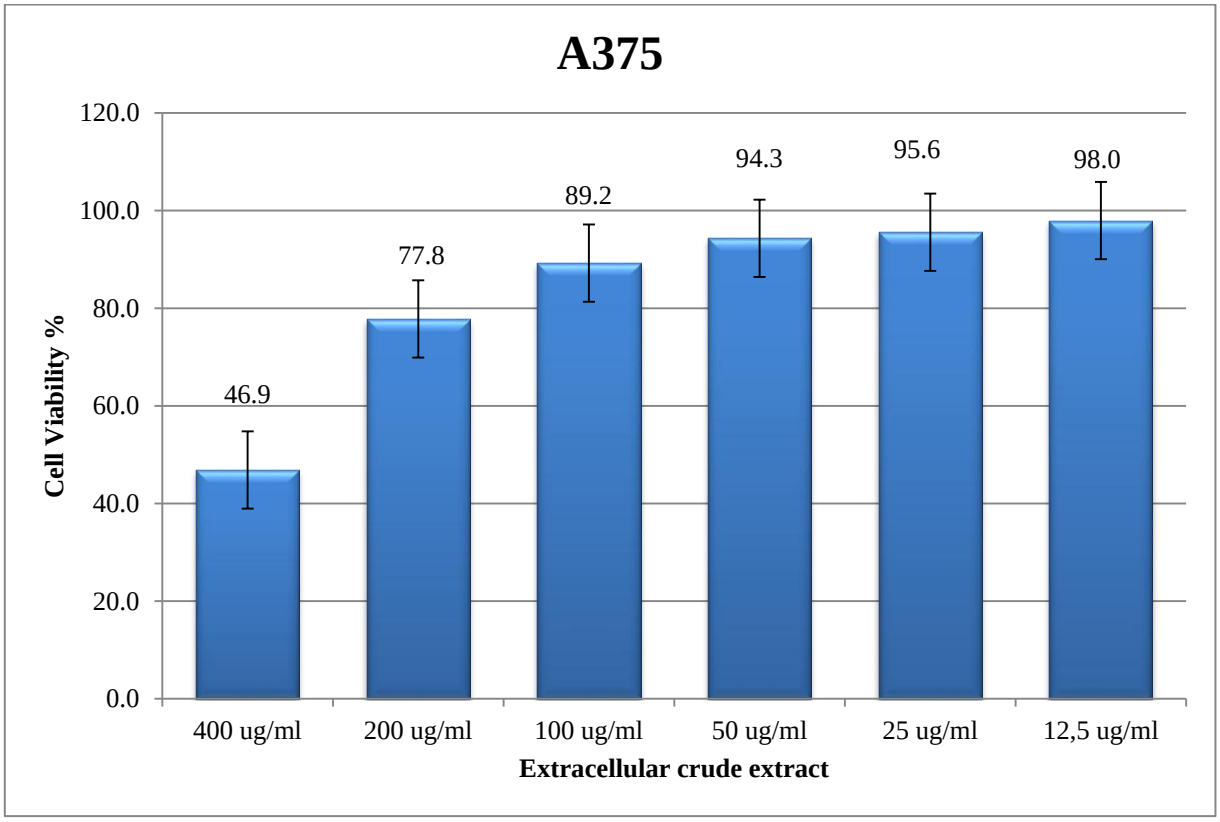


Figure 3.22: Antitumor activities of KH14 extracellular crude extract on A375 cancer cell lines.

The antitumor activities of KH14 extracellular crude extract among all cancer cell lines were studied. The MCF-7 was the most one that highly responded to the KH14 extracellular crude extract through inhibiting the metabolically active cancer cells, while, A375 and PC3 cancer cell lines became as second and third responded respectively. However, the MCF-7 cell lines were used to complete the study, through studying their high content screen (HCS) assay to determine the exact changes that occur during the incubation period at the levels of cell size, morphology and some internal components, increasing the extracellular crude extract concentration from 12.5 to 400 µg/mL against cancer

cell lines viability for PC3, MCF-7 and A375, their cytotoxicity was increased as well as the cell viability was decreased. Our results were in agreement with those obtained by Mohsen *et al.* (2015) from their study for screening new bioactive compounds against MCF-7 and A549 cell lines from crude extract fraction in which the effect was dose dependent.

Mueller *et al.* (2002) and Azambuja *et al.* (2005) stated that the Antitumor antibiotics (antitumorantibiotics / cytotoxic) are medications that prevent and fight the growth of tumors, and the antitumor antibiotics produced by *Streptomyces* species are precious in the medical field.

The results agreed with those described by Ravikumar *et al.* (2008) and Rashad *et al.* (2015) who evaluated some genus of actinomycetes especially *Streptomyces* to have energetically product compounds of functional bioactive metabolites with a broad range of pharmaceutically application such as antimicrobial metabolites, anthelminthic, antiviral and antitumor agents. Their in *vitro* applications of cytotoxicity assay from the secondary metabolites against different cancerous cell lines showed a significant antitumor activity, and this effect was dependent on dose, isolated strains and types of cancerous cell lines.

Ayuso *et al.* (2005) also confirmed that some isolated actinomycetes strain such *Streptomyces* are talented to produced dissimilar antitumor complexes with various chemical natures because they anchorage different gene clusters which encoded poly-ketide and non-ribosomal peptide synthases. Bode *et al.* (2002). On the other hand described the way which can be used to isolate and extract these bioactive metabolites by using different organic solvents because these compounds were secreted into culture broth. Finally, Wadkins *et al.* (1998) summarized the mechanism action of some of these compounds isolated and extracted from some species of *Streptomyces* through intercalating with genetic

materials especially with duplex DNA which cause harmful effects on fast multiplying cells by preventing the DNA dependent RNA polymerase activities.

3.10 Cytotoxicity Effect of KH14 Extracellular Crude Extract via High Content Screen on MCF-7

As described by Haskins et *al.* (2001); Diaz *et al.* (2007) and Abraham *et al.* (2008), analysis at the level of individual cells played key roles in finding toxicity and categorization of compounds based on their observed cellular damage and by flow cytometers and cellular imaging–based approaches using such a High Content Screening (HCS).

Six cellular parameters valid cell count, total nuclear intensity, cell membrane permeability, nuclear morphology, cytochrome c and mitochondrial membrane potential were detected after 24 hour of incubation at 37^{o}C with four different concentrations of KH14 extracellular crude extract 25, 50, 100 and 200 ug/ml. Positive control 5.0 μM of paclitaxel was used on MCF-7 cell lines. The results presented in Table (3.23) and Table (3.24) summarizes the data for extracellular crude extract concentrations and average intensities for each studied concentrations. Besides, each treatment was studied with duplicated trials.

The positive control paclitaxel used in the study was a tubulin targe which is considered as their mechanism of action. The Paclitaxel treated cells have a force with the spindle assembly, cell division and also chromosome segregation which was contrary in character to colchicine, a drug that also targets tubulin, whereas Paclitaxel exactly stabilizes and guards microtubule against disassembly as described by Bharadwaj and Yu (2004); Ganguly *et al.* (2010) and Priyadarshini and Keerthi (2012).

Table 3.23: Cytotoxicity effects of KH14 extracellular crude extract on MCF-7 for multi-cellular parameters.

Con. ug/ml	valid object count		Nuclear morphology		Total nuclear intensity	
	Mean± SD	P value	Mean± SD	P value	Mean± SD	P value
Positive control	2108± 155.56***	0.000	15.565± 0.07*	0.030	447.61± 9.1***	0.000
200	2884± 289.20**	0.004	16.225± 0.21	0.3790	341.24± 14.0	0.985
100	3265± 94.04	0.051	16.37± 0.16	0.085	360.5± 0.79	0.147
50	3297± 35.35	0.066	16.52± 0.02*	0.020	368± 8.79	0.051
25	3333± 45.25	0.090	16.47± 0*	0.031	361.8± 9.17	0.124
Vehicle (Negative control)	3754± 77.78		16.02± 0.02		336.95± 9.65	

* Significant differences when p value ≤ 0.05; ** p value ≤ 0.01 and *** p value ≤ 0.001

Macroscopical analysis of the studied parameters and images was taken by fluorescent Microscope Zeiss Axio Z1 with Photometrics X1 CCD processing via softwares that quantify the targets (intensity of appearances) of six cellular parameters, all summarized in Figure (3.23) which compares between 200 μg/ml of extracellular crude extract and 5.0 μM of paclitaxel on one side, and with untreated cell line (vehicle) on the other side. In the studies of Towne *et al.* (2012) and Joy *et al.* (2014), they reported that high content images (HCI) have been widely used for studying the change in the functional characteristics and morphological properties in cancer researches such as motility, growth, proliferation, and death by antitumor drugs.

Table 3.24: Cytotoxicity effects of KH14 extracellular crude extract on MCF-7 for multi-cellular parameters.

Con. ug/ml	Cell membrane permeability		Mitochondrial membrane potential		Cytochrome c	
	Mean± SD	P value	Mean± SD	P value	Mean± SD	P value
Positive control	37.61± 7.42	0.063	218.00±11.12***	0.000	462.04± 31.39**	0.004
200	47.04± 11.51	0.317	263.8± 11.58**	0.004	418.56± 3.95	0.059
100	56.33± 4.57	0.960	260.49± 3.61**	0.003	351.79± 15.51	0.928
50	86.42± 7.32*	0.038	278.97± 2.77*	0.021	354.85 4.06	0.977
25	59.31± 1.73	1.000	274.82± 2.30*	0.013	358.81± 1.73	0.998
Vehicle (Negative control)	60.48± 6.46		309± 4		363.42± 21.7	

* Significant differences when p value ≤ 0.05 **. p value ≤ 0.01 and *** p value ≤ 0.001

Ignatius and Hung (2007) stated that a main factor in the improvement of HCS technology is the capacity to recognize the exact proteins or cellular constructions with immune reagents, organic dyes, genetically encoded fluorescent proteins or quantum dots (QDs). Moreover, any fluorescent organic dye was used as a biosensor to identify the physiological alterations in the cell or to label exact organelles, including the nucleus, mitochondria, cytosol, endoplasmatic reticulum, lysosomes and Golgi apparatus. Furthermore, an accumulative diversity of fluorescent antibodies and probes are now available to

maintain many HCS applications, and for the kinetic HCS experiments with alive cells although they were not always suitable.

Davies *et al*. (2008) and Cheah et *al*. (2011) mentioned that all five commonly cellular multi-parameters including cell viability, membrane permeability, mitochondria membrane permeability, cytochrome c and nuclear intensity will afford information about the change within each cell at the level of quantitative.

The cellomics multi-parameter cytotoxicity 3 kit enables simultaneous measurements in the same cell of six independent parameters that monitor cell health, including cell loss (valid object count), nuclear size (total nuclear intensity) and nuclear morphological changes, mitochondrial potential changes, the changes in cell permeability and cytochrome c release. The Hoechst dye enables checking of cell loss, nuclear morphology changes and total nuclear intensity, which is relative to the total Hoechst intensity for each nucleus, while other three parameters are monitored by separate dyes, as summarized in Figure (3.23).

The results presented in Table (3.23) and Figure (3.24) summarizes the cytotoxicity effects and statistically analysis of different concentrations of KH14 extracellular crude extract on valid cell counts for MCF-7 after 24h of incubation. O'Brien *et al.* (2006) situate the verification that one of the most sensitive cell health indicators was cell proliferation and this had a direct correlation between the effects of toxicity of any substance and the changes in the viability of cell counts. However, there were significant differences between the vehicle (untreated) sample with a positive control and 200 ug/ml concentrations when (p value =0.000 and p=0.04 respectively), while the rest of other concentrations did not show any significant differences with the untreated

sample. Furthermore, Figure (3.25) summarize the correlation between the average intensity and KH14 extracellular crude extract activity, indicating that the decrease of valid cell numbers were found to be dose-dependent between the used concentrations with $R^2 = 0.8213$, which indicates the presence of a high correlation between the dose and the intensity. Besides, increasing the concentration will lead to decrease the intensity.

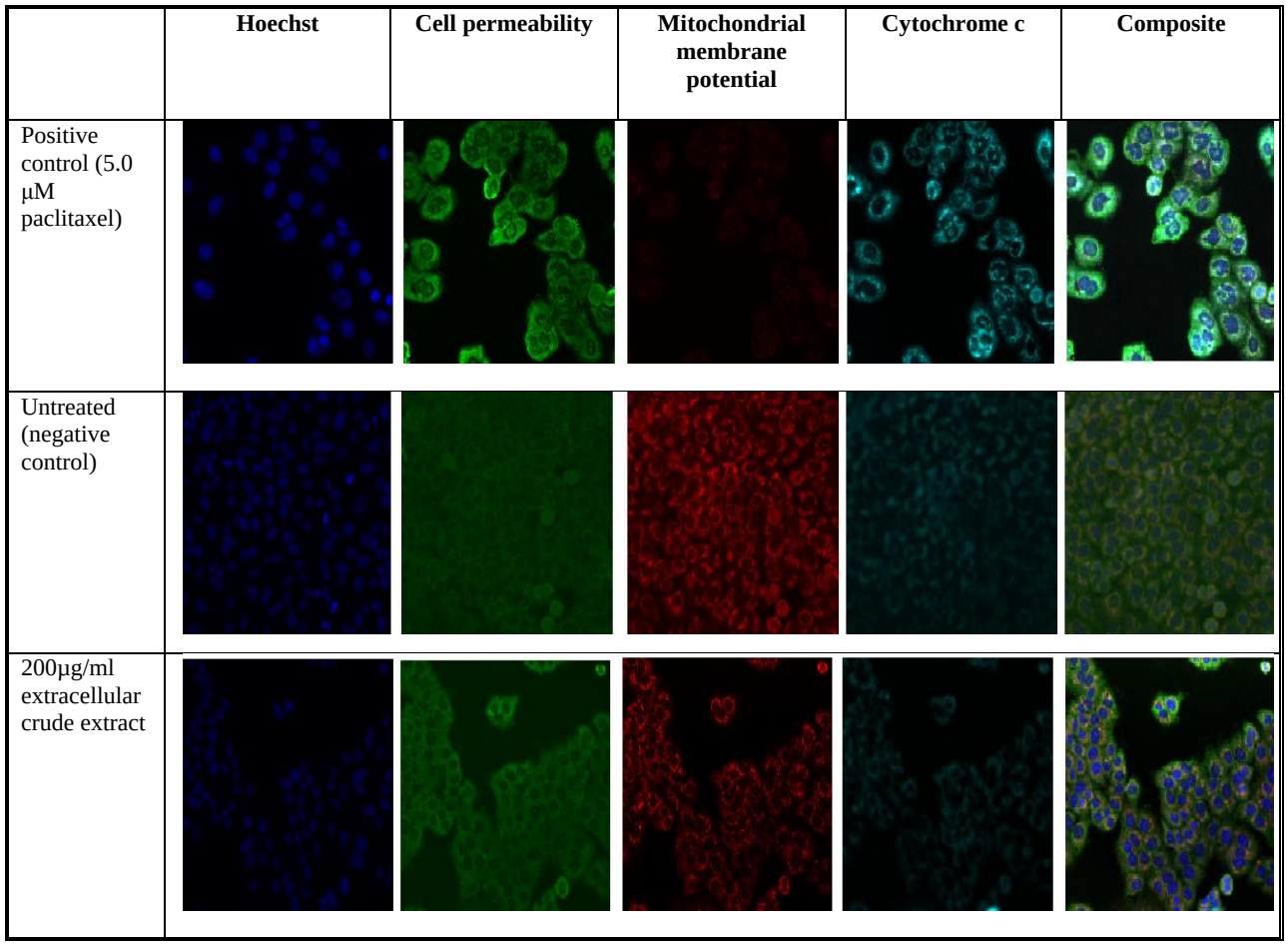


Figure 3.23: Entire cytotoxicity effect of KH14 extracellular crude extract with positive and negative control on MCF-7 cancer cell line multi-cellular parameters.

The results were in agreement with those obtained by Al Barzanchi (2014) and Hassan et *al.* (2015) who also found that the cytotoxicity effect was dose–dependent. The targeted organelle for cell viability assay was cytoplasm as

described by Donato *et al.* (2012) and Tolosa *et al.* (2012) when Propidium iodide fluorescent probe was used and the color appeared as red.

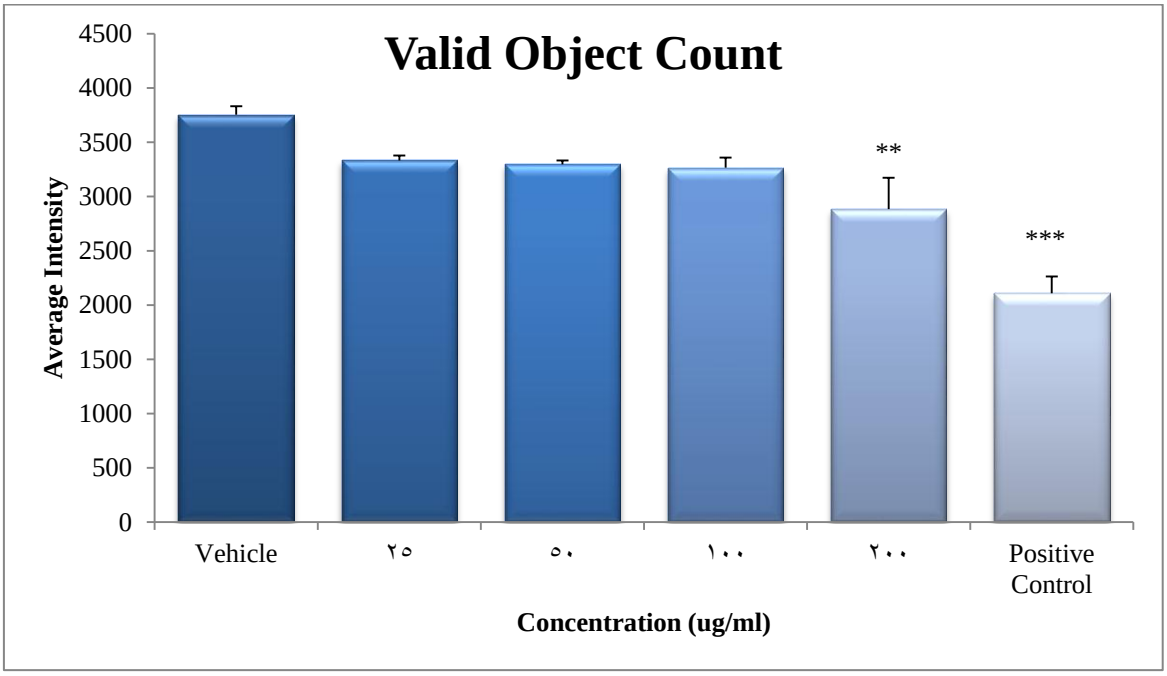


Figure 3.24: Cytotoxicity effects of KH14 extracellular crude extract on MCF-7 cancer cell line valid cell count

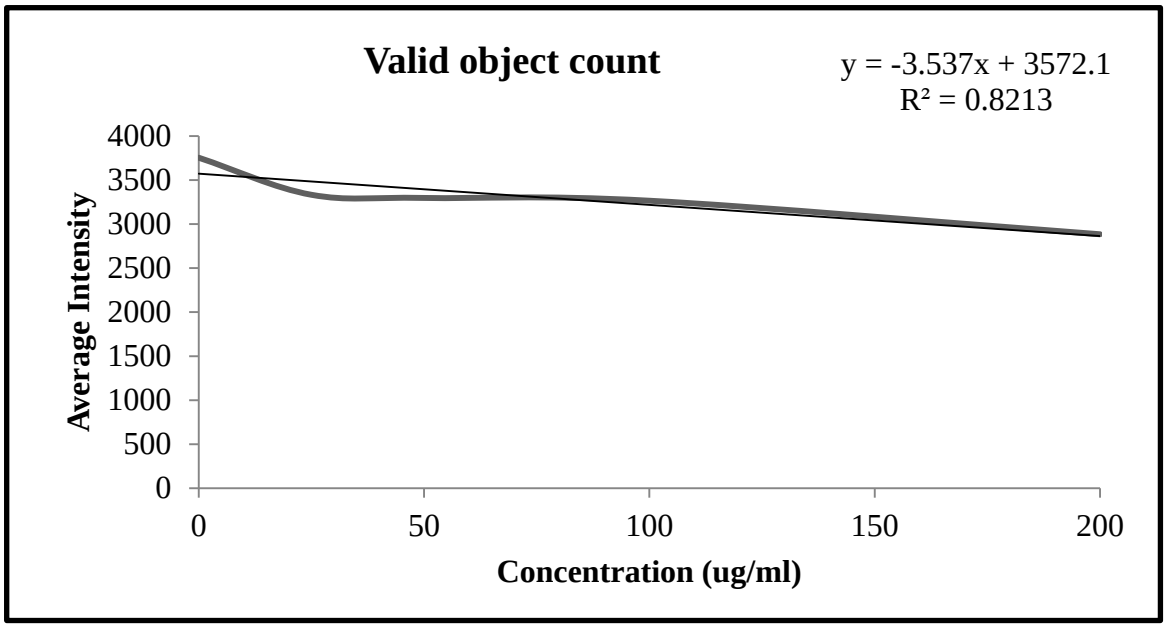


Figure 3.25: Correlation between concentrations of extracellular crude extract and average intensity for valid object count on MCF-7

The results presented in Table (3.23) and Figure (3.26) summarizes the cytotoxicity effects and the statistical analysis of different concentrations of KH14extracellular crude extract on nuclear morphology for MCF-7 after 24h of incubation. The positive control, 50 ug/ml and 25 ug/ml of extracellular crude showed significant differences with the vehicle (p value = 0.030, 0.020 and 0.031 respectively), while 200ug/ml and 100 ug/ml did not show any significant difference with the negative control. Nuclear morphology assay in HCS analysis, as described by Harrill *et al.* (2013) and Sirenko *et al.* (2014), was achieved throughout the nucleus which considered as the target organelles of it. On the other hand, it appeared blue in color when stained with the fluorescent probe Hoechest 33258. The lowest average intensity was obtained by positive control (15.565) when compared to the vehicle which recorded the average intensity (16.02). We concluded that each decrease in average intensity of nuclear morphology will lead to more destruction of the nuclear through changing its shape, as well as destroying the overall cell and finally producing apoptosis by the action of tested drugs. This agrees with Ranade *et al.* (2014) who demonstrated the platform for studying the drug responses against cancer cells via applying the high content images (HCI) such as nuclei morphology, nuclei density, apoptosis and cell proliferation.

The results presented in Figure (3.27) summarize the correlation between average intensity and KH14 extracellular crude extract, with a correlation R^2 = 1E-04, which explains that any increase in the extract concentration would affect the average intensity until 50 ug/ml concentration. While after 50 ug/ml, the correlation became the opposite an increasing concentration led of decreasing their average intensity.

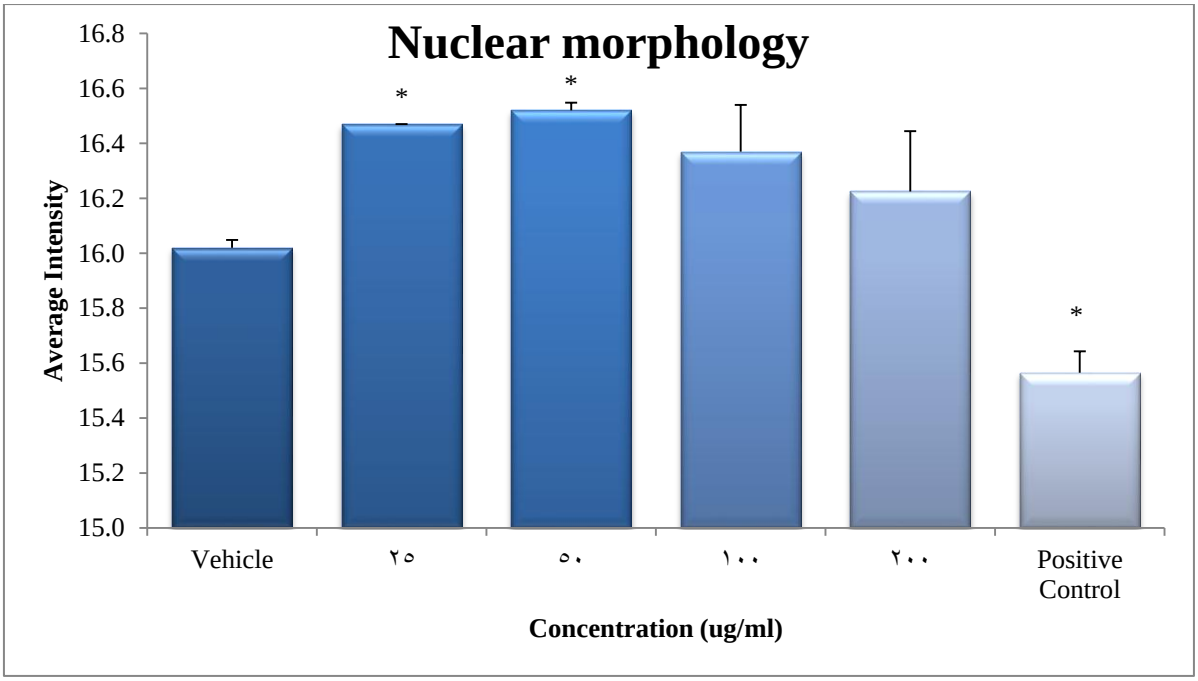


Figure 3.26: Cytotoxicity effects of KH14 extracellular crude extract on nuclear morphology of MCF-7 cancer cell line

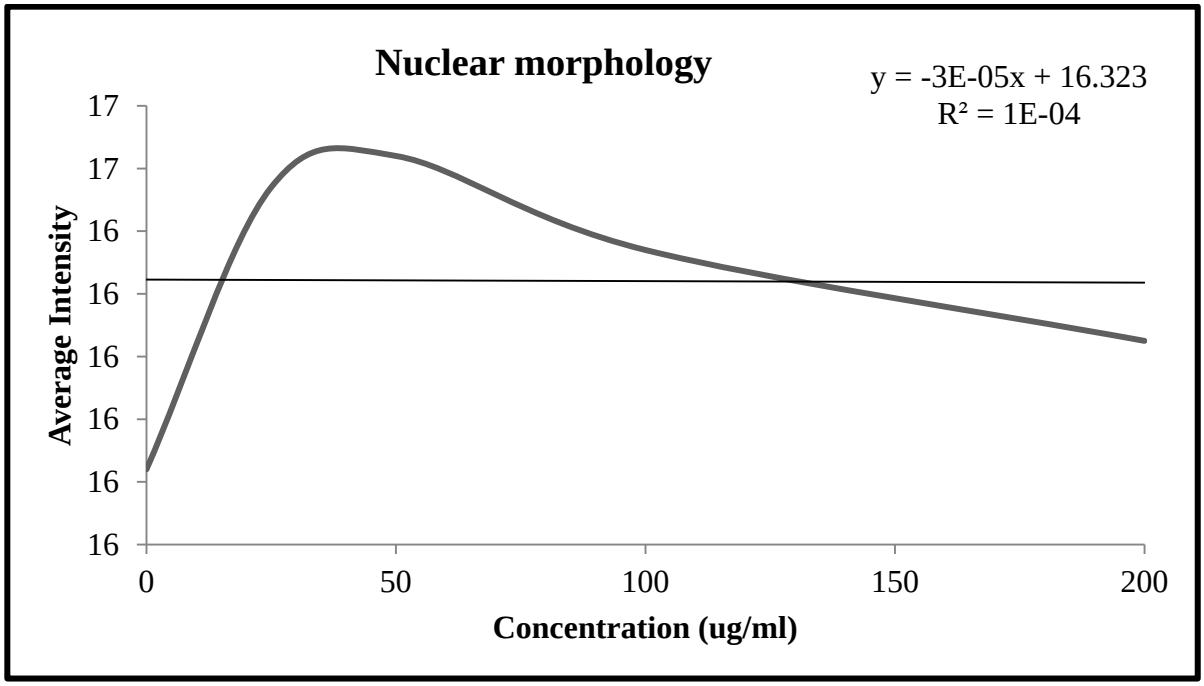


Figure 3.27: Correlation between concentrations of extracellular crude extract and average intensity for nuclear morphology on MCF-7.

The results presented in Table (3.23) and Figure (3.28) summarizes the cytotoxicity effects and the statistical analysis of different concentrations of KH14 extracellular crude extract on the total nuclear intensity for MCF-7 after 24h of incubation. The results showed that there were no significant differences of used concentrations against the negative control (vehicle), while only the positive control showed significant differences with untreated sample (vehicle) with (p value =0.000). The highest result among the used concentrations was achieved by 50 ug/ml. Furthermore, as the data presented in Figure (3.29), the correlation between the average intensity and KH14 extracellular crude extract recorded a correlation with $R^2 = 0.043$, in which after 50 ug/ml each increase in the concentration of extracellular crude extract would lead to decreasing the average intensity. However, this relationship between the concentration and the amount of the intensity will be in relation to dose- dependent. Our results in case of dose- dependent were in agreement with those obtained by Al-barazanchi (2014) in her study of antioxidant and cytotoxic effects of Lignan Purified from *Myristica fragrans* seeds and from that related with the total nuclear intensity.

According to Hassan *et al.* (2015) one of the most abundant hallmarks of the apoptosis was related to the nuclear condensation and fragmentation. However, any destruction in the nuclear will lead to increasing the average intensity as clearly noticed in the positive control which recorded the highest value 447.61.

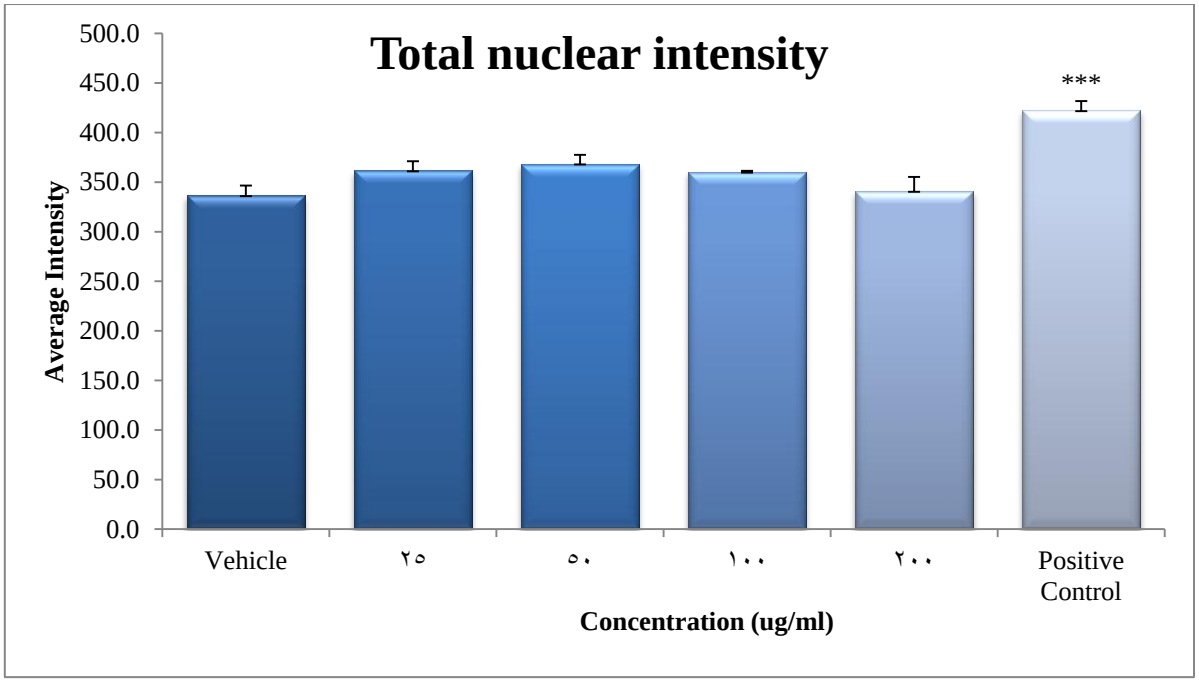


Figure 3.28: Cytotoxicity effects of KH14 extracellular crude extract on total nuclear intensity of MCF-7 cancer cell line.

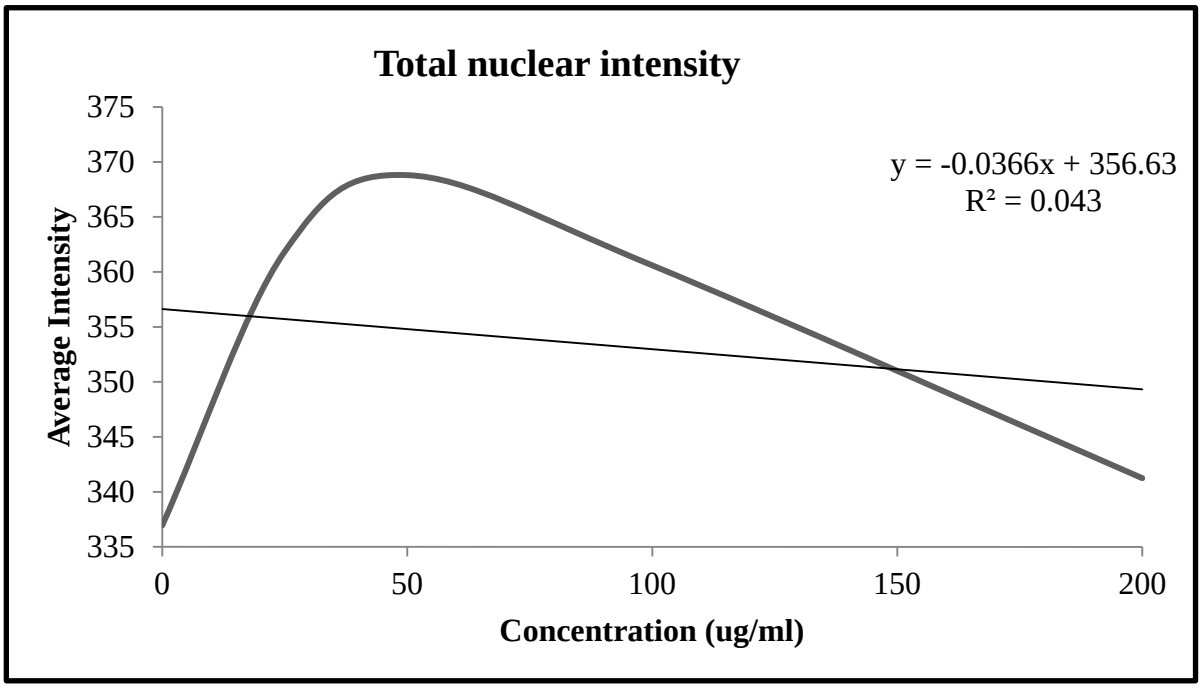


Figure 3.29: Correlation between concentrations of extracellular crude extract and average intensity for total nuclear intensity on MCF-7.

The results listed in Table (3.24) and Figure (3.30) summarizes the cytotoxicity effects and the statistical analysis of different concentrations of KH14 extracellular crude extract on the cell membrane permeability for MCF-7 after 24h of incubation. The analyzed data indicated that only 50ug/ml concentration showed highly significant differences with (p value =0.032) against untreated sample (vehicle) which recorded the highest data (86.42), while the rest of the concentrations did not show any significant differences. Furthermore, this was considered as one of the most important factors that facilitate the passage of effective material into the cancerous cell line via membrane only with this concentration. The results presented in Figure (3.31) summarize the correlation between intensity and extracellular crude extract KH14 concentration when recording the correlation with $R^2 = 0.2616$, which indicated that there was a very low correlation between used concentration and obtained intensity. In addition to this achievement, the target organelle for determining the cell membrane permeability was nucleus, and this was described by Hakanson *et al.* (2014) and Jahr *et al.* (2015) when staining with fluorescent probe TO-PRO-3 and the results appeared as red.

The role of cell membrane permeability in studying cytotoxicity was described by Abraham *et al*. (2008). In their study, they reported that any change in the cell membrane permeability as well as are often associated (related) with either an ongoing toxic (were occurring actually every day in our bodies) or apoptotic responses, and the common phenotypic feature of cytotoxicity noticeable lost cell membrane integrity.

The appearance of significant differences only with 50ug/ml concentration may be considered as a critical point to be discussed in details because as described by Bova et *al.* (2005) and Dykens *et al.* (2008) all assays

carried out for HCS can measure the cell's physiological replies to stimulus and these may be chemical or environmental motives, and relatively from simple measures of acute cytotoxicity, such as cell rounding and cell counting with extra detail measuring of organelle health, often as a multi-parameter assay where cross correlation of multiple endpoints describe slight toxic conditions.

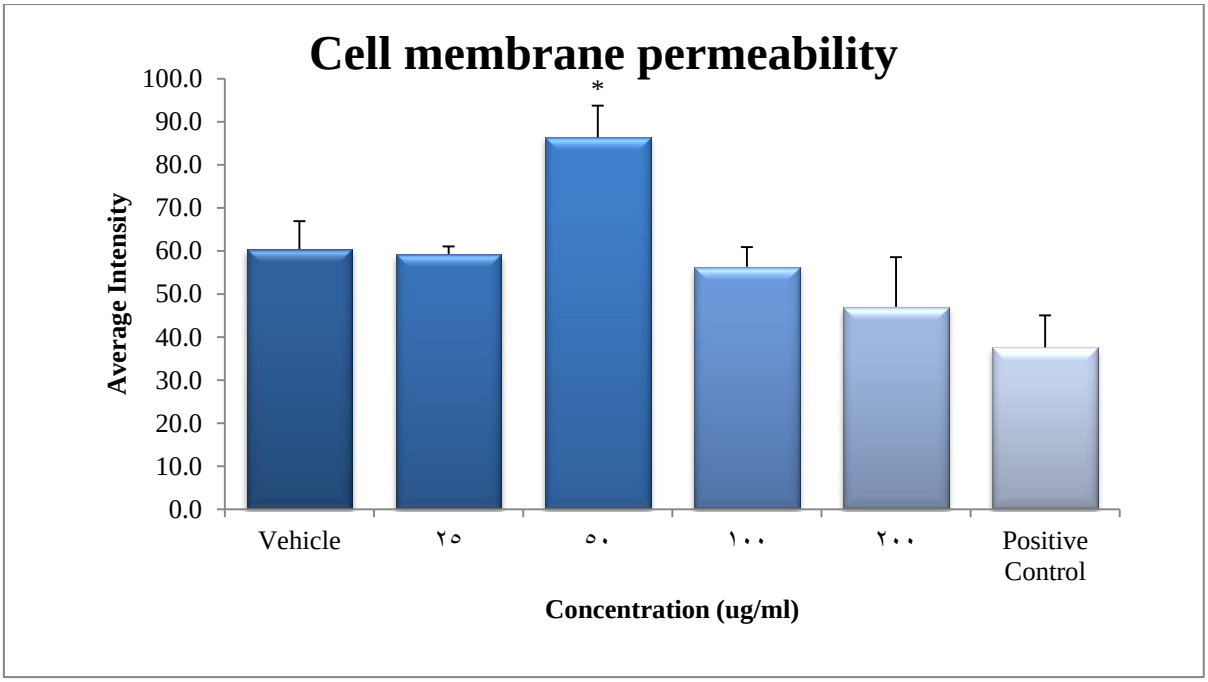


Figure 3.30: Cytotoxicity effects of KH14 extracellular crude extract on cell membrane permeability of MCF-7 cancer cell line.

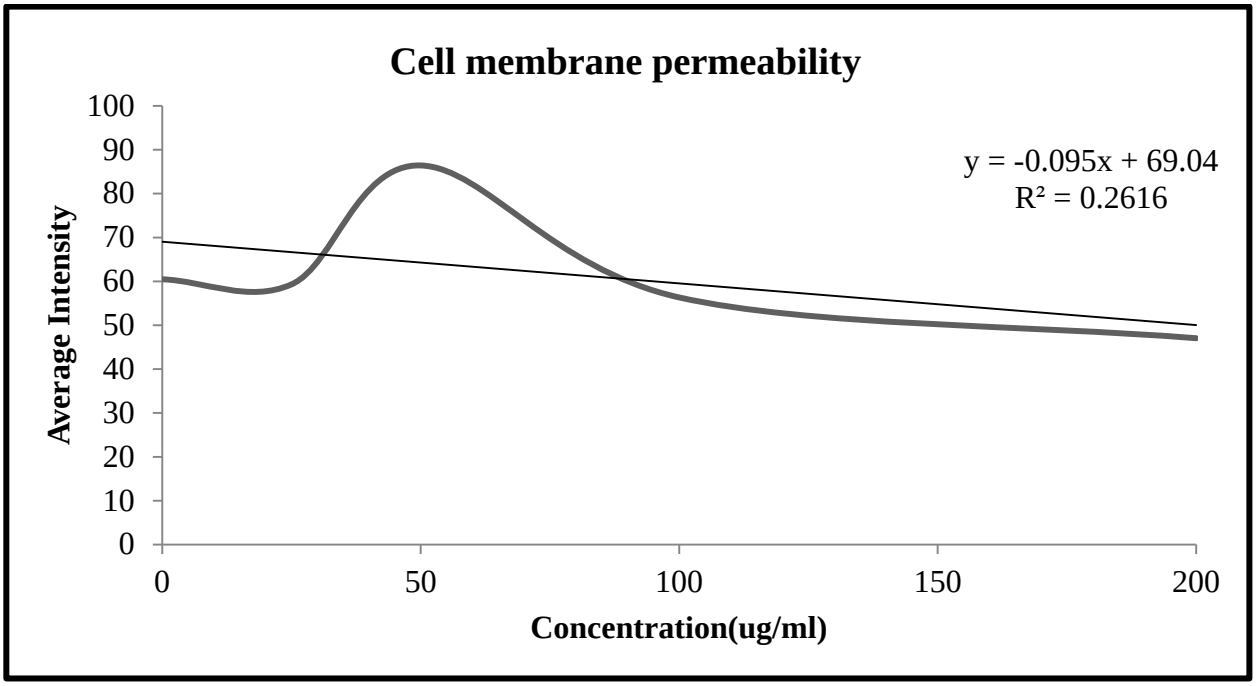


Figure 3.31: Correlation between concentrations of extracellular crude extract and average intensity for cell membrane permeability on MCF-7.

The results presented in Table (3.24) and Figure (3.32) summarizethe cytotoxicity effects and the statistical analysis of different concentrations of KH14 extracellular crude extract on mitochondrial membrane potential for MCF-7 after 24h of incubation. The results indicated that all concentrations showed significant differences against untreated sample (vehicle) with different p values which means that all used concentrations were able to penetrate the mitochondrial membrane, as well as changing the cancer cell intensity when compared to the untreated one. The positive control on the other hand has a powerful ability to change the intensity of the cancer cell line (MCF-7), when recording the lowest average intensity 218.005. The results presented in Figure (3.33) summarize the correlation between intensity and extracellular crude extract (KH14) concentration when recording $R^2 = 0.5023$. This indicates that there was a moderate correlation between the used concentrations and intensity.

Both Donato *et al.* (2012) and Anguissola *et al.* (2014) separately stated that the target organelle for determining the mitochondrial membrane permeability was mitochondria itself, and when it stained with fluorescent probe tetramethyl rhodamine methyl ester (TMRM), it would appear as red-orange in color. In this regard, Jan *et al*. (2008) and Williams *et al*. (2009) reported that in the case of mitochondria –mediated cell death (apoptosis) characterize the cell death signaling events in a very complex toxicity pathway. Besides, opening the mitochondrial permeability transition pore causes the release of cytochrome c from mitochondria into the cytosol, and finally causing destruction and death of the cell.

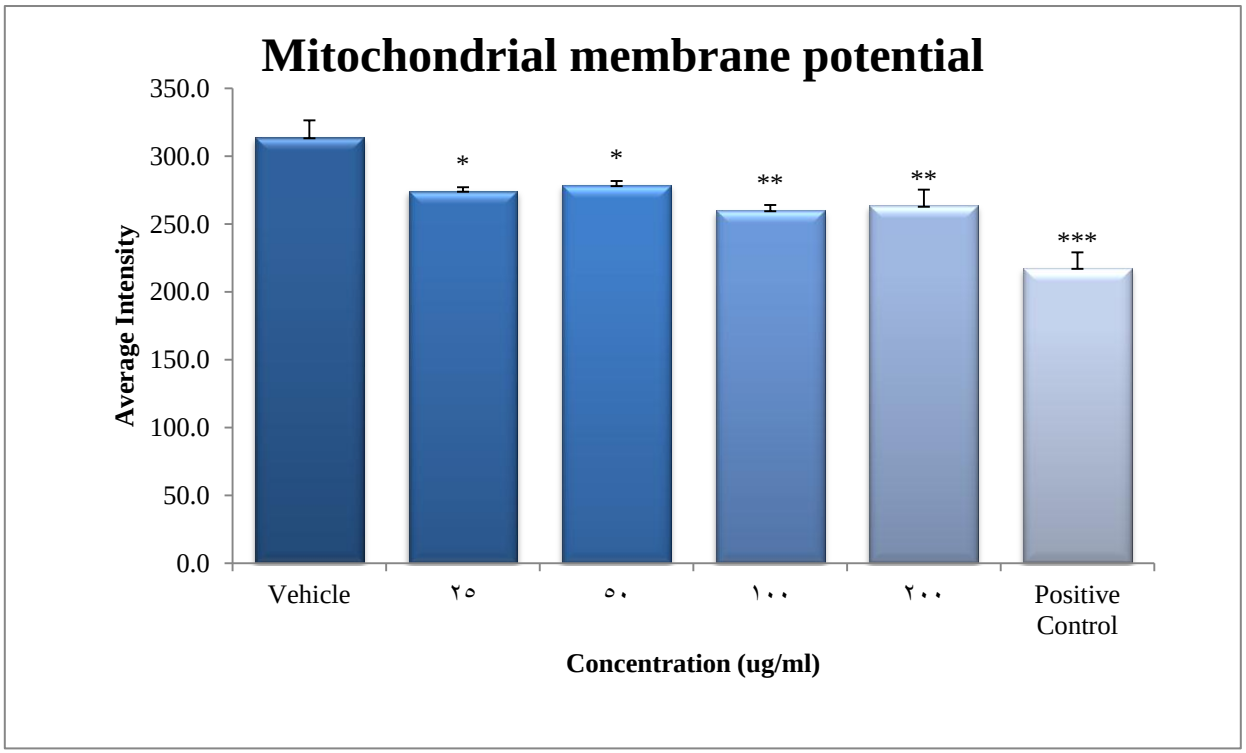


Figure 3.32: Cytotoxicity effects of KH14 extracellular crude extract on mitochondrial membrane permeability of MCF-7 cancer cell line.

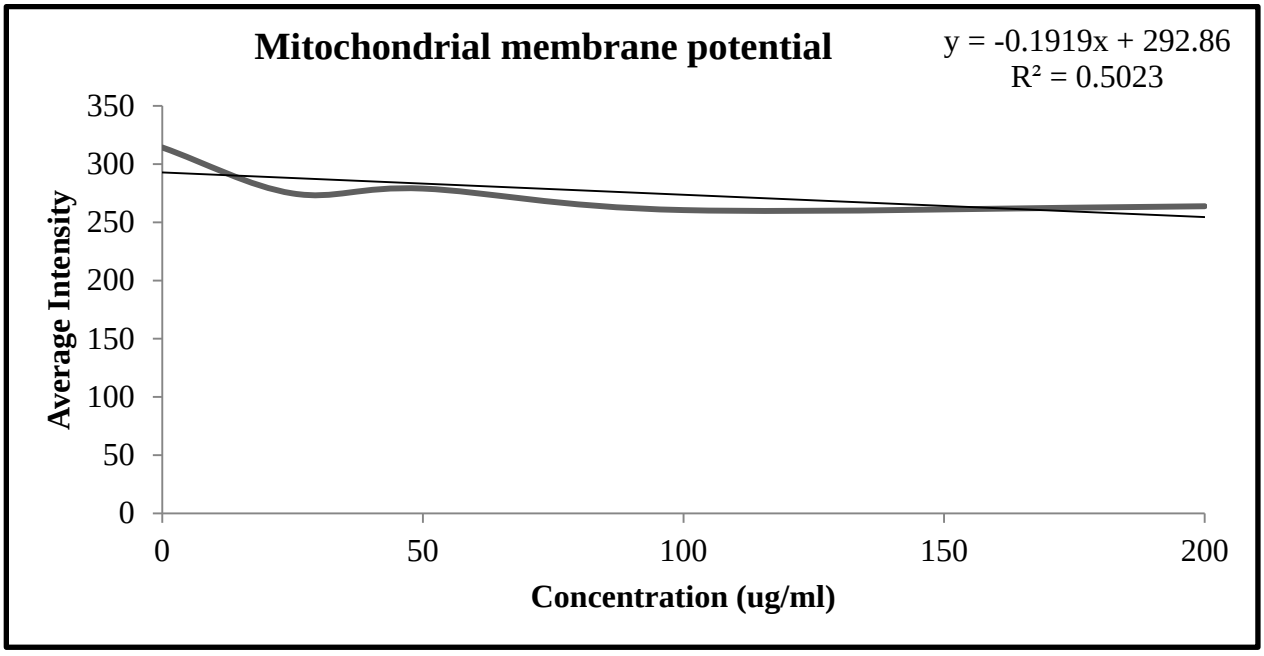


Figure 3.33: Correlation between concentrations of extracellular crude extract and average intensity for mitochondrial membrane potential on MCF-7.

Results listed in Table (3.24) and Figure (3.34) summarize the cytotoxicity effects and the statistical analysis of different concentrations of KH14 extracellular crude extract on cytochrome c released from MCF-7 after 24h of incubation. The results showed that there were no significant differences in the crude concentration used against negative control (vehicle), while the positive control which recorded the highest amount of average intensity 462.04, showed significant differences against the untreated sample (vehicle) which mrans that it was highly stained and released cytochrome c as presented in Figure (3.25). Therefore, it gave the highest intensity because of the extreme release of cytochrome c into cytosol, while the rest of the concentrations did not show any difference with the vehicle, the results presented in Figure (3.35) summarize the correlation between intensity and extracellular crude extract (KH14) concentration when recording the correlation with $R^2 = 0.6435$, which indicated that there was a moderate correlation between the concentrations used

and intensity. George *et al*. (2010) reported that cytochrome c was considered as an important mediator for apoptosis (programmed cell death), when the apoptotic signal stimulated the release of cytochrome c from the mitochondria to the cytosol.This would bind to Apaf-1 to form apotosome which finally caused the activation of the caspase cascade.

Sartore-Bianchi *et al.* (2007) stated that each identified anti-cancer compound through utilizing the high content screen went into clinical trials. On the other hand, the ability of understanding the effect of anti-cancer compounds on both cancerous and normal cell lines became a useful pattern for determining the cancer biomarkers function as described by O'Brien *et al.* (2008).

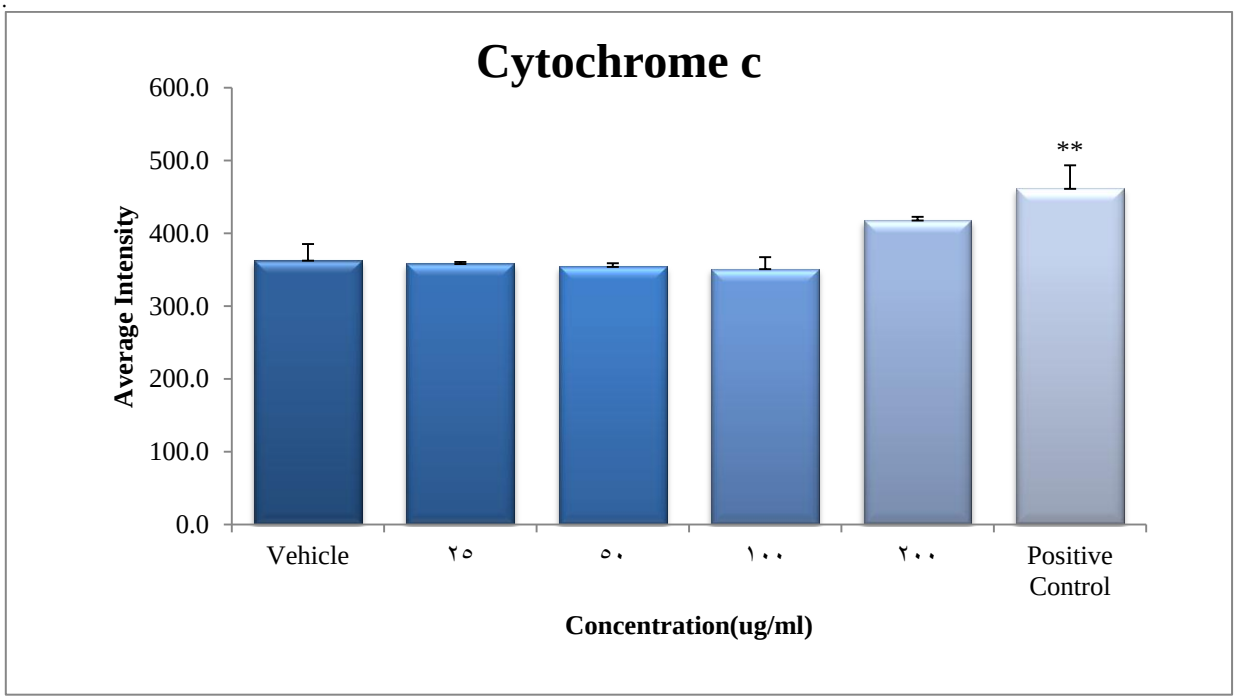


Figure 3.34: Cytotoxicity effects of KH14 extracellular crude extract on cytochrome c of MCF-7 cancer cell line.

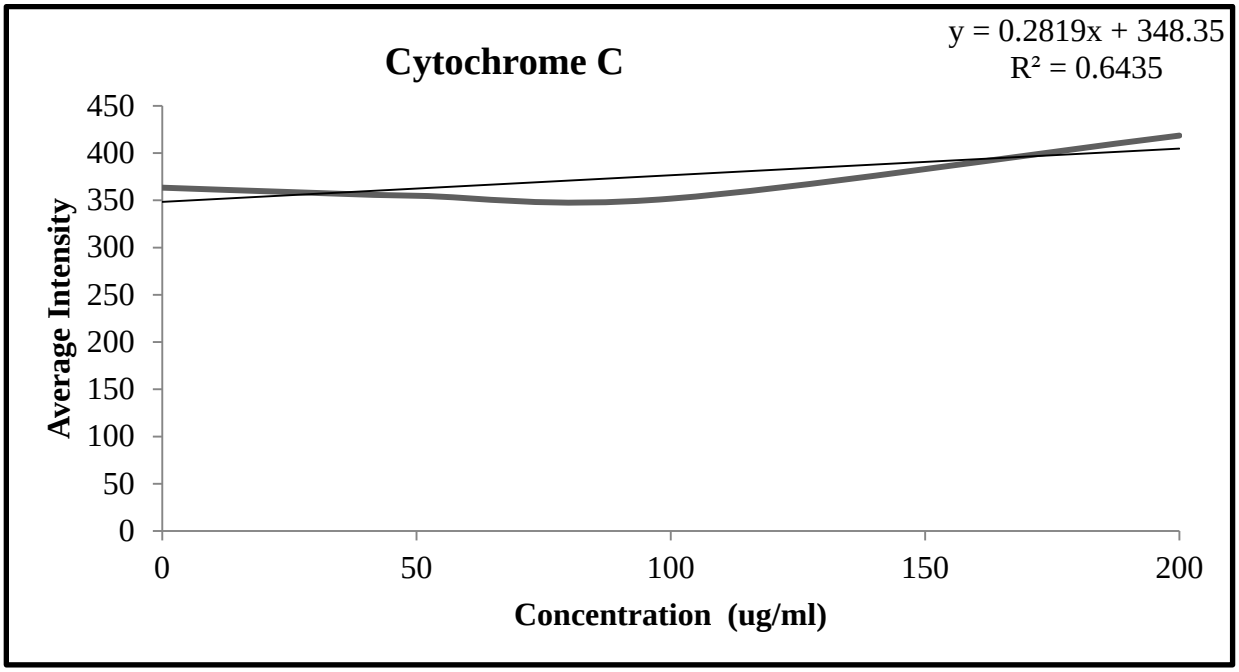


Figure 3.35: Correlation between concentrations of extracellular crude extract and average intensity for cytochrome c on MCF-7.

Conclusions and Recommendations

Conclusions and Recommendations

Conclusions

1. Screening for the actinomycetes spp. from Garmian soils was done, obtaining many isolates, with numerous bioactive properties, which emphasize the potential antimicrobial, antioxidant and antitumor activities.

2. Many of the isolated actinomycetes spp. exhibited a weak antagonistic activity against tested human microbial pathogens, while the isolates (KH14, KH16, KH18, KA19, KA38 and KA19), successfully exhibited somewhat broad spectrum activities against some Gram-positive, Gram-negative bacteria and yeast.

3. Gram-positive bacteria (*Staphylococcus aureus)* appeared more susceptible than both Gram-negative bacteria and yeast (*Escherichia coli* and *Candida albicans*).

4. Optimizing the production and extraction of the bioactive compounds have successfully completed, for the best media, the extraction with the best organic solvents, optimum incubation days, optimum pH and NaCl salt tolerance.

5. Analysis and separation of the nature of the bioactive compounds were achieved by the thin layer chromatography. Besides, determining the position of the active spots (antimicrobial metabolites) was carried out via bioautography.

6. Analyzing both ethyl acetate extract and scraped spot from bioautography by HPLC-MS, revealed the presence of large numbers of compounds which have different molecular weights with different UV absorbencies.

Conclusions and Recommendations

7. GC-Mass chromatograph analysis of ethyl acetate extract observed the presence of large numbers of biologically active compounds, which involved 12 identified compounds with different biological prosperities.

8. There are attractive biological features which achieve the powerful antitumor activities against various cancerous cell lines, especially for developing the potential antitumor drugs with precise cellular targets. However, this requires more research to identify its important therapeutic properties and determine their mechanisms on target cellular metabolisms.

9. Cytotoxicity and high content screen (HCS) study revealed significant differences between some of the cellular multi-parameters studied, especially in comparing with untreated sample (vehicle).

Conclusions and Recommendations

Recommendations

1. Screening protocol (survey) must includes 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.

2. Molecular analysis detection and phylogenic analysis are requisited for determining the genus and species of the suspected isolates.

3. An additional study is required for optimizing the production conditions and enhancing the bioactive compounds separation at the levels of best temperature, best agitation, best extraction with other organic solvents and chromatography techniques.

4. A further exploration should be done to study and determine the chemical structure of the bioactive compounds from all extracts, via obtaining a pure fraction, by means of studying various physiochemical properties, such as studying their solubility, melting points, Nuclear Magnetic Resonance(NMR) and Infra red (IR).

5. A further study is required to approve the toxicity of the extracellular extract against normal cell lines. Moreover, studies should also be conducted to clarify the safety aspect of the antitumor properties.

References

Reference

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 Appl Sci.* 6 (2): 1905-1911.

Abd-Elnaby, H.; Abo-Elala, G.; Abdel-Raouf, U.; Abdelwahab, A. and Hamed, M. (2016). Antibacterial and anticancer activity of marine *Streptomyces parvus*: optimization and application, *Biotechno and Biotechno Equip.* 30:1, 180-191

Abdulhameed, Z.T. (2013). The Isolation and Study of Morphological Characterization of *Streptomyces* Isolated from the Soil as a Source of Active Antibiotic. *Colle Bas Educ Rese J.* 12 (3).77-89

Abdullah, H.; May, E.; Bahgat, M. and Dewedar, M.(2008). Characterization of Actinomycetes isolated from ancient stone and their potential for deterioration. *Pol J Microbiol.,* 57(3): 213-220.

AbdulWahab, A.; Tabbassum, K.; Syed, A.; Subhan, Z. and Ahmed, P. (2015). Production, extraction and characterization of antimicrobial metabolites from *Streptomyces* sp. GZ024 isolated from Karachi soil. *Advan in Appl Scie Rese.* 6(1):38-44

Abou-Zeid, A. Z.; Salem, H. M. and Eissa, A. E. (1978). Production of gentamicins by Micromonospora purpurea. *Zent Bakt Natur.* 133: 261-275.

Abraham, V. C.; Danlil, T.; Jeffrey, F.; Waring, U.W. and David , J. B. (2008). Application of a High-Content Multiparameter Cytotoxicity Assay to Prioritize Compounds Basisd on Toxicity Potential in Humans. *J. Biomole Scr.* 13(6):527-37

Abussaud, M.J. And Saadoun, I. M. (1991). *Streptomyces* flora of some Jordan valley soils, characteristics and seasonal distribution. *Dirasat.* 18:66-75.

Aghaie-Khouzani, M.; Forootanfar, H.; Moshfegh, M.; Khoshayand, M. R. and Faramarzi, M. A. (2012). Decolorization of some synthetic dyes using

optimized culture broth of laccase producing ascomycete Paraconiothyrium variabile. *Biochem Eng J.* 60: 9-15.

Al Barzanchi, Sh. I. (2014). Antioxidant and Cytotoxic Effects of Lignan Purified from Myristica fragrans Seeds. A ph.D thesis Submitted To the Council of the Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, University of Baghdad.

Al-Hulu, S.; Al-Charrakh, A.H. and Jarallah, E.M. (2011).Antibacterial activity of *Streptomyces gelaticus* isolated from Iraqi soils. *Baby Medi J.* 8(3):404-411.

Anansiriwattana, W.; Tanasupawat, S.; Amnuoypol, S.and Suwanborirux, K. (2006). Identification and Antimicrobial Activities of Actinomycetes from Soils in Samed Island, and geldamycin from strain PC4-3. *Thai J Pharma Sci.* 30: 49-56.

Anderson, A.S. and Wellington, E.M.H. (2001). The taxonomy of *Streptomyces* and related genera. *Int. J. Syst Evolu Microbiol.*, 51:797-814.

Andrews, J. M. (2001). Determination of minimum inhibitory concentrations. *The J antimicr chemoth.* 48 (1):5-16.

Anguissola, S.; Garry, D.; Salvati, A.; Brien, P.J.O. and Dawson, K.A. (2014). High Content Analysis Provides Mechanistic Insights on the Pathways of Toxicity Induced by Amine-Modified Polystyrene Nanoparticles. *PLoS ONE.* 19 (9): 1-16

Ara, I.; Najat, A.; Bukhari, N. M.; Aref Mahera, M. A. and Bakir M. A. (2012). Antiviral activities of streptomycetes against tobacco mosaic virus (TMV) in Datura plant: Evaluation of different organic compounds in their metabolites, *Afri J Biotech.* 11: 2130- 2138.

Asatian, M. D.; Elisashvili, V. I.; Wasser, S. P. and Reznick, A. K. (2007). Free-Radical Scavenging Activity of Submerged Mycelium Extracts from Higher basidiomycetes Mushrooms. *Biosci Biotechnol Biochem.* 71:3090-3092.

Athlete, M.; Lacey, J. and Goodfellow, M. (1981).Selective isolation and enumeration of Actinomycetes using Rifampicin. *J Appl Bacteriol. 51, 289-229.*

References

Attimarad, S.L.; Ediga, G.N.; Karigar, A.A.; Karadi R.; Chandrashekhar, N. and Shivanna, Ch. (2012). Screening, isolation and purification of antibacterial agents from marine actinomycetes. *Intern Current Pharma J.*1(12): 394-402.

Attwell, R.W. and Colwell, R.R (1984). Thermoactinomycetes as terrestrial indicators for estuarine and marine waters. In: Biological, biochemical and biomedical aspects of actinomycetes (L. Ortiz – Ortiz and L.F. Bojalil, eds.) *Academic Press Inc.*, 441-472.

Augustine, S.K.; Bhavsar, S.P. and Kapadnis, B.P. (2005). A non polyene antifungal antibiotic from Streptomyces albidoflavus Pu 23. *J. Biosci*, 30(2): 201-211.

Awais, M.; Shah, A.; Hammed, A. and Hasan, F. (2007). Isolation, identification and optimization of bacitracin produced by *Bacillus* sp. *Pak J Bot.* 39:1303-1312.

Ayakkanu, K. and Chandramohan, D. (1971). Occurance and distribution of phosphate solubilizing bacteria and phosphatase in marine sediments at portonovo. *Mar Biol.*, 11: 201-205.

Ayuso, A.; Clark, D.; Gonzlez, I.; Salazar, O.; Anderson, A. and Genilloud, O. (2005). A novel actinomycete strain de-replication approach based on the diversity of polyketide synthase and nonribosomal peptide synthetase biosynthetic pathways. *Appl Microbiol Biotechnol.* 67(6):795–806.

Azambuja, E.; Fleck, J. F.; Batista, R. G. and Barreto, S. S. M. (2005). Bleomycin lung toxicity: who are the patients with increased risk? *Pulm Pharma and Therap.*, 18(5): 363-366.

Azeez, S. N. (2013). Identification of soil map units and vegetation indices using geoinformatics techniques for Garmyan, Kurdistan Region Iraq. Ph D, Dissertation thesis, University of Sulaimani.

Babalola , O. O. and Kirby, B. M.; Le Roes-Hill, M.; Cook, A. E.; Cary, S. C. Burton, S. G. and Cowan, D. A. (2009). Phylogenetic analysis of actinobacterial populations associated with Antarctic dry valley mineral soils. *Environ Microbiol.* 11:566-576.

References

Badji, B.; Riba, A.; Mathieu, F.; Lebrihi, A. and Sabaou, N. (2005). Antifungal activity of a saharan Actinomadura strain against various pathogenic and toxinogenic fungi. *J Med Mycol.* 15(4):211-219.

Baltz, R. H. (2008). Renaissance in antibacterial discovery from actinomycetes. *Curr. Opin Pharmacol.* 8: 557-563

Basilio, A.; González, I.; Vicente, M.F.; Gorrochategui, J.; Cabello, A.; González, A. and Genilloud, O. (2003). Patterns of antimicrobial activities from soil actinomycetes isolated under different conditions of pH and salinity. *J. Appl. Microb.,* 95: 814-823.

Bentley, S. D.; Chater, K. F.; Cerdeño-Tárraga, A. M.; Challis, G. L.; Thomson, N. R.; James, K. D.; Harris, D. E.; and et al., . (2002). Complete genome sequence of the model actinomycete Streptomyces coelicolor A3(2). *Nature* 417(6885):141-7.

Bérdy, J. (2005). Bioactive Microbial Metabolites A Personal View. *J Antibiot* 58: 1-26

Bharadwaj, R. and Yu, H. (2004). The spindle checkpoint, aneuploidy, and cancer. *Oncogene*. 23(11): 2016-2027.

Bhawani. S. A.; Ibrahim, M. N. M,; Hashim, O. S. R.; Mohammad, A. and Hena. S. (2012). Thin layer chromatography of amino acid : A review. *J Liq Chromat Related Technol.* 35: (11). 1497-1516.

Black, R. E.; Brown, K. H.; Becker, S. and Yunusm M. (1982). Longitudinal Studies of Infectious Diseases and Physical Growth of Children in Rural Bangladesh. I. Patterns of Morbidity. *Ameri J Epidemiol.* 115:305–314.

Bode, H. B.; Bethe, B.; Hofs, R. and Zeeck, A.(2002). Big effects from small changes: possible ways to explore nature's chemical diversity. *Chem Bio Chem.* 3(7):619–27.

Boonlarppradab, C.; Kauffman, C.A.; Jensen, P.R. and Fenical, W. (2008). Marineosins A and B, cytotoxic spiroaminals from a marine-derived actinomycete. *Org Lett.* 10:5505-5508.

References

Boudjelal, F.; Zitouni1, A.; Mathieu, F.; Lebrihi, A. and Sabaou, N. (2011). Taxonomic study and partial characterization of antimicrobial compounds from a moderately halophilic strain of genus actinoalloteihus. *Brazi J Microb.* 42: 835-845.

Bouras, N.; Meklat, A.; Toumatia, O.; Mokrane, S.; Holtz, M.D.; Strelkov, S.E. and Sabaou, N. (2013). Bioactive potential of a new strain of *Streptomyces* sp. PP14 isolated from Canadian soil. *Afr J Microbiol Res.* 7(25):3199-3208.

Bova, M. P.; Tam, D.; McMahon, G.; Mattson, M. N. (2005). Troglitaone induces a rapid drop of mitochodrial membrane potential in liver HepG2 cells. *Tox Lett.* 155: 41-50.

Braca, A.; De Tommasi, N.; Di Bari, L.; Pizza, C.; Politi, M.; and Morelli, I. (2001). Antioxidant principles from Bauhinia terapotensis. *J Nat Prod.* 64:892–895.

Breier, J. M.; Nicholas, M. R.; William, R. M. and Timothy, J. Sh. (2008). Development of a High-Throughput Screening Assay for Chemical Effects on Proliferation and Viability of Immortalized Human Neural Progenitor Cells. *Toxol sci.* 105(1): 119–133.

Bringmann, G.; Münchbach, M.; Feineis, D.; Faulhaber, K. and Ihmels, H. (2001), Studies on single-strand scissions to cell-free plasmid DNA by the dopaminergic neurotoxin 'TaClo' (1-trichloromethyl-1,2,3,4-tetrahydro--carboline). *Neurosci Lett.* 304: 41-44.

Bull, A. T. and Stach, J. E. M. (2007). Marine actinobacteria: new opportunities for natural product search and discovery. *Trends Microbiol.* 15: 491-499

Burman, N. P. (1973). The occurrence and significance of actinomycetes in water supply. *Soc. Appi. Bacteriol Symp Ser.* 2:219-230

Bushell, M. E. (1988). Growth, metabolism and fermentation technology. In Biotechnology of the Actinomycetales, pp. 185-215. Edited by M. Goodfellow, S. T. Williams & M. Mordarski. London : Academic Press

References

Bushell, M. E. (1993). A method for increasing the success rate of duplicating antibiotic activity in agar and liquid cultures of *Streptomyces* isolates in new antibiotics screens. *J of Ferment Bioen.* 76 (2): 89-93

Busti, E. and Yushi, O. (2006). Media conditions for growthing Actinomycetes. *Microbial Res.* 424-427.

Busti, E.; Monciardini, P.; Cavaletti, L.; Bamonte, R.; Lazzarini, A.; Sosio, M. and Donadio, S.(2006). "Antibiotic-producing ability by representatives of a newly discovered lineage of actinomycetes", *Microbiol.,,* 152: 675–683.

Cai, P.; Kong, F.; Fink, P.; Ruppen, ME.; Williamson, R.T. and Keiko, T. (2007). Polyene antibiotics from *Streptomyces* mediocidicus. *J Nat Prod.,* 70(2):215–219.

Carlsen, M.; Nielsen, J. and Villadsen, J. (1996). Growth and α-amylase production by Aspergillus oryzae during continuous cultivations. *J Biotechnol.* 45: 81–93.

Ceylan, O.; Okmen, G. and Ugur, A. (2008). Isolation of soil Streptomyces as source antibioticsactive against antibiotic-resistant bacteria. *Eur Asian J Bio Sci.* 2(1): 73-82.

Challis, G. L. and Hopwood, D. A. (2003). Synergy and contingency as driving forces for the evolution of multiple secondary metabolite production by Streptomyces species. *Proc Natl Acad Sci USA100 Suppl.* 2:14555-14561.

Chang, C.; Yang, M.; Wen, H. and Chern, J. (2002) .Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *J Food Drug Analaysis.* 10: 178-182.

Charoensopharat, K.; Thummabenjaponem, P.; Sirithorn, P. and Thammasirirak, S. (2008). Antibacterial substance produced by Streptomyces sp. No. 87. *Afr J Biotechnol.* 7(9): 1362-1368.

Chaudhary, H. S.; Soni, B.; Shrivastava, A. R. and Shrivastava, S. (2013). Diversity and Versatility of Actinomycetes and its Role in Antibiotic Production. *J Appl Pharm Scie.* 3 (8): 83-94

References

Chavan Dilip V.; Mulaje S. S. and Mohalkar R.Y. (2013). A review on actinomycetes and their biotechnology application. *Int J Pharma Scie Reas.* 4 (5):1730-1742

Chawawisit, K.; Bhoopong, P.; Phupong, W. and Lertcanawanichakui, M. (2015). Antimicrobial and cytotoxic activities of bioactive compounds produced by Streptomyces Sp. KB1. *Intern J of Pharma and Pharma Sci.* 7:11

Cheah, S. C.; Appleton, D.R.; Lee, S. T.; Lam, M. L.; Hadi, A. H. A. and Mustafa, M. R. (2011). Panduratin A inhibits the growth of A549 cells through induction of apoptosis and inhibition of NF-κB translocation. *Molecules.* 16: 2583-2598.

Cheraiti, N. and Gacemi Kirane, D. (2012). Isolation of actinomycetes strains producing new antifungal molecules. *Rev. Microbiol Ind San Environn.* 6(1):18-34.

Clardy, J.; Fischbach, M. A. and Walsh, C. T. (2006). New antibiotics from bacterial natural products. *Nat Biotechn.* 24: 1541– 1550

Clawson, H.; Small, K.; Go, E. and Myles, B. (2004). Needs assessment for service providers and trafficking victims. National Institute of Justice, Washington, DC: Government Printing Office.

Collins, C. H.; Lyne, P. M. and Grange J. M. (1995). Microbiological methods. 7th edition, Butterworth Heinemann Ltd. London.

Colquhoun, J. A.; Mexson, J.; Goodfellow, M.; Ward, A .C.; Horikoshi, K. and Bull, A.T. (1998). Novel rhodococci and other mycolate actinomycetes from the deep sea. *Antonie van Leeuwenhoek.* 74:27-40.

Considinet, J. M. and Mallette, M. F. (1965). Production and partial purification of antibiotic materials formed by Physarum gyrosum. *Appl Microbiol.* 13:464-468.

Crawford, D. L.; Lynch, J. M.; Whipps, J. M. and Ousley, M. A. (1993). Isolation and characterization of actinomycete antagonists of a fungal root pathogen. *Appl Environ Microbiol.* 59:3899-3905.

References

Cremen, P. A. and Zeng, L. (2002).“High-throughput analysis of natural product compound libraries by parallel LC–MS evaporative lightscattering detection”. *Anal Chem.* 74:5492–5500.

Cummins, C. S. and Harris, H. (1956). A comparison of cell-wall composition in Nocardia, Actinomyces, Mycobacterium and Propionibacterium. *J Gen Microbiol.* 18, 173-189

Das, S.; Lyla, P. S. and Khan, S. A. (2008). Distribution and generic composition of culturable marine actinomycetes from the sediments of Indian continental slope of Bay of Bengal. *Chin J Oceanol Limnol.* 26:166- 77.

Davenport, R. J.; Curtis, T. P.; Goodfellow, M.; Stainsby, F. M. and Bingley, M.(2000). Quatitative use of fluorescent In situ hybridization to examine relationships between mycolic acid-containing actinomycetes and foaming in activated sludge plants. *Appl Environ Microbiol.* 66:1158-66.

Davies, A.M.; Volkov, Y. and Spiers, J.P. (2008). Drug discovery automation: Development of cardiac cell based assays to study drug efficacy and toxicity. *Screening-Trends Drug Discovery*, 9: 21-24.

Dehnad, A.; Parsa, L.; Bakhshi, R. ; Abdi Soofiani, S. and Mokhtarzadeh, A. (2010). Investigation antibacterial activity of *Streptomycetes* isolates from soil samples, West of Iran. *Afr J Microb Res* .4(14):1542-9.

Desai, S.S. and Phatak, A.A. (2010). Study of growth pattern of actinomycetes species in various nutrient medium. *Int J Pharm Pharm Sci,* 2 (4): 42-45

D'Esposito, M .; Aguirre, GK.; Zarahn, E.; Ballard, D.; Shin, RK. and Lease J.(1998). Functional MRI studies of spatial and nonspatial working memory. *Brain Res Cogn Brain Res.,* 7(1):1-13.

Dharumadurai D. and Yi J. (2016). Biochemistry, Genetics and Molecular Biology » "Actinobacteria - Basics and Biotechnological Applications", ISBN 978-953-51-2248-7, chapter 2, pp 13

Diaz, D.; Scott, A.; Carmichael, P.; Shi, W. and Costalesm, C. (2007).Evaluation of an automated *in vitro* micronucleus assay in CHO-K1 cells. *Mutat Res.* 630:1-13.

References

Dietz, A. (1994). The lives and times of industrial actinomycetes. *ASM News.* 60: 366-369.

Diraviyam, T.; Radhakrishnan, M. and Balagurunathan, R.(2011). Antioxidant activity of melanin pigment from Streptomyces species D5 isolated from Desert soil, Rajasthan, India. *Drug Invent Today*. 3:12-13.

Donadio, S.; Maffioli, S.; Monciardini, P.; Sosio M. ; Jabes, D. and Milano, N.(2010). Antibiotic discovery in the twenty-first century: current trends and future perspectives. *The J of Antibio.* 63: 423–430

Donato, M.T.; Tolosa, L.; Jiménez, N.; Castell, J.V. and Gómez-Lechón, M. J. (2012). High-content imaging technology for the evaluation of drug-induced steatosis using a multiparametric cell-based assay. *J Biomol Screen.* 17(3):394-400.

Drouin, C. M. and Cooper, D. G. (1992). Biosurfactant and aqueous two-phase fermentation. *Biotechnol Bioeng*. 40: 86-90.

Dykens, J. A.; Jamieson, J. D.; Marroquin, L. D.; Nadanaciva, S.; Xu, J. J.; Dunn, M. C.; Smith, A. R. and Will, Y. (2008). *In vitro* assessment of mitochondrial dysfunction and cytotoxicity of nefazodone, trazodone, and buspirone. *Toxicol Sci.* 103(2): 335-345.

El-Naggar, M.Y.; El-Assar, S. A. and Abdul-Gawad, S. M. (2006). Meroparamycin Production by Newly Isolated *Streptomyces* sp. Strain MAR01: Taxonomy, Fermentation, Purification and Structural Elucidation. *J Microbiol.* 44: 432-438.

El-Nakeeb, M. A. and Lechevalier, H. A. (1963). Selective isolation of aerobic actinomycetes. *Appl Microbiol.* 11: 75-77.

El-Tayeb, O. M.; Salama, A.; Hussein, M. and El-Sedawy, H. F. (2004). Optimization of industrial production of rifamycin B by Amycolatopsis mediterranei I. The role of colony morphology and nitrogen sources in productivity. *Afr. J. Biotechnol.,* 3:266–272.

References

El-Waziry, A.M.; Tomita, Y.; Ling, J.R. and Onodera, R. (1996). Measurement of total and separate stereoisomers of diaminopimelic acid in rumen bacteria by high-performance liquid chromatography. *J Chromat B.* 677: 53-59.

EUCAST. (2000). Terminology relating to methods for the determination of susceptibility of bacteria to antimicrobial agents. *Clin Microbiol Infect.* 6: 503-508

Euzéby, J. P. (2014). List of Bacterial Names with Standing in Nomenclature: a folder available on the Internet. http://www.bacterio.net

Eva, S. M.; Baraka, S. and Ken, H. M. M. (2014). Cytotoxicity and antioxidant activity of a *Streptomyces* sp. From mangrove sediment of dar es salaam, TANZANIA. *Int J Pharm Pharm Sci.* 6 (6): 563-566

Ganguly, A.; Yang, H. and Cabral, F.(2010). Paclitaxel-dependent cell lines reveal a novel drug activity. *Mol Cancer Ther*. 9: 2914-2923.

Gebreyohannes, G.; Moges, F.; Sahile, S. and Raja, N. (2013). Isolation and characterization of potential antibiotic producing actinomycetes from water and sediments of Lake Tana, Ethiopia. *Asian Pac J Trop Biome.* 3(6): 426-435.

Genilloud, O.; González, I.; Salazar, O.; Martín, J.; Tormo, J. R. and Vincente, F. (2011). Current approaches to exploit actinomycetes as a source of novel natural products. *J Ind Microbiol Biotechnol*. 38(3):375–389

George, M. Anjumol, A.; George, G.; and Mohamed Hatha A. A. (2012). Distribution and bioactive potential of soil actinomycetes from different ecological habitats. *Afr J Microbiol Res.* 6:2265-71.

George, S.; Pokhrel, S.; Xia, T.; Gilbert, B.; Marco,J.; Andreas,S.; Robert , R.; Kenneth, D.; Mädler, L. and E Nel, A. (2010). Use of a rapid cytotoxicity screening approach to engineer a safer zinc oxide nanoparticle through iron doping. *Acs Nano.* 4: 15-29.

References

Goodal, R. R.; Levi, A. A. (1946). A Microchromatographic Method for the Detection and Approximate Determination of the Different Penicillins in a Mixture .*Nature.* 158: 675-676

Goodfellow, M. and Williams, S.T. (1983). Ecology of Actinomycetes. *Annu Rev Microbiol.* 37:189-216.

Goodfellow, M. and Fiedler, H. P. (2010). A guide to successful bioprospecting: informed by actinobacterial systematics. *Antonie van Leeuwenhoek*. 98:119–142.

Goodfellow, M.; Stainsby, F. M.; Davenport, R.; Chun, J. and Curtis, T. (1998). Activated sludge foaming: the true extent of actinomycete diversity. *Water Sci Technol.* 37:511-518

Goodfellow, M.; Williams, S. T. and Mordarski, M.(1988). Actinomycetes in Biotechnology. *Academic Press, London*.

Gopi, R. and Ramakrishna, R. (2011) Optimization of culture conditions of *Streptomyces rochei* (MTCC 10109) for the production of antimicrobial metabolites. *Egyptian J Biol* 13: 21-29.

Gullo, V. P.; McAlpine, J.; Lam, K. S.; Baker, D. and Petersen, F. (2006). Drug discovery from natural products. *J Indust Microbiol Biotechnol.* 33: 523–531.

Gurung, T.D.; Sherpa, Ch.; Prasad, V.A. and Lekhak, B. (2009). Isolation and Characterization of Antibacterial Actinomycetes from Soil Samples of Kalapatthar, *Mount Everest Region Nepal Journal of Science and Technology* 10: 173-182.

Gust, B.; Challis, G.L.; Fowler, K.; Kieser, T. and Chater, K.F. (2003). PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. *Proc Natl Acad Sci USA.,* 100:1541-1546

Håkanson, M.; Cukierman, E.; Charnley, M. (2014). Miniaturized pre-clinical cancer models as research and diagnostic tools. *Adv Drug Deliv Rev.* 69(70), 52–66,

References

Hamburger, M. O. and Cordell, G. A. (1987). A direct bioautographic assay of compounds possessing antimicrobial activity. *J Nat Prod.* 50: 19-22.

Hamedi, J.; Imanparast, S. and Mohammadipanah. F. (2015). Molecular, chemical and biological screening of soil actinomycete isolates in seeking bioactive peptide metabolites *Iran J Microbi.* 7 (1):23-30

Haq, I. and Ali S. (2006). Innovative effect of illite on improved microbiological conversion of L-tyrosine to 3, 4 dihydroxy phenyl L-alanine (L-dopa) by *Aspergillus oryzae* ME2 under acidic reaction conditions. *Curr Microbiol.* 53: 351–357.

Harrill, J. A.; Robinette, B. L.; Freudenrich, T. and Mundy, W. R. (2013). Use of high content image analyses to detect chemical-mediated effects on neurite sub-populations in primary rat cortical neurons. *Neurotox.* 34: 61–73

Haskins, J. R.; Rowse, P.; Rahbari, R. and de la Iglesia, F. A. (2001).Thiazolidinedione toxicity to isolated hepatocytes revealed by coherent multiprobe fluorescence microscopy and correlated with multiparameter flow cytometry of peripheral leukocytes. *Arch Toxicol*. 75:425-438.

Hassan, F.; Ayah, A.; Ahemd, A.; Bashar, M.A; Hasniza Z.H., Nany, H. and Emad Y. (2015). Antitumor Activity for Gold (III) Complex by High Content screening Technique (HCS) and Cell Viability Assay. *Asian J Biochem*. 10 (6): 252-266

Hayakawa, M. (2008). Studies on the Isolation and Distribution of Rare Actinomycetes in Soil. *Actinomycetol*. 22:12-19

Hemashenpagam, N. (2011). Purification of secondary metaboloities from soil actinomycetes .*Intern J Microb Res.* 3(3): 148-156

Hites, R. A. and Biemann, K. (1970). "Computer evaluation of continuously scanned mass spectra of gas chromatographic effluents". *Analyt Chemi.* 42 (8): 855-860

References

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Stanley, J. T., Williams, S. T. (1994): Bergey's Manual of Determinative Bacteriology, 9th ed. Williams & Wilkins, Co., Baltimore.

Hopp, D. C.; Milanowsk,i D. J.; Rhea, J.; Jacobsen, D.; Rabenstein, J.; Smith, C.; Romari, K.; Clarke, M.; Francis, L.; Irigoyen, M.; Luche, M.; Carr, G. J. and Mocek, U. (2008) Citreamicins with potent grampositive activity. *J Nat Prod*. 71(12):2032–2035

Hostettmann, K.; Wolfender, J. and Terreaux, C. (2001).Modern screening techniques for plant extracts. *Pharm. Biol.* 39: 18–32.

Hozzein, W. N. and Goodfellow, M. (2011). Actinopoly sporaegyptensis sp. nov., a new halophilic actinomycete. *Afr J Microbi. Res.* 5:100-105.

Hozzein, W. N.; Rabie, W. and Ali, M. A. (2011). Screening the Egyptian desert actinomycetes as candidates for new antimicrobial compounds and identification of a new desert *Streptomyces* strain. *African J Biotech.* 10(12): 2295-2301

Huyen. T.; Pham, Nhue P. N.; Tien Q.; Phi, P. T. and Hy G. L. (2014).The Antibacterial and Anticancer Activity of Marine Actinomycete Strain HP411 Isolated in the Northern Coast of Vietnam. *Intern J Med Heal Biom Bioen and Pharmac Engi.* 8(12):842-846

Ignatius, M. J. and Hung, J. T. (2007) Physiological indicators of cell function. *Methods Mol Biol.* 356:233–244

Ili, S.B.; Konstantinovi, S. S. and Todorovi, Z. B. (2005). UV/VIS Analysis and Antimicrobial Activity of Streptomyces Isolates. *Facta universitatis-series: Medi and Bio.* 12(1):44-46.

Ilic, S. B.; Konstantinovic, S. S.; Todorovic, Z. B.; Lazic, M. L.; Veljkovic, V. B. and Jokovic, N. (2007). Characterization and antimicrobial activity of the bioactive metabolites in streptomycete isolates. *Mikrobiologiia.* 76(4):480-487.

References

Islam, N.; Parveen, S. A.; Nakazawa, N.; Marston, A. and Hostettmann, K. (2003). Bioautography with thefungus Valsa ceratosperma in the search for antimycotic agents. *Pharm. Biol.*, 41: 637-640.

Itokawa, H.; Morris-Natschke, S. L.; Akiyama, T. and Lee, K. H. (2008). Plant-derived natural product research aimed at new drug discovery. *J Nat Med.* 62:263–280.

Jahr, W.; Schmid, B.; Schmied, C.; Fahrbach, F. O. and Huisken, J. (2015). Hyperspectral light sheet microscopy. *Nat Commun.* 6: 1–7.

Jalaluldeen, A. M.; Sijam, K.; Othman, R. and Ahmad, Z. (2015). Growth characteristics and production of secondary metabolites from selected *streptomyces* species isolated from the Rhizosphere of Chili Plant. *Inter J of Enh Res in Sci Tech & Eng.* 4 (1): 1-8.

Jan, E.; Byrne, S. J.; Cuddihy, M.; Davies, A. M.; Volkov, Y.; Gun'ko, Y. K. and Kotov, N. A. (2008). High-content screening as a universal tool for fingerprinting of cytotoxicity of nanoparticles. *ACS Nano.* 2: 928-938.

Jarallah, E. M. and Noor, H. R. (2014).Extraction and purification of antimicrobial agent produced from Actinomycetes isolated from agricultural soils. *J. Baby. Univ.* 22(2):749-758.

Jensen, P. R.; Mincer, T. J.; Williams, P. G. and Fenical, W. (2005). Marine actinomycete diversity and natural product discovery. *Antonie Van Leeuwenhoek.* 87: 43-48.

Joy, M. E.; Vollmer, L. L.; Hulkower, K.; Stern, A. M.; Peterson, C. K.; Boltz, R. C.; Roy, P. and Vogt, A. (2014). A high-content, multiplexed screen in human breast cancer cells identifies profilin-1 inducers with anti-migratory activities. *PLoS ONE.*9: 1–12.

Kalakoutswl, V. and Agre, N. S. (1973). Endospores of actinomycetes: dormancy and germination. In The Actinomycetales: *Characteristics and Practical Importance.* pp. I79-195.

References

Kariminik, A. and Baniasadi, F. (2010). Pageantagonistic activity of Actinomycetes on some gram negative and gram positive bacteria. *World Appl Sci J*. 8(7): 828-832.

Kekuda, P. T. R.; Shobha, K. S. and Onkarappa, R. (2010). Studies on Antioxidant and Antihelmintic activity of two *Streptomyces* species isolated from Western Ghat soil of Agumbe, Karnataka. *J. Pharma Res.* 3(1): 26-29.

Kekuda, P. T. R.; Shobha, K. S.; Onkarappa, R.; Gautham, S. A. and Raghavendra, H. L. (2012). Screening Biological Activities of a Streptomyces Species Isolated from Soil of Agumbe, Karnataka, India. *Intern J Drug Devel and Res.* 4(3):104-114.

Khalaf, N. A.; Shakya, A. K.; Al-Othman, A.; El-Agbar, Z. and Farah, H. (2008). Antioxidant activity of some common plants. *Turk J Biol.* 32: 51-55.

Khan, J. A. and Patel, A. S. (2011). Extraction and purification of antibacterial metabolites from actinomycetes spp. isolated from soil sample. *intern J pharma res and devel.* 3(10):63-71

Khan, M. and Tripathi, C. K. M. (2011). Optimization of fermentation parameters for maximization of Actinomycin D production. *J Chem Pharma Res.* 3(5): 281-289.

Khanna , M.; Renu, S. and Rup, L. (2011). Selective isolation for rare actinomycetes producing novel antimicrobial compound. *Int J of Adva Biotech and Res.* 2 (3): 357-375

Kojiri, K.; Nakajima, S.; Suzuki, H.; Kondo, H. and Suda, H. (1992). A new macrocyclic lactam antibiotic, BE-14106. I. Taxonomy, isolation, biological activity and structural elucidation. *J Antibiot.* 45:868-874.

Koleva, II.; van Beek, T. A.; Linssen, J. P. H.; de Groot, A. and Evstatieva, L. N. (2001). Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. *Phytochem Anal.* 13: 8 17.

References

Kontro, M.; Lignell, U.; Hirvonen, M. R. and Nevalainen, A. (2005). pH effects on 10 *Streptomyces* spp. growth and sporulation depend on nutrients. *Lett Appl Microbiol.* 41:32-38.

Korn-Wendisch, F. and Kutzner, H.J. (1992). The family Streptomycetaceae. In: Balows A, Truper HG, Dworkin M, Harder W, Schleifer KH,Editors. The prokaryotes. New York: *Springer-Verlag Inc*., 921-95

Kudo, T.; Matsushima, K.; Itoh, T.; Sasaki, J. and Suzuki, K. (1998). Description of four new species of the genus Kineosporia: *Kineosporia succinea* sp. nov., *Kineosporia rhizophila* sp. nov., *Kineosporia mikuniensis* sp. nov. and *Kineosporia rhamnosa* sp.nov., isolated from plant samples, and amended description of the genus Kineosporia. *Int J of Syst Bact.* 48:1245-1255.

Kumar, S. K. (2010). Diversity and Optimization of process parameters for the growth of *Streptomyces* VITSVK9 spp. isolated from Bay of Bengal. *Ind J Nat Environ Sci.* 1(2): 56-65.

Kumar, P.; Preetam, R. J.; Duraipandiyan, V. and Ignacimuthu, S. (2012). Antibacterial activity of some actinomycetes from Tamil Nadu, India. *Asian Pac J Trop Biomed.* 2(12): 936-943.

Kurtböke, D. I. (2010). Bacteriophages as tools in drug discovery programs. *Microbiol Australia.* 31:67-70

Kurusu, K.; Ohba, K. (1987). New peptide antibiotics LI-FO3 and characterization. *J Antibiot.* 40 (11): 1506-1514.

Kuster, E. (1968). Taxonomy of soil actinomycetes and related organisms. In: Gray S, Parkinson T, Editors. Ecology of soil bacteria. Liverpool: *Liverpool University Press.*

Kutzner, H. J. (1986).The family Streptomycetaceae. In M.P. Starr, H. Stolp, H.G. Trüper, A. Balows, and H.G. Schlegel (ed.), The prokaryotes, a handbook on habitats, isolation, and identification of bacteria, *Springer-Verlag, New York* 2:2028-2090.

References

Lang, G.; Mayhudin, N. A.; Maya, I.; Mitova, M. I.; Sun, L.; Sun, L.; van der Sar, S.; Blunt, J. W.; Cole, A. J. L.; Ellis, G.; Laatsch, H. and Munro, M.H, G. (2008). "Evolving trends in the dereplication of natural product extracts: new methodology for rapid, small-scale investigation of natural product extracts". *J Nat Prod.* 71:1595–1599.

Lechevalier, H. and Lechevalier, M. P. (1981). Introduction to the order Actinomycetales. In: Starr MP, Stolp H, Trüper HG, Balows A, Schlegel HG,Editors. The Prokaryotes. Germany: *Springer-Verlag Berlin.* 2:1915-22.

Lertcanawanichakul, M.; Pondet, K. and Kwantep, J. (2015).*In vitro* antimicrobial and antioxidant activities of bioactive compounds (secondary metabolites) extracted from Streptomyces lydicus A2. *J Appl Pharma Sci.* 5 (2): 017-021

Linos, A.; Mahmoud, M.; Berekaa, A.; Steinbüchel, K. and Kyu Kim, C. S. (2002). Gordonia westfalica sp. nov., a novel rubber-degrading actinomycete.Reiner M Kroppenstedt. *Int J Syst Evol Microbiol.* 52: 1133-1139.

Locci, R. (1989). Streptomycetes and related genera. Bergey's Manual of Systematic Bacteriology, Williams and Wilkins Company, *Baltimore.* 4:2451-2508.

Lucas, X.; Senger, C.; Erxleben, A.; Grüning, B. A.; Döring, K.; Mosch, J.; Flemming, S. and Günther, S. (2013). Streptome DB: a resource for natural compounds isolated from Streptomyces species. *Nucl Acids Res.* 41: 1130–1136

Maataoui, H.; Iraqui, M.; Jihani, S.; Ibnsouda, S. and Haggoud A. (2014). Isolation, characterization and antimicrobial activity of a *Streptomyces* strain isolated from deteriorated wood. *Afr J Microb Res.* 8(11):1178-1186.

Maguelez, E. M.; Hardisson, C. and Manzanal, M. B. (2000). Streptomycetes: a new model to study cell death. *Int microbial.* 3:153–158.

Mahalaxmi, Y.; Sathish, T.; SubbaRao, C. and Prakasham, R. S. (2010) . "Corn husk as a novel substrate for the production of rifamycin B by isolated Amycolatopsis sp. RSP 3 under SSF," *Process Biochemistry*, 45(1):47–53.

References

Maitland, P. D and Maitland, D. P. (2010). Chromatography: Are we getting it right. *J Biolog Edu.* 37(1): 6-8.

Manteca, A.; Jung, H. R.; Schwämmle, V.; Jensen, O. N. and Sanchez, J. (2010). Quantitative proteome analysis of Streptomyces coelicolor Non sporulating liquid cultures demonstrates a complex differentiation process comparable to that occurring in sporulating solid cultures. *J Proteome Res.* 9(9):4801-11.

Manuselis, G. and Mahon, C. R (2007). In: Textbook of diagnostic microbiology. In: Manon CR, Lehman DC, Mauselis G, Editors. Saunders, 3-13.

Martín, J. F.; Casqueiro, J. and Liras, P. (2005). Secretion systems for secondary metabolites: how producer cells send out messages of intercellular communication. *Curr Opin in Microbio.* 8: 282-293.

Mason, M.; Ball, A.; Reeder, B.J.; Silkstone, G.; Nichollis, P.; Wilson, M. T. (2001). Extracellular Heme Peroxidases in Actinomycetes: A case of mistaken identity. *Appl and Environ Microbiol.* 67: 4512–9.

Mbwambo, Z.H.; Moshi, M.J.; Masimba, P.J.; Kapingu, M.C. and Nondo, R.S.O. (2007). Antimicrobial activity and brine shrimp toxicity of extracts of Terminalia browniiroots and stem, *BMC Compl and Alter Med*. 7:9.

McCarthy, A. J. and Williams, S. T. (1992). Actinomycetes as agents of biodegradation in the environment, a review. *Gene.* 15:115(1-2):189-92.

Medvedovici, A.; Sandra, P.; Kot, A. and Kolodziejczyk, A. (1996). Study on the separation of D, L- and meso-N,N0 -di (carbobenzy loxy)-2,6 -diaminopimelic acid by SFC. *J of High Resol Chromat.* 19: 227-228.

Miao, V. and Davies, J. (2010). Actinobacteria: the good, the bad, and the ugly. *Antonie Van Leeuwenhoek*. 98: 143–150.

Mohsen, M. S. A. Osama, H.; El-Sayed Seham, M. S. and Shimaa R. H. (2015). Isolation, Structure elucidation and Biological Activity of Di- (2-ethylhexyl) phthalate Produced by *Penicillium janthinellum* 62 *.Int J Chem Tech Res.* 8(1): 58-66.

References

Moncheva, P.; Tishkov, S.; Dimitrova, N.; Chipera, V.; Nikolova, S. A. and Bogatzevska, N. (2002) Characteristics of soil Actinomycetes from Antarctica. *J of cult coll.* 3:3 – 14.

Mosmann, F. R. (1983) Colorimetric assay for cellular growth and survival: Application and cytotoxicity assay. *J Immunol Methods.* 65(55):63-69

Motta, A. and Brandelli, D. (2006) Characterization of an antimicrobial peptide produced by Brevibacterium linens. *J Applied Microbiol.* 52(6):357-63.

Mueller, U. G. and Nicole, G. (2002). Fungus farming insects: multiple origins and diverse evolutionary histories. *Section of Integ Biol*. 99: 15247-15249.

Muiru, W. M.; Mutity, E. W. and Mukunya, D. M. (2007). Characterization of antibiotic metabolites from actinomycete isolates .*African Crop Sci Confe Proce.* 8: 2103-2107

Mukai, A.; Fukai, T.; Matsumoto, Y.; Ishikawa, J.; Hoshino, Y.; Yazawa, K.; Harada, K-I. and Mikami, Y. (2006). Transvalencin Z, A New Antimicrobial Compound with Salicylic acid Residue from Nocardia transvalensis IFM 10065. *J Antibiot.* 59(6):366-369.

Nagasawa, T.; Ling, J. R. and Onodera, R. (1993) Chiral high-performance liquid chromatographic separation of the three stereoisomers of 2,6 - diaminopimelic acid without derivatisation. *J Chromat A.* 653: 336-340.

Naine, S. J.; Devi, C. S.; Mohanasrinivasan, V. and Vaishnavi. B. (2015) Antimicrobial, Antioxidant and Cytotoxic Activity of Marine *Streptomyces parvulus* VITJS11 Crude Extract. *Braz Arch Biol Technol.* 58(2): 198-207.

Nanjwade, B. K.; Chandrashekhara, S.; Goudanavar, P. S.; Shamarez, A. M. and Manvi, F. V. (2010). Production of Antibiotics from Soil-Isolated Actinomycetes and Evaluation of their Antimicrobial Activities. *Trop J Pharma Res.* 9 (4): 373-377

Navarro, G. F.; Pérez-Díaz, R. M.; Negredo, A. I.; Pla J. and Nombela, C. (1998). Cloning and sequence of a 3.835 kbp DNA fragment containing the

HIS4 gene and a fragment of a PEX5-like gene from *Candida albicans. Yeast.* 14(12):1147-57.

Nawaz, K.; Hussain, K.; Majeed, A.; Khan, F.; Afghan S. and Ali. K. (2010). Fatality of salt stress to plants: Morphological, physiological and biochemical aspects. *Afri J of Biotech.* 9: 5475-5480.

Newman, D. J. and Cragg, G. M. (2007). Natural products as sources of new drugs over the last 25 years. *J Nat Prod.* 70: 461−477.

Nguyen, D.T.; Nyugen, D. H.; Lyun, H. L.; Lee, H. B.; Shin, J. H. and Kim, E. K.. (2007). Inhibition of melanogenesis by diocyl phthalate isolated fromNigella glandulifera Freyn. *J Microbio Biotechno.* 17:1585-1590.

Nicolaou, K. C.; Chen, J. S. and Dalby, S. M. (2009).From nature to the laboratory and into the clinic. *Bioorg Med Chem*. 17(6):2290–2303

Nisbet, L. J. and Fox, F. M. (1991). The importance of microbial biodiversity to biotechnology, In, The biodiversity of microorganisms and invertebrates: its role in sustainable Agriculture, ed.D.L. *Hawksworth, 224-229, CAB International.*

Nonoh, J.; Lwande, W.; Masiga, D.; Herrmann, R.; Presnail, J.; Schepers, E.; Okech, MA.; Bagine, R.; Mungai, P.; Bernard Nyende, A. and Boga H.I. (2010). Isolation and characterization of *Streptomyces* species with antifungal activity from selected national parks in Kenya. *Afri J Microb Res.* 4(9): 856-864.

O'Brien, P. J.; Irwin, W.; Diaz, D.; Howard-Cofield E.; Krejsa, C. M.; Slaughter, M. R.; Gao, B.; Kaludercic, N.; Angeline, A.; Bernardi, P.; Brain, P. and Hougham, C. (2006). High concordance of drug-induced human hepatotoxicity with *in vitro* cytotoxicity measured in a novel cell-based model using high content screening. *Arch Toxicol*. 80:580-604.

Olano, C.; Méndez, C. and Salas, J. A. (2009). Antitumor compounds from actinomycetes: from gene clusters to new derivatives by combinatorial biosynthesis. *Nat Prod Rep.* 26:628-660.

References

Ortiz, M.; Neilson, J. W.; Nelson, W. M.; Legatzki, A.; Byrne, A.; Yu, Y.; Wing, R. A.; Soderlund, C.A.; Pryor, B. M.; Pierson, L.S. and Maier, R. M. (2013). Profiling Bacterial Diversity and Taxonomic Composition on Speleothem Surfaces in Kartchner Caverns, AZ. *Microbiol Ecol.* 65: 371-383.

Oskay, M.; Tamer, U.A. andAzeri, C. (2004). Antibacterial activity of some actinomycetes isolated from farming soils of Turkey. *Afr. J. Biotechnol.*, 3: 441-446.

Oskay, M. (2009). Antifungal and antibacterial compounds from *Streptomyces* strains. *Afr J Biotechnol.* 8(13): 3007 – 3017.

Pallavi, S.; Manasa, M.; Kambar, Y.; Asha, M.; Chaithra, M.; Vivek, M. and Mallikarjun, N. (2013). Anti-Staphylococcus aureus and Anti-yeast activity of *Streptomyces* species isolated from rhizosphere soil of Sahyadri Science College, Shivamogga, Karnataka. *Asian J Biom and Pharma Sci.* 3(24): 7-11.

Pandey, B.; Ghimire, P. and Agrawal, V. P. (2004). Studies on the Antibacterial Activity of the Actinomycetes Isolated from the Khumbu Region of Nepal. *J. Biol Sci.* 23:44-53.

Pankey, G. A. and Sabath, L. D. (2004). Clinical Relevance of Bacteriostatic versus Bactericidal Mechanisms of Action in the Treatment of Gram-Positive Bacterial Infections. *Oxf J.* 38(6): 864-870.

Pathom-aree, W.; Stach, J. E.; Ward, A. C.; Horikoshi, K.; Bull, A. T. and Goodfellow, M. (2006). Diversity of actinomycetes isolated from Challenger Deep sediment (10,898 m) from the Mariana Trench. *Extremophiles.* 10:181-189.

Peczynska-Czoch, W. and Mordarski M. (1988). Actinomycete enzymes In: M. Goodfellow, S.T. Williams & M. Mordarski (eds.) *Actinomycetes in Biotechnology. Academic Press, London,* pp. 220 – 283.

References

Pei, G.; Dai, H.; Ren, B.; Liu, X. and Zhang, L. (2010). Exploiting bioactive Enediynes from marine microbe based on activity and gene screening. *Wei Sheng Wu Xue Bao*. 50(4):472–477.

Pervez, M. R.; Mohammed, M.; Prashant, V. T. and Ashutosh K. (2015). Characterization of Bioactive compound isolated from *Myrothecium spp.* with UV, FTIR and HPLC Analysis, *Indian J Pharm Biol Res.* 3(1):1-5

Porter, J. N. (1971). Prevalence and distribution of antibiotic –producing actinomycetes. *Adva in Appl Micro.* 14: 73-92.

Portillo, M. C.; Saiz-Jimenez, C. and Gonzalez, J. M. (2009). Molecular characterization of total and metabolically active bacterial communities of "white colonizations" in the Altamira Cave, *Spain Res Microbiol.* 160: 41-47.

Prakash, A.; Rigelhof, F.; Miller, E. (2001). Medallion Laboratories Analytical Progress. *Antioxidant Activity.* 19(2): 1-6.

Pridham, T. G.; Hesseltine, C. W. and Benedict, R. G. (1958). A guide for the classification of streptomycetes according to selected groups; placement of strains in morphological sections. *Appl Microbiol.* 6: 52–79.

Priyadarshini, K. and Keerthi Aparajitha U. (2012). Paclitaxel Against Cancer: A Short Review. *Med chem.*. 2:139-141

Rafieenia, R. (2013). Effect of nutrients and culture conditions on antibiotic synthesis in Streptomycetes. *Asi J of Pharma and Heal Scie.* 3(3): 810-821.

Rahalison. L.; Hamburger, M.; Hostettmann, K.; Monod, M. and Frenk E. A. (1991). A bioautographic agar over lay method for the detection of antifungal compounds from higher plants. *Phytochem. Anal.* 2: 199-203.

Rahman, H.; Austin, B.; Mitchell, W. J.; Morris, P. C.; Jamieson, D. J.; Adams, D. R.; Spragg, A. M. and Schweizer, M. (2010). Novel anti-infective compounds from marine bacteria. *Mar Drugs.* 5(3):498–518.

References

Rahman, M. A.; Islam, M. Z. and Islam, M. A. (2011). Antibacterial activities of actinomycete isolates collected from soils of Rajshahi, *Bangladesh Biotech Res Int.* 6.18-24

Raja, A.; Prabakaran, P.; Gajalakshmi, P. and Rahman, A. H. (2010). A Population Study of Psychrophilic Actinomycetes Isolated from Rothang Hill-Manali Soil Sample. *J Pure Applied Microbiol.* 4:847-51.

Raja, A. and Prabakarana, P. (2011). Actinomycetes and Drug- An overview, *Amer J of Drug Disco and Devel.* 1(2): 75-84.

Rajesh, R .O.; Mary Helen, P. A. and Jaya Sree, S. (2013). Screening of Antibiotic Producing Actinomycetes from Coconut Husk Retting Sample. *Intern J Res in Pharma and Biom*. 4:11-18

Ramazani, A.; Moradi, S.; Sorouri, R.; Javani, S. and Garshasbi, M. (2013). Screen for antibacterial activity of Streptomyces Species isolated from Zanjan province, Iran. *IJPCBS.* 3(2): 342-349.

Rana, S. and Salam, M. D. (2014) Antimicrobial Potential of Actinomycetes Isolated from Soil Samples of Punjab, *India J Microbiol Exp.* 1(2): 1-6

Ranade, A. R.; Wilson, M.S.; Mcclanahan, A. M. and Ball, A. J. (2014). High Content Imaging and Analysis Enable Quantitative in Situ Assessment of CYP3A4 Using Cryopreserved Differentiated HepaRG Cells. *J Toxicol*.1–12,

Rashad, F. M.; Nadia H A.; Insaf, E. D. and Fatima, H. M. (2015). Isolation and Characterization of Antibiotic / Antitumor Producing Streptomyces. *RJPBCS.* 6(2) :1917

Ravel, J.; Wellington, E. M. And Hill, R. T. (2000). Interspecific Transfer of StreptomycesGiant Linear Plasmids in Sterile Amended Soil Microcosms. *Appl. Envir. Micro.* 66: 529-534.

Ravi, R.K.; Vasantba, J.; Bhoomi, M.; Bonisha, T. and Bhumika, C. (2015). Antibacterial Potentials of Actinomycetes Isolated from Gujarat. *Int J Pharm Sci Rev Res.* 30(1): 78-83.

References

Ravikumar, S.; Inbaneson, S. J.; Uthiraselvam, M.; Priya, S. R.; Ramu, A. and Banerjee, M. B. (2011). Diversity of endophytic actinomycetes from Karangkadu mangrove ecosystem and its antibacterial potential against bacterial pathogens. *J Pharm Res.* 4:294-6.

Ravikumar, Y. S.; Mahadevan, K. M.; Kumaraswamy, M. N.; Vaidya, V. P.; Manjunatha, H.; Kumar, V.(2008). Antioxidant, Cytotoxic and Genotoxic evaluation of alcoholic extract of Polyalthia cerasoides (roxb) Bedd. *Environ Toxicol Pharmacol.* 26: 142-146.

Reddy, N. G.; Ramakrishna, D. P. N. and Raja Gopal, S.V. (2011). A Morphological, Physiological and Biochemical Studies of Marine Streptomyces rochei (MTCC 10109) Showing Antagonistic Activity Against Selective Human Pathogenic Microorganisms. *A J Bio Sci.* 4: 1-14.

Reiner, R. (1982). Antibiotics: An Introduction. Roche Scientific Service, *Switzerland.* 2: 21.

Ren, X.; Sun, J.; Housden, B. E.; Hu, Y.; Roesel, C.; Lin, S.; Liu, L .P.; Yang, Z.; Mao, D.; Sun L. et al. (2013). Optimized gene editing technology for Drosophila melanogaster using germ line-specific Cas9. *Proc Natl Acad Sci U S A.* 110: 19012–19017.

Rifaat, H.; Nageib, Z. and Ahmed, Y. (2005). Production of xylanases by *Streptomyces* species and their bleaching effect on rice straw pulp881. *Applied Ecol Environ Res.* 4 (1): 151-160.

Rios, J. L.; Recio, M.C. and Villar, A. (1988). Screening methods for natural products with antimicrobial activity: A review of the literature. *J Ethnopharmacol.* 23: 127-149.

Rioseras, B.; López-García, M. T.; Yagüe, P.; Sánchez, J. and Manteca, A. (2014). Mycelium differentiation and development of *Streptomyces coelicolor* in lab-scale bioreactors: Programmed cell death, differentiation, and lysis are closely linked to undecylprodigiosin and actinorhodin production. *Bioresour Technol Jan*. 151:191-8.

References

Saadoun, I.; Hameed K. M. and Moussauui, A. (1999) Characterization and Analysis of Antibiotic Activity of Some Aquatic Actinomycetes. *Microbios.* 99(394):173-179.

Saadoun, I.; AL-Joubori, B. and Al-Khoury, R. (2015). Testing of Production of Inhibitory Bioactive Compounds by Soil Streptomycetes as Preliminary Screening Programs in UAE for Anti-Cancer and Anti-Bacterial Drugs. *Int J Curr Microbiol App Sci*. 4(3): 446-459.

SaravanaKumar, P.; Al-Dhabi, N.; Duraipandiyan, V.; Balachandran, C.; Praveen Kumar, P. and Gnacimuthu, S. (2014). *In vitro* antimicrobial, antioxidant and cytotoxic properties of *Streptomyces lavendulae* strain SCA5. *BMC Microbio.* 14:291-299.

Sarkonen, N.; Kononen, E.; Summanen, P.; Kononen, M. and Jousimies-Somer, H. (2001).Phenotypic identification of Actinomyces and related species isolated from human sources. *J Clin Microbiol.* 39:3955- 3961.

Sartore-Bianchi, A.; Gasparri, F.; Galvani, A.; Nici, L.; Darnowski, J.W.; Barbone, D.; Fennell, D.A.; Gaudino, G.; Porta, C.; Mutti, L. (2007). Bortezomib inhibits nuclear factor-kappa B dependent survival and has potent *in vivo* activity in mesothelioma. *Clin Cancer Res.* 13(19): 5942-5951.

Sastry, V. M. V. S. and Rao, G. R. K. (1995). Dioctyl phthalate and antibacterial compound from the marine brown alga Sargassum wightii. *J App Phy.* 7:185-186.

Schabereiter-Gurtner, G.; Saiz-Jimenez, C.; Pinar, G.; Lubitz, W. and Rolleke, S. (2002) Altimira cave Paleolithic paintings harbor partly unknown bacterial communities. *FEMS Microbiol Lett*. 211: 7-11

Schneider, G.; Murray D.; Silverstone, D.; Hines, C.; Augereau, J.; Pinte, CH.; Menard, F.; Krist, J.; Clampin, M.; Grady, C.; Golimowski, D.; Ardila, D.; Henning, T.; Wolf, S. and Rodmann,J. (2006).Discovery of an 86 AU Radius Debris Ring Around HD 181327. *The Astrophysical J.* 650(1): 414-431.

References

Schöller, C. E.; Gürtler, H.; Pedersen, R.; Molin, S. and Wilkins, K. (2002). Volatile metabolites from actinomycetes. *J Agric Food Chem.* (9):2615-21.

Schulz, D.; Nachtigall, J.; Riedlinger, J.; Schneider, K.; Poralla, K.; Imhoff, J. F.; Beil, W.; Nicolson, G.; Fiedler, H. P. and Sussmuth R. D. (2009).Piceamycin and its N-acetylcysteine adduct is produced by Streptomyces sp. GB 4-2. *J Antibi.* 62:513-518.

Scudiero, D. A.; Shoemaker, R. H.; Paull, K. D.; Monks, A.; Tierney, S.; Nofziger, T. H.; Currens, M. J.; Seniff, D. and Boyd, M. R. (1988). Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. *Cancer Res.* 48(17):4827–33.

Selvakumar, D.; Arun, K.; Suguna, S.; Kumar, D. and Dhevendaran , K. (2010). Bioactive potential of *Streptomyces* against fish and shellfish pathogens. *Iran J Microbiol.* 2(3): 157-164.

Selvameenal, L.; Radakrishnan, M. and Balagurunathan, R.(2009) .Antibiotic pigment from desert soil actinomycetes; biological activity, purification and chemical screening, *Indian J Pharma Sci.* 71: 499–504.

Shahverdi, A. R.; Abdolpour, F.; Monsef-Esfahani, H. R. and Farsam, H.(2007). A TLC bioautographic assay for the detection of nitrofurantoin resistance reversal compound. *J Chromato B.* 850:528–530.

Sharma, D., Mayilraj, S. and Manhas, R.K. (2014). *Streptomyces amritsarensis* sp. nov., exhibiting broadspectrum antimicrobial activity. *Antonie Van Leeuwenhoek.,* 4(5), 943–949.

Sharon, F. B.; Daniel, R. R.; and Shenbagarathai, R. (2014).optimaziation of antibiotic production by marine actinomycetes Streptomyces sp. K)D10S . *Int J Pharm Pharm Sci.* 6(2):506-510

Shirling, E. B. and Gottlieb, D. (1966). Methods for characterization of *Streptomyces* species. *IJSB*, 16: 313-340

Shomurat, T.; Yoshida, J.; Amano, S.; Kojina, M. and Niida, T. (1979). Studies on actinomycetal producing antibiotics only in agar culture. I. Screening

taxonomy and morphology – productivity relationship of Streptomyces halstedii, strain SF. *J Antibiot.*32: 427-435.

Silverstein, R. M.; Bassler, G. C.; Morril, T.C. (1981). Spectrometric Identification of Organic Compounds. New York: John Wiley and Sons, 4th edition .116-118.

Singh, S. B.; Zink, D. L.; Dorso, K.; Motyl, M.; Salazar, O.; Basilio, A.; Vicente, F.; Byrne, K. M.; Ha, S. and Genilloud, O. (2009). Isolation, structure and anti-bacterial activities of lucensimycins D-G, discovered from *Streptomyces lucencis* MA7349 using an antisense strategy. *J Nat Prod*. 72(3):345–352

Singh, S.; Kushwaha, B. P. ; Nag, S. K. ; Mishra, A. K. ; Singh, A. and Anele, U. Y. (2012). *In vitro* ruminal fermentation, protein and carbohydrate fractionation, methane production and prediction of twelve commonly used Indian green forages. *Anim Feed Sc. Technol.* 178 (1/2): 2-11.

Singh, L. Shantikumar, S. h.; Hemant. T. and Narayan, C. (2014). Production of potent antimicrobial agent by actinomycete, *Streptomyces sannanensis* strain SU118 isolated from phoomdi in Loktak Lake of Manipur, India. *BMC Microbiol.*14:278.

Sirenko, O.; Hesley, J.; Rusyn, I. and Cromwell, E. F. (2014). High-content assays for hepatotoxicity using induced pluripotent stem cell-derived cells. *Assay Drug Dev. Technol.* 12: 43–54.

Song, Q.; Huang, Y. and Yang, H. (2012) Optimization of fermentation conditions for antibiotic production by Actinomycetes YJ1 strain against Sclerotinia sclerotiorum. *J Agr Sci.* 4(7): 95-102.

Sprusansky, O.; Stirrett, K.; Skinner, D.; Denoya, C.; Westpheling, J. (2005). The bkdR gene of Streptomyces coelicolor is required for morphogenesis and antibiotic production and encodes a transcriptional regulator of a branched-chain amino acid dehydrogenase complex. *J Bacteriol.* 187:664-671.

Srinivasan, M. C.; Laxman, R. S. and Deshpande, M. V. (1991). Physiology and nutrition aspects of actinomycetes – An overview. *World J Microbial Biotechnol.* 7:171-184.

References

Stackebrandt, E .; Witt, D. ;Kemmerling, C. ;Kroppenstedt, R. ; and Liesack, W. (1991). Designation of Streptomycete 16S and 23S rRNA-based target regions for oligonucleotide probes. *Appl Environ Microbiol.* 57(5):1468-77.

Stackebrandt, E.; Rainey, F.A.; Ward-Rainey, N.L.(1997). Proposal for a new hierarchic classification system, Actinobacteria classis nov., *Int. J SystBacteriol.,* 47:479–491.

Striegel, M. F., and Jo H. (1996). Thin-Layer Chromatography for Binding Media Analysis. Scientific Tools for Conservation. Los Angeles, CA: Getty Conservation Institute. http://hdl.handle.net/10020/gci_pubs/thin_layer_chromatography

Sudha, S. and Masilamaniselvam, M. (2013). *In vitro* cytotoxic activity of bioactive metabolite and crude extract from new actinomycetes. *Streptomyces avidinii* strain SU4. *Int J Pharm Pharm Sci.* 5(3):612-616.

Sunil, C.; Agastian, P.; Kumarappan, C. and Ignacimuthu, S. (2012). *In vitro* antioxidant,antidiabetic and antilipidemic activities of Symplocos cochinchinensis(Lour.) S. Moore bark. *Food Chem Toxicol.* 50: 1547 – 1553.

Suthindhiran, K. and Kannabiran, K. (2009). Cytotoxic and Antimicrobial Potential of Actinomycete Species *Saccharopolyspora salina* VITSDK4 isolated from the Bay of Bengal Coast of India. *Ameri J Infect Dise.* 5(2):90-8.

Suthindhiran, K. and Kannabiran, K. (2010). Diversity and exploration of bioactive marine actinomycetes in the Bay of Bengal of the Puducherry coast of India. *Indian J. Microbiol.* 50(1):76-82.

Taddei, A.; Rodriguez, M. J.; Marquez-Vilchez, E. and Castelli, C. (2006). Isolation and identification of *Streptomyces* spp. from Venezuelan soils: Morphological and biochemical studies. I, *Microb Res.* 161:222-231.

Takahashi, Y. and Omura, S. (2003). Isolation of new actinomycete strains for the screening of new bioactive compounds. *J Gen Appl Microbiol.* 49:141-54.

Takami, H.; Inoue, A.; Fuji, F. and Horikoshi, K.(1997). Microbial flora in the deepest sea mud of the Mariana Trench. *FEMS Microbiol Lett.,* 152:279-85.

References

Takizawa, M.; Colwell, R. R. and Hill, R. T. (1993) Isolation and diversity of actinomycetes in the Chasapeake Bay. *Appl Environ Microbiol*. 59: 997-1002

Tan, G.Y.A.; Robinson, S.; Lacey, E.; Brown, R.; Kim, W. and Goodfellow, M. (2007). *Amycolatopsis regifaucium* sp. nov., a novel actinomycete that produces kigamicins. *Int J Syst Evol Microbiol.*, 57:2562–2567.

Thakur, D.; Yadav, A.; Gogoi, B. K. and Bora, T. C. (2007). Isolation and screening of Streptomyces in soil of protected forest areas from the state of Assam and Tripura, India for antimicrobial metabolites. *J mycol and med.* 17: 242-249.

Tolosa, L.; Pinto, S.; Donato, M. T.; Lahoz, A.; Castell, J. V.; O'Connor, J. E. and Gómez-Lechón, M. J. (2012). Development of a multiparametric cell-based protocol to screen and classify the hepatotoxicity potential of drugs. *Toxicol Sci.* 127(1):187-98

Tondeur, F.; Brack, M.; Farine, M. and Pearson, J.M. (1984). Static nuclear properties and the parametrisation of Skyrme forces. *Nucl Physi A.* 420(2): 297–319.

Tormo, J. R.; Garcıa, J. B.; DeAntonio, M.; Feliz, J.; Mira, A.; Dıez, M. T.; Hernandez, P. and Pelaez, F.(2003). A method for the selection of production media for actinomycete strains based on their metabolite HPLC profiles. *J Ind Microb Biotechnol.* 30: 582–588.

Towne, D. L.; Nicholl, E. E.; Comess, K. M.; Galasinski, S. C.; Hajduk, P. J.; Abraham, V. C. (2012). Development of a high-content screening assay panel to accelerate mechanism of action studies for oncology research. *J. Biomol. Screen.* 17: 1005–1017

Tresner, H. D.; Hayes, J. A. and Backns, E. J. (1968). Differential tolerance of *Streptomyces* to sodium chloride as a taxonomic aid. *Appl Microbiol.* 16: 1134-1136.

References

Valli, S.; Suvathi Sugasini S.; Aysha, O. S.; Nirmala, P.; Vinot Kumar, P. and Reena, A. (2012).Antimicrobial potential of actinomycetes species isolated from marine environment. *Asian Pacific J Trop Biomed*, 9: 416-473.

Van Wezel, G. P. and McDowall, K. J. (2011). the regulation of the secondary metabolism of *Streptomyces*: new links and experimental advances. *Nat Prod Rep.* 28(7):1311-13333.

Veiga, M.; Esparis, A. and Fabregas, J. (1983). Isolation of cellulolytic actinomycetes from marine sediments. *J Appl Environ Microbiol*. 46:286-297.

Ventura, M.; Canchaya, C.; Tauch, A.; Chandra, G.; Fitzgerald, G.F. and Chater, K.F. (2008). Genomics of Actinobacteria: Tracing the evolutionary Das S, Lyla PS, Khan SA. Distribution and generic composition of culturable marine actinomycetes from the sediments of Indian continental slope of Bay of Bengal. *Chin J Oceanol Limnol*. 26:166- 177.

Vijakumar, R.; Murugesan, S. and Panneerselvam, A. (2010). Isolation, characterization and antimicrobial activity of actinobacteria from point calimere coastal region, east coast of India, *Int Res J Pharam.*1: 358–365.

Vimal, V.; Mercy, R.B. and Kannabiran, K. (2009). Antimicrobial activity of marine actinomycete, Nocardiopsis sp. VITSVK 5 (FJ973467). *Asian J. Med. Sci.*, 1: 57-63.

Wadkins, R.M.;, Vladu, B. and Tung, C.S.(1998). Actinomycin D binds to metastable hairpins in single-stranded DNA. *Biochem.*, 37(34):11915–1923.

Waksman, S.A. (1940). On the classification of Actinomycetes. *J. Bacteriol*., 39(5):549–558

Waksman, S.A.; Schatz, A. and Reynolds, D.M. (2010). Production of antibiotic substance by actinomycetes .*Ann. N.Y. Acad. Sci.* 1213:112–124

Walker, D. and Colwell, R.R. (1975).Factors affecting enumeration and isolation of actinomycetes from Chesapeake Bay and South eastern Atlantic Ocean sediments. *Mar. Biol.* 30:193-201.

References

Williams, S.T.; Goodfellow, M.; Alderson, G.; Wellington, E.M.H.; Sneath, P.H.A. and Sackin, M.J. (1983). Numerical classification of *Streptomyces* and related genera. *J. Gen. Microbiol.* 129:1743–1813

Williams, S.T.; Locci, R.; Beswick, A.; Kurtböke, D.I.; Kuznetsov, V.D.; Le Monnier, F.J.; Long, P.F.; Maycroft, K.A.; Palmit, R.A.; Petrolini, B.; Quaroni, S.; Todd, J.I. and West, M. (1993). Detection and identification of novel actinomycetes. *Rese. in Microbi.* 144: 653-656.

Williams, Y.; A. Sukhanova, M. Nowostawska, A.M. Davies and S. Mitchell *et al.*, (2009). Probingcell-type-specific intracellular nanoscale barriers using size-tuned quantum dots. *Small,* 5: 2581-2588.

Wood, S.A.; Kirby, B.M.; Goodwin, C.M.; Le Roes, M. and Meyers, P.R. (2007). PCR screening reveals unexpected antibiotic biosynthetic potential in Amycolatopsis sp. strain UM16. *J. Appl. Microbiol.*, 102:245-253.

Xi, Y.; Chen, R.; Si, S.; Sun, C. and Xu, H. (2007). A new nucleosidyl peptide antibiotic, sansanmycin. *J Antibiot (Tokyo)*, 60(2):158–161

Xie, Y., Chen, R., Si, S., Sun, CH. and Xu, H. (2007). A New Nucleosidyl-peptide Antibiotic, Sansanmycin. *J. Antibiot.* 60(2):158-161.

Yunt, Z.; Reinhardt, K.; Li, A.; Engeser, M.; Dahse, H.M.; Gütschow, M.; Bruhn, T.; Bringmann, G. and Piel, J. (2009). Cleavage of 4 carbon- carbon bonds during biosynthesis of griseorhod in a spiroketal pharmacophore. *J. Am. Chem. Soc.* 131(6):2297–2305

Zgurskaya, H.I. and Nikaido, H. (2000). Multidrug resistance mechanisms: drug efflux across two membranes. *Mol. Microbiol.* 37(2):219-25.

Zhi, X.Y.; Li, W.J. and Stackebrand, E. (2009). An update of the structure and 16S rRNA gene sequence-based definition of higher ranks of the class Actinobacteria, with the proposal of two new suborders and four new families and emended descriptions of the existing higher taxa. *Int. J. Syst. Evol. Microbiol*. 59:589-608.

References

Zhou, J.; Gu, Y.; Zou, C. and Mo, M. (2007). Phylogenetic diversity of bacteria in an earth-cave in Guizhou province, southwest of China. *J. Microbiol.* 45: 105-112.

Zhou, W. and Zimmerman, W. (1993). Decolorization of industrial effluents containing reactive dyes by actinomycetes. *Microbiol . Lett. FEMS .*, 107: 157-162

Appendices

Apendices

Appendice A: Growth curves (Growth kinetics) study of locally isolated actinomycetes (KH14), which was growing on malt extract yeast extract broth for 6 days at 29±1 °C, measuring the absorbance of growth at 600nm.

No.	Times in (days)	OD at 600nm	
		Blank	Absorbance
1	1	0.042	0.067
2	2	0.042	0.315
3	3	0.042	0.619
4	4	0.042	0.447
5	5	0.042	0.484
6	6	0.042	0.471

Appendice B: Estimating optimum pH value for achieving maximum growth of isolated strain (KH14), which was growing on ISP2 broth with different pH value, spectrophotometrically evaluated.

No.	pH of media	OD at 600nm	
		Blank	Absorbance
1	4	0.015	0.027
2	5	0.082	0.119
3	6	0.023	0.156
4	7	0.071	0.211
5	8	0.024	0.409
6	9	0.031	0.249
7	10	0.080	0.186

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Appendice C: Estimating of isolated strain (KH14) NaCl salt tolerance, growing on malt extract yeast extract broth (ISP2) for 4 days of incubation at 29±1 °C, spectrophotometrically measured the absorbance.

No.	NaCl concentration	OD at 600nm		Means of OD	Growth state
		Ab. 1	Ab.2		
1	1%	0.113	0.128	0.121	++
2	2%	0.106	0.119	0.113	++
3	3%	0.113	0.105	0.109	++
4	4%	0.084	0.076	0.080	+
5	5%	0.076	0.075	0.075	+
6	6%	0.033	0.034	0.033	-
7	7%	0.037	0.039	0.038	-
8	8%	0.032	0.032	0.032	-
9	9%	0.034	0.032	0.033	-
10	Negative control (blank) (Without bacteria)	0.043	0.040	0.0415	-
11	Positive control (Without salt)	0.142	0.123	0.132	+++

Appendice D: Antioxidant scavenging activities of extracellular crude extract (KH14) were achieved through DPPH assay, after 30 minutes incubation with duplicated, 517nm OD for measuring absorbance.

Conc. mg/ml	OD at 517nm		Mean OD	Blank	Log concentration	Scavenging activity %
	Ab1	Ab2				
1	0.626	0.7	0.663	0.805	0	17.63975
3	0.621	0.658	0.6395	0.805	0.477121	42.99242
5	0.545	0.521	0.533	0.805	0.69897	46.9697
10	0.071	0.172	0.121	0.805	1	76.98864
20	0.050	0.035	0.045	0.805	1.30103	91.95076
30	0.052	0.051	0.0515	0.805	1.477121	90.24621

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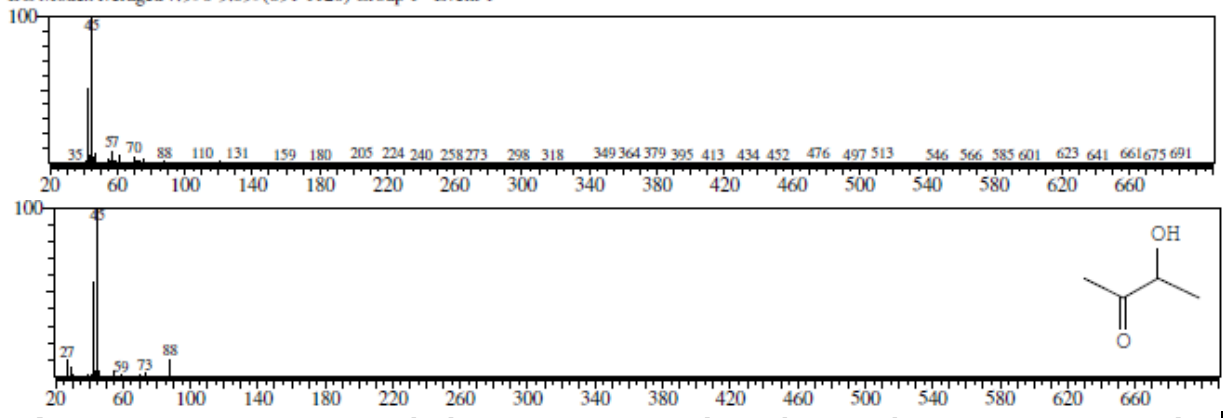
Appendice E: Antioxidant scavenging activities of ascorbic acid (standard) were achieved through DPPH assay, after 30 minutes incubation,

Ascorbic acid concentration mg/ml	OD at 517 nm	Log concentration mg/ml	Scavenging activity %
1	0.211	0	53.21508
0.5	0.235	-0.30103	47.89357
0.25	0.241	-0.60206	46.56319
0.125	0.271	-0.90309	39.91131
0.031	0.359	-1.50864	20.39911
0.015	0.426	-1.82391	5.543237

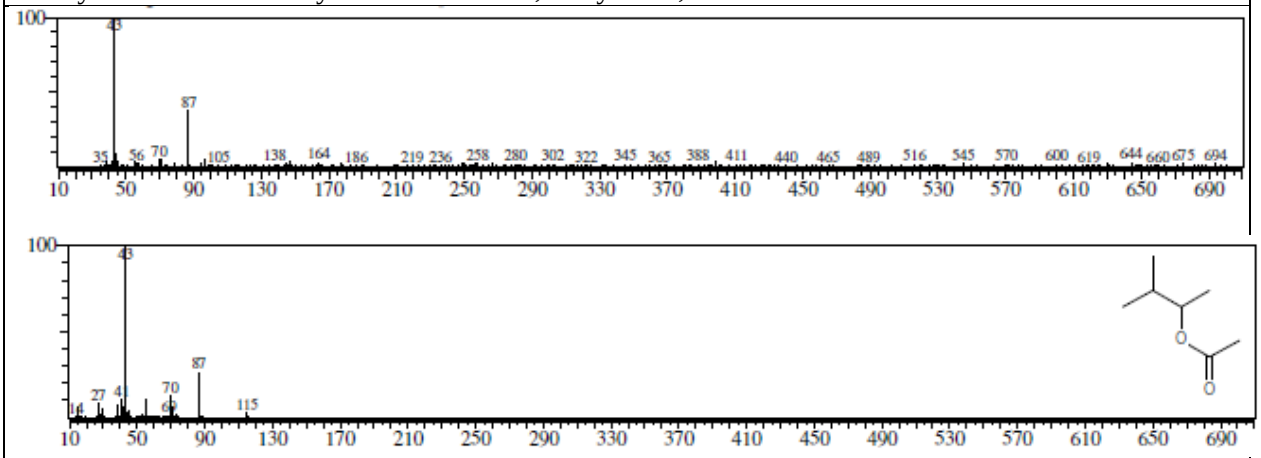
Apendices

Appendice F: Detail documents of compounds analysis from ethyl acetate extract of (KH14) by GC-MS for all obtained 12 peaks; each compounds with their details evidence such (molecular structure, companies name and mass peaks) ; plate **A**. peaks 1 to 3; plate **B** peaks 4to 6; plate **C** peaks 7to 9 and plate **D** peaks 10 to 12.

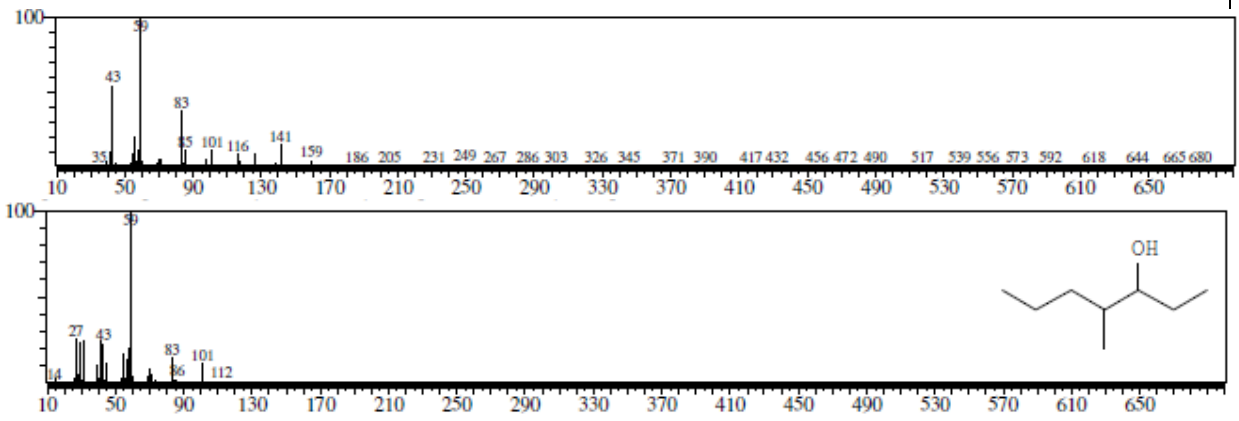
A



Peak 1: CompName:2-Butanone: 3-hydroxy- **:** .gamma.-Hydroxy-.beta.-oxobutane **:** Acetoin **:**Acetyl methyl carbinol **:** Dimethylketol **:** Methanol, acetylmeth; MassPeaks:513



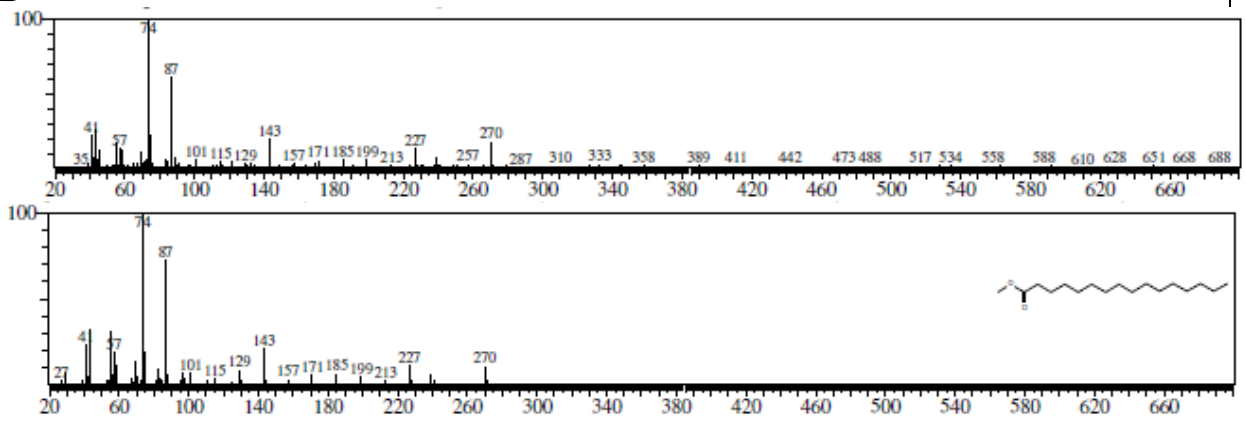
Peak 2: Comp Name: DL-3-Methyl-2-butanol, acetate: MassPeaks:348



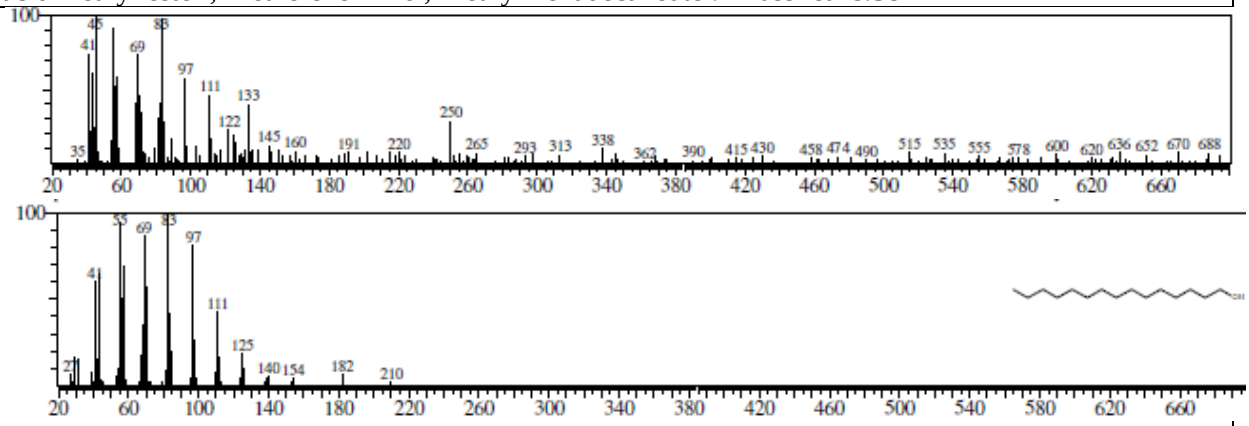
Peak 3: CompName:3-Heptanol, 4-methyl- : 4-Methyl-3-heptanol : 4-Methyl-5-heptanol; MassPeaks:461

Apendices

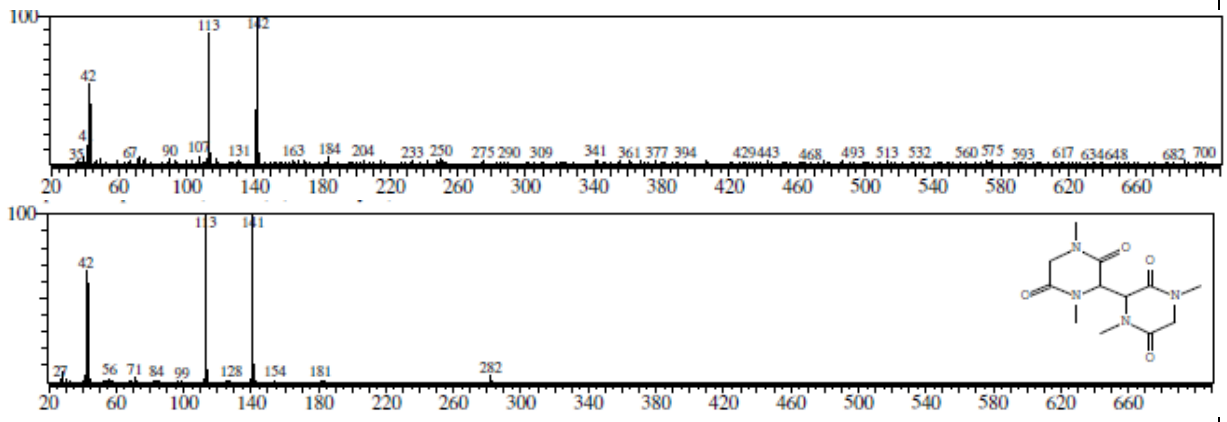
B



Peak 4: Comp Name: Hexadecanoic acid, methyl ester ;Palmitic acid, methyl ester ; n-Hexadecanoic acid methyl ester ; Metholene 2216 ; Methyl hexadecanoate : MassPeaks:387



Peak 5: Comp Name :n-Pentadecanol ; n-1-Pentadecanol ; Pentadecanol ; Neodol 5 ; 1-Pentadecanol ; Pentadecan-1-ol ; Pentadecyl alcohol : MassPeaks:223



Peak 6: CompName:Piperazine-2,5-dione, 1,4-dimethyl-3,3'-bis- : MassPeaks:296

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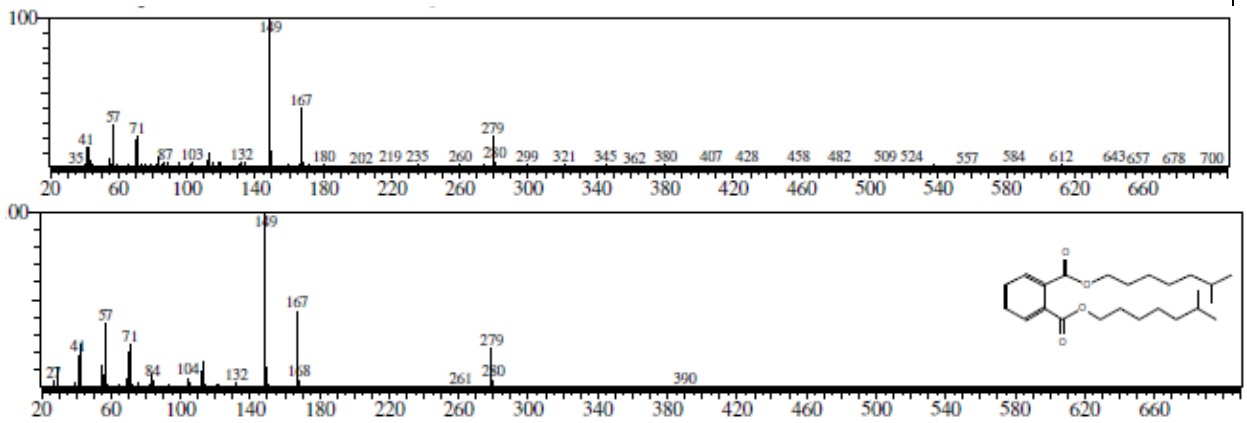
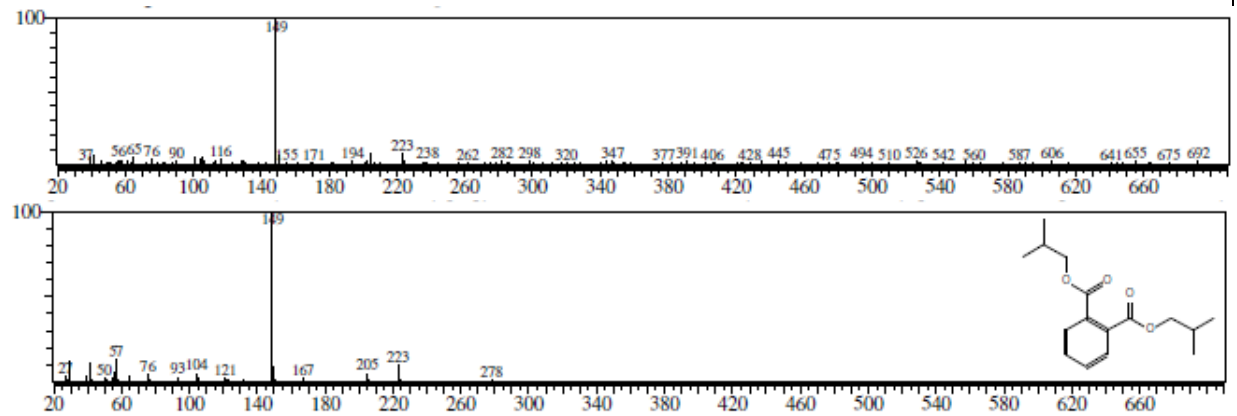
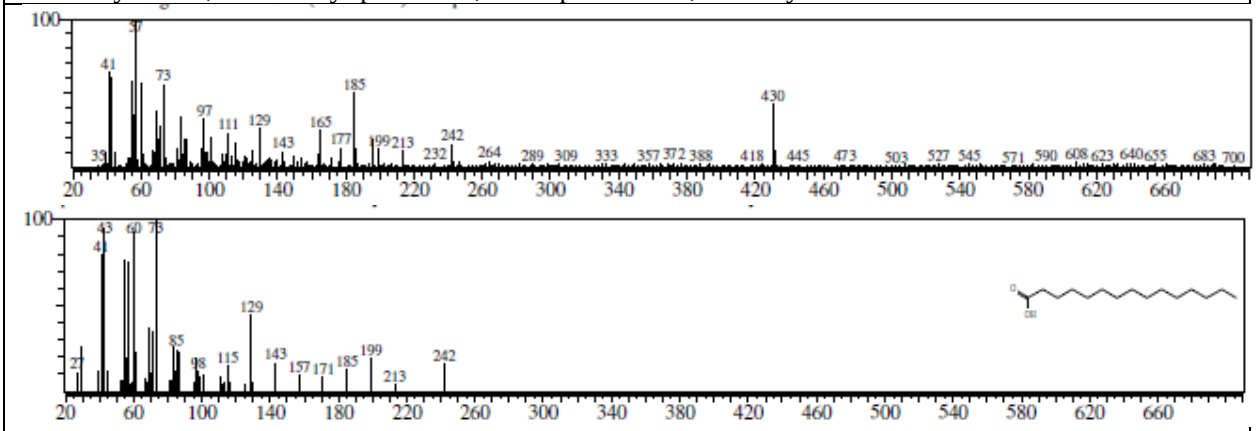
C



Peak 7: CompName:1,2-Benzenedicarboxylic acid, diisooctyl ester ; Diisooctyl phthalate ; Hexaplas M/O ; Isooctyl phthalate ; Corflex 880; DIOP ; Flex : MassPeaks:337



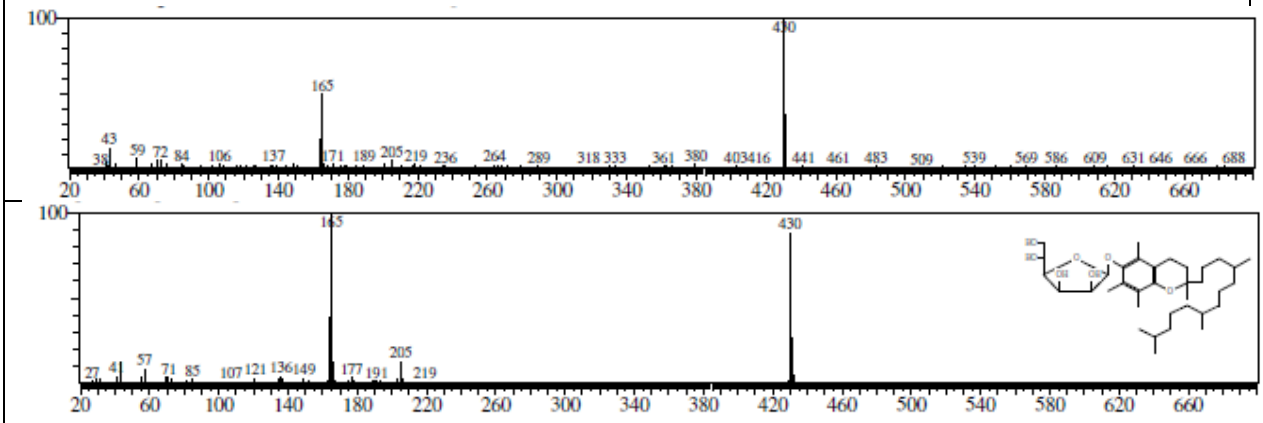
Peak 8: CompName:1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester ; Phthalic acid; diisobutyl ester ; Diisobutyl phthalate ; Hexaplas M/1B ; Isobutyl: MassPeaks:307



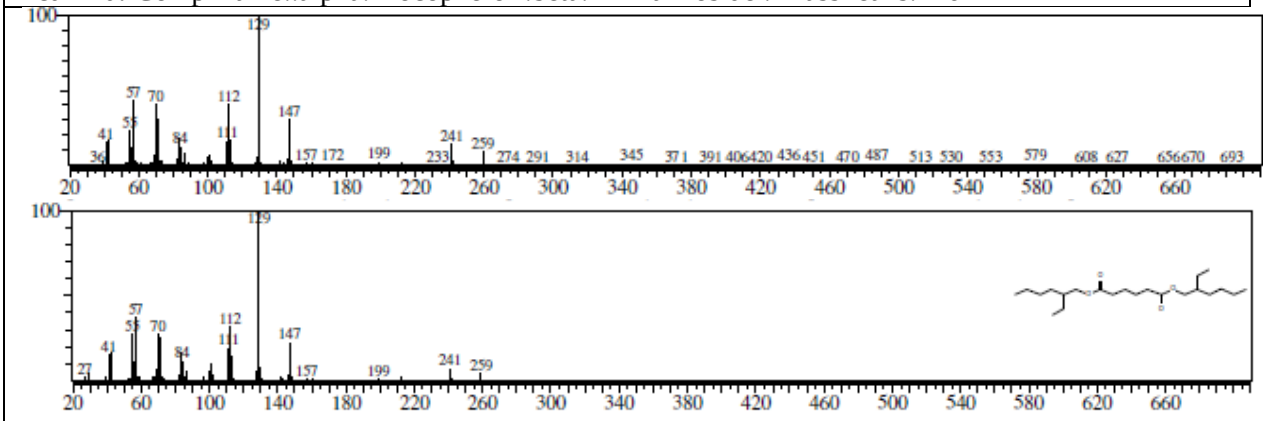
Peak 9: Comp Name: Pentadecanoic acid ; Pentadecylic acid ; n-Pentadecanoic acid ; n-Pentadecylic acid :MassPeaks:516

Apendices

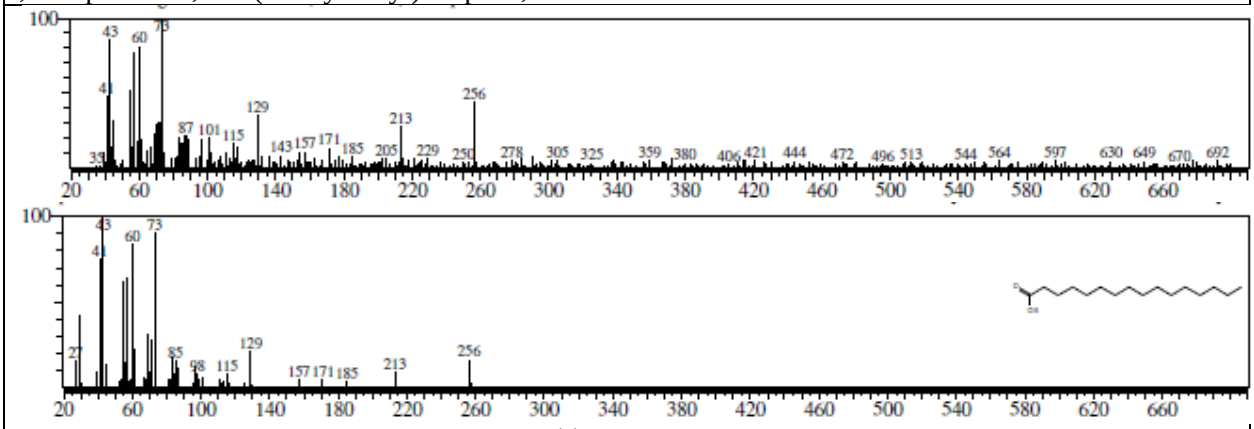
D



Peak 10: Comp Name:.alpha.-Tocopherol-.beta.-D-mannoside : MassPeaks:276



Peak 11: Comp Name: Hexanedioic acid, bis(2-ethylhexyl) ester; Adipic acid, bis(2-ethylhexyl) ester ; Adipol 2EH ; Bis(2-ethylhexyl) adipate ; Bisoflex DOA :MassPeaks:362



Peak 12: Comp Name: n-Hexadecanoic acid $$ Hexadecanoic acid; n-Hexadecoic acid ; Palmitic acid ; Pentadecanecarboxylic acid ; 1-Pentadecanecarboxylic : MassPeaks:396

وأشارت الفعالية المضادة للسرطان والتي تم قياسها عن طريق الفحص *MTT* بان مستخلص العزلة KH14 تمتلك اقوى تأثير ضد خطوط الخلايا السرطانية المستخدمة، وخصوصا ضد MCF-7 وذلك بتثبيط ٧٥.٨٤٪ (٤٠٠ ميكروغرام تركيز) من الخلايا السرطانية بعد ٧٢ ساعة من الحضن، بينما اظهرت فعالية معتدلة ضد ما تبقى من خطوط الخلايا سرطانية الأخرى المستخدمة (PC3وA375). وقد أجريت فحص المحتوى العالي لستة قيم خلوية ضد خط الخليةMCF-7 ، بالإضافة إلى ذلك، فإن المقارنة التي أجريت بين تراكيز مختلفة من استخراج خارج الخلية الخامة ل KH14 اظهرت نتائج التحليلات الإحصائية عند احتماليات مختلفة للخطا (قيمP) وجود اختلافات معنوية بشكل ملحوظ بين عينات (السيطرة) مع المستخلصات خارج خلوية الخام وخصوصاً في حالة بقاء الخلية، المظهر النووي و نفاذية غشاء الخلية وكفاءة غشاء الميتوكوندريا عند تراكيز مختلفة، في حين أن كل من إجمالي الكثافة النووية و Cytochrome C لم تدون اية اختلافات معنوية عند مقارنتها مع معاملة السيطرة السلبة (مركبة).

اقل تركيز لتثبيت النمو (MICs) ضد كل من *Staphylococcus aureus*, *Escherichia coli* و *Candida albicans* كانت ٦٤ و ٥٠٠ و ٥٠٠ مايكروليتر /مل على التوالي ، وبينت النتائج بانه المستخلص الخارج خلوي الخام كان اكثر نشاطا ضد البكتيريا موجبة للصبغة جرام وذو فعالية تثبيطية معتدلة ضد كل من البكتيريا السالبة لصبغة جرام والخميرة.

بالإضافة إلى فعالية المضادة للجراثيم، تم إجراء فحص *DPPH* لتحديد الفعالية المضادة للأكسدة ، أظهرت النتائج بان المستخلص الخارج خلوي للعزلة KH14 اعطت قيمة تثبيطية 5.24 IC_{50} ملغم / مل الذي ازالت ٩١٪ من الجذور الحرة المتكونه لل *DPPH* . تم تحديد طبيعة المركبات المضادة للميكروبات باستخدام كروماتوكرافيا الطبقة الرقيقة *TLC*،والتي أشارت إلى وجود أربع بقع منفصلة، اظهرت واحدة منهما فقط نشاطاً مضاداً للميكروبات، وكذلك تم تحديد موقعها بواسطة التصوير الحيوي الذاتي bioautography ، وذلك باستخدام عدد مختلف من المذيبات للطور المتحرك ، والتي اعطت قيم مختلفة ل R_f . وأشار التحليل بواسطة كروماتوكرافيا الحالة السائلة العالية الاداء *HPLC-MS* للبقع النشطة والمقشطة من *TLC* والاستخلاص باستخدام ethyl acetate وجود العديد من المركبات، بالإضافة إلى ذلك أظهرت النتائج بأن معظم هذه المركبات ذو كتلة وزنية مماثلة. اما من ناحية الامتصاصية للأشعة فوق البنفسجية المعينة اظهرت وجود قمتين فقط عند طول موجي 221.85و 264.85 نانوميتر للبقع المقشطة من الكروماتوكرافيا الطبقة الرقيقة، في حين وجود ثلاث قمم متميزة ٢٢٢.٨٥ ، ٢٦٤.٨٥ و ٤٤٢.٨٥ نانوميتر للمستخلص باستخدام ethyl acetate. وعلاوة على ذلك، تم تحقيق التشابه بين القمم المركبين على أساس الامتصاصية لأشعة فوق البنفسجية في القمة الثانية لكل منهما وهو الطول الموجي ٢٦٤.٨٥ نانوميتر ، وبهذا يعتبر هذا الامتصاصية عبارة عن امتصاصية المادة الفعالة. بينت نتائج التحليل بواسطة *GC-MS* للعينة KH14 المستخلصة باستخدام ethyl acetate extract ظهور 12 قمة امتصاصية، حددت الاوزان الجزيئية والصيغ الجزيئية لها بأعتماد المكتبة الرقمية *NIST*.

الخلاصة

ازدادت في الآونة الأخيرة عدد المُمرضات المقاومة المضادة للجراثيم والتي ادت الى مشاكل صحية، لذلك هدفت الدراسة الحالية الى عزل الفطريات الشعاعية(الاكتينومايستات) المحلية ، والتي لها القدرة على إنتاج مركبات جديدة فعالة حيوياً ، والتحري عن فعاليتها البايولوجية (كمضاد للجراثيم ومضاد للسرطان)، بالاضافة الى الوصف و التنقية الجزئيةللمركبات الايضية المضادة للجراثيم. اجري مسح وجمع لأربعين مصدر من التربة واحتوت (٦٥٪) منها على الفطريات الشعاعية كامنة الحيوية، وتم عزل ٥٥ مستعمرة مختلفة من الناحية المظهرية . اختبرت جميع العزلات للكشف عن انشطتها المضادة للجراثيم بالاختبار ألاولي ضد سلالات معروفة. ومن بين جميع الانواع المستحصل عليها وجدت ستة عزلات كانت لها القدرة على كبح نمو ما لا يقل عن اثنين من الاحياء المجهرية التي تم اختبارها وهي KH14، KH16، KH18، KA19، KA38 وKA39. تم دراسة تكوين اوساط زراعية مثلى لنمو وتعزيز إنتاج مركبات الايضية مفيدة كمضادات للجراثيم وذلك باستخدام خمسة اوساط زرعية مختلفة ISP1 ، ISP4, ISP2، ISP5 والكليسيرول ومستخلص الخميرة استخدمت ايضا كاوساط زرعية. تبين بأن الوسط ISP2 اعطت أفضل النتائج (منطقة التثبيط) بالمقارنة مع الاوساط الغذائية الاخرى المستخدمة. وعلاوة على ذلك، تم استخدام خمسة مذيبات عضوية مختلفة للحصول على الحد الأقصى للمستخلص مركبات الايضية خارج الخلية و المضادة للجراثيم ، كما أظهرت النتائج بأن الاستخلاص عن طريق n-butanol، methanol، ethyl acetate، n - hexaneو chloroform ، سجلت مناطق تثبيط (٤٦.٦٪، ٣٣.٣٪، ٢٠٪، ١٣.٣٪ و ٦.٦٪ على التوالي) ضد الكائنات الدقيقة التي تم اختبارها.و اظهرت الفعالية المضادة للجراثيم للمستخلصات الخامة بأن المستخلص الخارج خلوي أكثر قوة و فعالية من الداخل خلوية. واستخدمت خمسة عشر نوع مختلف من أقراص المضادات الحيوية المصنعة لمقارنة فعاليتها مع المستخلصات خارج الخلوية الخامة ، ومن الجدير بالذكر، ان المستخلص الخارج خلوي اظهرت فعالية عالية ضد المسببات المرضية بالمقارنة مع الاقراص المصنعة المذكورة انفاً. بينت نتائج الدراسة من ناحية تحقيق القيم المثلى لميكانيكية النمو وعدد ايام التحضين و درجة الحموضة و القدرة التحملية لملح كلوريد الصوديوم بأن اجراء التحضين لمدة ٣ أيام، ودرجة الحموضة ٨ ونسبة ٥٪ من كلوريد الصوديوم هي المثلى للنمو خصوصاً للعزلة (KH14). اظهرت النتائج بان

بسم الله الرحمن الرحيم

(فَانْظُرْ إِلَى آثَارِ رَحْمَتِ اللَّهِ كَيْفَ يُحْيِ الْأَرْضَ بَعْدَ مَوْتِهَا
إِنَّ ذَلِكَ لَمُحْيِ الْمَوْتَى وَهُوَ عَلَى كُلِّ شَيْءٍ قَدِيرٌ)

الروم: ٥٠

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وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم
قسم التقانة الأحيائية

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اطروحة مقدمة

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ماجستير تقانة الاحياية، جامعة يرموك، اربد الاردن،٢٠١١

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