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Antitumor agent produced by *Streptomyces*

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By

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Eveen

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Summary

This study included determining the activity of crude secondary metabolites produced by *Streptomyces* and their effect on the growth cancer cells *in vitro*.

Twenty samples of soil were collected from different places of Baghdad, and five isolates referred as *Streptomyces*, were cultivated using Gauza agar medium. The isolates went through microscopical and cultural examinations as well as biochemical tests, growing at different salt concentrations on SY- media, and growing at different incubation temperatures.

Results showed that none of the isolates were not tolerant to neither high nor low temperature (28- 50ºC), also it was noticed that these isolates grow better when pH value range between 7- 8.

The ability to produce antibacterial substances was studied by determining the biological activity against gram +ve and gram –ve bacteria and eukaryotes, the results showed that two of the five isolates had the ability to inhibit the growth of these microorganisms because of their ability to produce antibiotic- like substances, they were called ERM1 and ERM2.

These results were also strengthened by thin layer paper chromatography, and it was found that these two isolates were able to produce Neomycin, Streptomycin, Doxorubicin, Bleomycin and Tetracycline.

When the factors affecting the productivity of these isolates were determined, it was found the growth at 28°C and pH 7.0 for five days was the best to produce antibiotics.

Cytotoxic activity was tested for every crude extract of ERM1 and ERM2 (10, 20, 30, 40, 50 and 60) µg/ml on the cancer line (Hep-2) for 28 hrs, the result indicated that the crude extract showed inhibitory activity on the growth of cancer cells.

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Chapter One

Introduction and Literature Review

1: Introduction& Literature Review

1.1: Introduction

There is a common belief that tumors “cancer” is considered as one of the most dangerous problem that faces medicine and one of the incurable diseases because it is almost accompanied by death of the patient.

Tumors are abnormal growth of cells that cannot be controlled by the body, tumors are classified into two types, the first type called Benign tumor it is a relatively slow growth restricted in one region, not evade neighboring tissues and not widely spreaded through the body and not considered as danger for life.

The other type is called Malignant tumor, this is characterized by the rapid growth which can not be controlled and it is evades neighboring tissues

(السعد والزبيدي 1990,الزبيدي وجماعته1980) .

The break through in drugs filed had an important role in prolonging the age of patients, it was noticed that most antibiotics which are clinically beneficial in treating cancer are produced from *Streptomyces* bacteria, these bacteria were characterized by their large economical importance for their ability to produce different (many) types of antibiotics which represent more than half of the known antibiotics produce by different microorganism like antibacterial, antiviral, antialgal, and antifungal in addition to antitumor, for this, looking for drug to the

patients of cancer with the second world war, studies of researcher Charles Hagnis showed some result telling that feminine hormone (Estrogen) blocks prostate cancer in males, since then, many trials were attempted to get antitumor drugs, the source of these antitumor drugs is microorganisms that have ability to inhibit carcinogenic cells selectively without any damages in normal tissues; the result was that most of these drugs were produced by *Streptomyces* (Hopwood and Charles, 1982).

In 1940, the antibiotic Dactinomycin was isolated, it is used now a day as antitumor drug from *S. parvulus*, in 1958 the antibiotic Mitomycin C was isolated from *S. caespitaasus*, then isolation of bleomycin from *S. verticillus* in 1962, followed by isolation Daunomycin in 1963 from *S. peucetiuscaesius* .

The antibiotic Adriamycin was produced by the latter bacteria was used to treat cancer in 1958 (Haskell, 1980; and Kahn, 1983).

It is known the importance of *Streptomyces* bacteria in medicine filed,therefore, this study was carried out to isolate of *Streptomyces sp*., capable of producing an antitumor agent like bleomycin and studying factors affecting production of this substance.

Aims of the Study

- Isolation and Identification of local *Streptomyces* sp.
- Identification of antitumor agents produced by *Streptomyces*.
- Factors effecting on production of antitumor drugs (culture media, pH, Temperature).

1.2: Literature Review

1.2.1: Actinomycetes

Actinomycetes comprise an extensive and diverse group of Gram positive, aerobic, mycelia bacteria with high (G+C) content (>55%), and play an important ecological role in soil cycle (Kuster, 1968).

They were considered as a group intermediate between the bacteria and fungi, but were recognized as prokaryotic unicellular microorganisms that are characterized by difference in appearance and metabolic compounds (Waxman, 1959).

In 1875, Ferden and Cohn observed a filamentous organism in the tear duct of human eye, two years later (1877-1878); Harz described an aerobic species *Actinomyces bovis* that causes actinomycosis, the "ray-fungus disease" of cattle in the lamp jaw. This group was known for more than a hundered years as actinomycetes are considered as filamentous bacteria by the researcher Globing in 1888.

First who used the term actinomycetes are the researcher Gasperini and Lachner in 1890 and they are placed in actinomycetales order in 1917 by Buchanan (Waksman, 1967).

Bacteriologists considered them as bacteria while mycologists considered them as fungi (Syskes, 1973); this era is now over that actinomycetes are generally accepted as bacteria. It was suggested that they form original prototypes from which both fungi and bacteria have been derived.

Actinomycetes are unicellular bacteria belong to order actinomycetales, and are separated into two subgroup, oxidative forms which found mostly in soil habitat, and fermentative forms living in natural cavities of man and animals (Lechevalier and Pine, 1997).

Reproduction is usually a sexual, though sexual reproduction process has been shown to occur by conjugation. The majority of actinomycetes are free living, saprophytic bacteria found widely distributed in soil, water , humans, plants and animals (Kuster, 1868), also found in compost sites , river, lake sediments and atmosphere (Alexander, 1977).

Actinomycetes population has been identified as one of the major group of soil population. Actinomycetes belong to order Actinomycetales (Superkindom: Bacteria, phylum: Firmicutes, Class: Actinobacteria, Subclass: Actinobacteridae) (Cross and Good fellow, 1973; Bunchanan and Gibbons, 1974), and they comprise 63 genera (Nisbet and Fox, 1991).

It produces several antibiotics including amino glycosides, anthracyclines, polyether, glycopeptides, β- lactams, macrolides, nucleosides, peptides, and tetracycline (Sahin and Ugur, 2003).

Actinomycetes were described as the largest source for producing antibiotics of biological activity and treating benefit, and became most fruitful source for antibiotics. In 1960ʹs and 1970ʹs of 20th century 85-90% of all discovered antibiotics derived from the order actinomycetales (Mancheva, 2002).

Asurvy of literature from 1988-1992 revealed more than new secondary metabolites detected in actinomycetes (Euverink, 1994), which are essential for health such as enzymes, antibiotics, immunodultors (Moncheva, 2002).

No doubt soil is the natural habitat for most of the microorganisms where vast array of bacteria, actinomycetes, fungi and other organisms exists and provided with suitable growth condition and ability to proliferate. Thus, most actinomycetes contributing to antibiotic production are screened from soil (Williams and Cross, 1971).

Following in the work of Gratia and Dath in Belgium and of Rosen in France on the affect of actinomycetes upon disease-producing bacteria, the systematic isolation of antibiotics begun in 1940.

Actinomycin was the first antibiotic isolated in crystalline. Streptomycin in 1943, the last soon became an important therapeutic agent in the treatment of various infectious diseases of man and animals, and which was the beginning of new generation is known the medicinal and economical importance of this group (Waksman and Wood druff, 1983).

1.2.2: Genus *Streptomyces*

The members of this genus *streptomyces* belong to order Actinomyctes, and was first described by the scientists Henrici and Waksman in 1940, they are filamentous gram positive bacteria, aerobic that are produce wide variety of secondary metabolites, may with important application as antibiotics or other useful compounds in human, medicine and agriculture (Chakrabur,1997; White and Bibb, 1997).

They are saprophytes, obtaining nutrients and energy by solubilizing organic materials in soil through production of extra cellular hydrolytic enzymes (Vanwezel *et al*., 1997).

This genus mainly spread in soil, they form spores with grown on culture medium forming what is known as substrate mycelium, these mycelia penetrate the medium surface or grow as parallel to it, they

adhere to the surface of the solid medium giving a skin-like shape (Davis and Chater1990).

Actinomycetes form two types of mycelia; the first one is the substrate mycelium that grows on and into the culture medium, and as the colony matures the second type of mycelium which grow into the air and will finally form the spores that called aerial mycelium (Mendez and Hardission, 1985).

Fragmentation of substrate mycelia is rare (Gorden and Mihm, 1962) and spores are rarely produced on substrate hyphae.

The aerial mycelium usually bears long chains of spores, but in certain species relatively short chains may be found, these spores are arthospores formed by regular septation of hypha enclosed within a fibrous sheath (Wildermuth, 1970; Hopwood and Wildermuth, 1971).

Substrate mycelium characterized by forming filamentous branches, they differ in color from one species to another, some are grey, white, brown and they might produce pigments in the medium according to *Streptomyces* species , culture medium ,incubation time (Kutzner,1981;William *et al.*,1989).

Also, substrate mycelium has the ability to destroy organic substrate insoluble in water and transform it soluble substrate (Chater and Merrick, 1979; Vanwezel *et al.*, 1997).

Aerial mycelia are projected vertically to substrate mycelia and are branched and filamentous. The formatting of these mycelia, influced by various factors, like temperature of incubation, constituents of culture media and other (Lysons, and Pridham, 1965; Nodwell *et al.*, 1999).

This type of mycelium (aerial) bearing long chains of spores which may reach (50 spores), these spores are non-motile (Cross, 1973), this

genus characterized by large variations in morphology and physiology (Benigni and Garere, 1975; Zakrzewska and Mordarski, 1991).

It is characterized by high genomic (G+C) content, and LL-DAP in its peptidoglycan, where as DL-DAP was found in all other actinomycetes then known.

It thus, appears that *Streptomyces* are now primarily recognized by the structure of their peptidoglycan which is characterized not only by the LL-DAP mentioned above, but also by inter Peptide Bridge consisting of glycin molecules (Hopwood and Chater, 1982).

1.2.3: *Streptomyces* Genetics

Interest in *Streptomyces* genetics started in 1095; they are characterized by large variation morphology and physiology, the first genetic study in this field was by experimenting recombination to the ability of forming new compounds, in 1970 a study on the role of plasmids in genetic control to some traits fertilization, production of antibiotics and resistance.

Many studies were made on genetic engineering and gene cloning, gene replacements by using protoplasts which had led in return to increasing interest in *Streptomyces* (Chater and Hopwood, 1984; Smith *et al*., 1999).

Another study used the genetic material to classify *Streptomyces* and of irrelativeness among groups by using restriction endonuclease analysis (REA) by treating DNA of *Streptomyces* with restriction enzymes then using electrophoresis using agarose gel, DNA bands appear by different sizes and numbers according to restriction spots specific to each enzymes (Zakzewska *et al*.; 1991).

1.2.4: Chromosomal DNA

The chromosomal DNA as well as numerous plasmids is linear in the genus *Streptomyces,* they belong to the class of genetic elements called “Invertrons”, which are characterized by terminal inverted repeats (TIRS) and protein covalently attached to their 5´-ends (Sakaguchi, 1990).

As shown in table (1-1), linearity of chromosome in the filamentous *Streptomyces* soil bacteria was first discovered in *Streptomyces lividans* (Lin, 1993), typically this chromosomal DNA is about 8 MB long and contains terminal inverted repeats (TIRS) ranging from 24 to 500 Kb and terminal protein (TP) covalently bound to the 5´- end of the DNA (Loblond, 1996).

An origin of replication (*Ori C*) is located in approximately the center of chromosome. Here bi- directional replication is initiated and proceeds to the telomeres (Bey *et al*., 2000). However, size of the chromosomal DNA is high in the comparison with the well known microorganisms such as *E. coli* and *Bacillus subtilis*, *Streptomyces* has high G+C content (more than 70%) than nearly all other organisms as . Thus *Streptomyces* chromosome is unique in its structure and size (Omura, 2001).

Table (1-1): General features of *Streptomyces* chromosome.

Component of the chromosome	
Total Size	8,667,507bp
Terminal inverted repeats	21,653bp
G+C content	72.12%
Coding sequences	7,825bp
Coding density	88.9%
Average gene length	991kb
Ribosomal RNAs	6x(16S-23S-5S)
Transfer RNA	63s
Other Stable RNAs	3s

1.2.5: Importance of *Streptomyces* in Biotechnology and in Antibiotics Production

The study of *Streptomyces* genome came from the importance of these bacteria in the industry and treatment in attempt to analyze the functional parts responsible for the production of many types of antibiotics for which it can be considered as protective source of more than half of known antibiotics.

This genus also produces many other biological compounds such as extracellular enzymes, vitamins, and pigments according to this millions of *Streptomyces* bacteria were isolated from various parts of the earth looking for metabolic substances of high economical income (Chater and Merrick, 1979).

These bacteria also had an active role in dissolving the problems of environmental pollution because for their ability to lyses hydrocarbon compounds, rubber and plastic (Lacy, 1988).

It was discovered more than 1000 types of these bacteria in period range (1940-1957), but in 1970 it reached about 3000 type (Trejo, 1983; Pridham *et al*., 1958 and Floriano *et al.,* 1996).

This type of bacteria considered the most important type of other types of industrial microorganisms. It could be produce (4000) type of antibiotics (Chen, 1995) such as pipalamycin, viomycin, and kakadumycin (Uchihata *et al.,* 2002; Castillo *et al.,* 2003; Yin *et al.*, 2003).

Other products which produce by *Streptomyces* are antibacterial (Neal and Chater, 1991; Gramajo *etal*., 1993; Rattiet *et al*., 1997), antifungal (Bibb, 1996), antitumor (Iwami, *et al*., 1989; Remsing *et al.*, 2003), insecticide (Deshpande *et al.*, 1988), Cytotoxine (Sanchez *et al*., 2003), antiparasite (White & Bibb, 1997; Grubbs, 1999), also this bacteria have the ability to produce extracellular enzymes (Chen, 1995;and Vanwezel, 1999) such as amylase(Guad,1996), (DNase,RNase) (Vujakija *et al*., 1996; and Price *et al*., 1999), Protease (Renko *et al*., 1989), Dextranase(العليلي, 2001) , and Urase (Demnerova, *et al*., 1986).

On the basis, the study of *Streptomyces* genome came from the importance of these bacteria in the industry and treatment in attempt to analyze the functional parts responsible for the production of many types of enzymes, antibiotics and other secondary metabolites.

These studies showed the biggest chromosome size among prokaryotes with specific criteria of change and evolution (Redenbach *et al*., 1996; Omura *et al*., 2001; Bentley *et al*., 2002).

In addition, *Streptomyces* may bear the largest plasmids in their genome that may range from (200-800) Kb in size, these plasmids may

have an important function in secondary metabolites production, antibiotics production, and in some cases may involve in differentiation and development of the organism by transferring genes not only among streptomycetes, but with other genera also (Bibb and Hopwood, 1981; Aguilar and Hopwood, 1982; Chater and Hopwood, 1993; Hopwood and Kieser, 1993), his may make *Streptomyces* an interesting organism to be studied as a model for other bacteria and development (Hopwood, 1999).

1.2.6: Antibiotic Production by *Streptomyces*

Antibiotics are compounds produced by some microorganisms that inhibit the growth of other microorganisms by some specific interference in their normal biochemistry.

The specificity of action of antibiotics for particular targets, and hence for particular groups of organisms, in the reason why many of them are so valuable in medical, veterinary, and agricultural practice, and therefore, as industrial products (Martin and Demain, 1980).

The biochemical analysis of the end products had revealed that many *Streptomyces* might produce more than one type of antibiotics during their growth and development (Rudd and Hopwood, 1979).

This may indicate the presence of complex biochemical and regulatory mechanisms involves production of such secondary metabolites.

1.2.7: Biochemical Synthesis of antibiotics in *Streptomyces*

Over the last years, biochemical techniques have spearheaded significant advances in the understanding of the mechanism of antibiotic production in *Streptomyces* (Sheves and Mazur, 1975*)*.

Much insight into these fascinating multifunctional enzymes had already been obtained by chemical and biochemical approaches,

including establishment of mechanistic relationship between carbon and fatty acid utilization, and antibiotic biosynthesis.

Biochemical analysis had shown that these antibiotics are derivatives of less complex molecules obtained by bacterial nutrition. Chemical modification and dimerization-polymerization may lead to produce more complex compounds with different function. (Charles *et* ., 1981).

Such biochemical reactions obtain their energy from specific molecules that affect specifically not in antibiotic production only, but also in *Streptomyces* differentiation. These molecules were identified as guanosine 5'-diphosphate 3'-diphosphate ((ppGpp)) and guanosine 5'-triphosphate 3'-diphosphate ((pppGpp)).

During the growth, concentration of (ppGpp) and (pppGpp) were several times higher in the logarithmic phase than in the stationary phase, they fell sharply when exponential growth ends; hence, antibiotic synthesis was found to begin several hours after exponential phase has started.

This may indicate the specific interference of these molecules in antibiotic production, since mutants that are defective in ppGpp and pppGpp were also found to be defective in antibiotic production (An and Vining, 1978). Also, there is other studies found that other molecules may have adverse effect in antibiotic production, the A-factor (2-s-isocapryloyl-3-s-hydroxymethyl-y -butyrolacton) is a potent auto regulatory factor essential for antibiotic production and spore formation in *Streptomyces* (Khokhlov, 1973).

Moreover, mutants lacking A-factor (2-s-isocapryloyl-3-s-hydroxymethyl-y- butyrolacton) were found to lose the ability to produce antibiotics and spore formation. However, exogenous supplementation of

this factor to the culture of these mutants restored all these phenotypes (Hara, 1983).

The major problems in the analysis of antibiotic biosynthesis pathways by traditional biochemical and chemical procedures have been caused by the low concentrations of the pathway enzymes, their liability in cell-free preparations, and the chemical instability of many highly reactive intermediates of the pathways.

As a result, the pathways even of important antibiotics that have been of industrial products for decades, such as β-lactams, aminoglycosides, and macrolides still yet poorly understood a wealth of new information is coming from molecular genetic analysis. Notable example is provided by chemical class of natural products called polypeptides, which includes macrolides, tetracycline, avermectins, and many others. A mechanistic correspondence between their biosynthesis and that of fatty acids has long been postulated (Chater and Hopwood, 1994).

Sever difficulties had, however, been met in trying to understand a key issue in the field, namely how the enzymes are “programmed”, this term has been introduced to describe control of the variables that determine the structure of the product. These variables are: (I) choice of the start unit; (II) choice of the natural and the number of the chain extender units; (III) control of the reductive cycle, (IV) stereochemistry of the compound (Amy *et al*., 1996). For better understanding of antibiotic production in *Streptomyces*, all studies had focused on the genetics of antibiotic production in these organisms; hence, they provided a wide knowledge to illustrate the precise biochemical pathways for the most studied polyketide antibiotics (Hopwood, 1997).

Chapter Two

Materials and Methods

2-Materials and Methods

2.1: Material

2.1.1: Apparatus

The following apparatus were used during this study.

Apparatus	Country	Origin
Autoclave	Tomy	Japan
Balance (analytic)	Ohaus	U.S.A
Bench centrifuge	Hermal	Germany
Millipore Filter Unit	Millipore Corp.	U.S.A
Micropipette	Volac	England
Light microscope	Olympus	Japan
Magnetic stirrer/hot plate	IKA	U.S.A
Oven	IKA	U.S.A
pH meter	WTW	Germany
Sensitive balance	Merttler	Switzerland
Incubator	Gallenkamp	(U.K)
Laminar flow hood	Gelair class 100 gelman instrument	(U.K)
Incubator CO_2	Gallenkamp	(U.K)
Inverted microscope	Gallenkamp	(U.K)

2.1.2: Chemicals and biological materials

The following chemicals were used during this study.

2.1.2.1: BDH/England

Acetic acid	Ammonium hydroxide
Chloroform	Ethyl acetate
Glycerol	Glucose
Trypticase Soya broth	KH_2PO_4
Ninhydrine	NaCl
Yeast extract	Methanol
HCl	K_2HPO_4
Sucrose	Peptone

2.1.2.2: Fluka/Switzerland

Ammonia	NaOH	Isopropanol	KCL
Ammonium acetate	Acetone	KNO_3	Nutrient agar
$CaCO_3$	Butanol	$MgSO_4.7H_2O$	Piperdine

2.1.2.3: Oxoid/England

Muller-Hinton broth	Malt extract
Agar	Soya bean meal

2.1.3: *Streptomyces* Isolates

No.	Streptomyces Isolates	Source
1.	ERM1	Soil
2.	ERM2	Soil
3.	ERM3	Soil
4.	ERM4	Soil
5.	ERM5	Soil

2.1.4: Standard bacteria

Microorganism	Source
Staphylococous aureus *ATCC21933*	Central Public Health Laboratories
Candida albican ATCC 31622	AL-Kadhmeya Teaching Hospital Clinical Diagnostic laboratories.
Bacillus sphaericus *ATCC 21951*	Central Public Health Laboratories
E.coli ATCC 25955	AL-Kadhmeya Teaching Hospital Clinical Diagnostic laboratories.
Bacillus thurengensis *ATCC 21954*	Central Public Health Laboratories.
Pseudomonas aeroginosa ATCC 27853	AL-Kadhmeya Teaching Hospital Clinical Diagnostic laboratories.
Klebsiela pneumonia *AT CC 31683*	AL-Kadhmeya Teaching Hospital Clinical Diagnostic laboratories.

2.1.4.1: Cell Line

Cancer cell Line HEP-2 passag 328 used in this study was obtained from Iraqi Centre for Cancer and Medical genetic research AL-Mustansuiyia University.

Human Epidermoid Carcinoma (HEP-2) cell line was established in 1952 by Moore and his partner from tumors that had been produced in irradiated cortisonized wealing rats after injection with epidermoid carcinoma tissue from the larynx of 65 year old of male (Moore *et al*., 1955).

This cell line was adapted to grow on RPMI-1640 medium supplemented with 10% fetal calf serum in Iraqi Centre for Cancer and Medical Genetic Research instead of the original medium MEM which also supplemented with 10% fetal calf serum.HEP-2 characterized as a hard cell line, resist temperature, nutrientional and environmental changes without loss of viability (Toolan,1954).

2.1.5: Culture media

The following media were used for cultivation and experimentation of *Streptomyces* isolates during this study.

2.1.5.1: Muller -Hinton agar medium

Muller-Hinton medium (36.5g/L) was prepared according to the manufacturer instructions.

2.1.5.2: Nutrient agar

Nutrient agar medium (28g/L) was prepared according to the manufacturer instructions.

2.1.5.3: Nutrient broth

Nutrient broth medium (8g/L) was prepared according to the manufacturer instruction.

2.1.5.4: Gauza agar (Komagata, 1986)

KNO_3	1g
Soluble strach	20g
NaCl	0.5g
Mg $SO_4.7H_2O$	0.5g
$FeSO_4.7H_2O$	0.01g
K_2HPO_4	0.5g
Agar	20g
D.W	Up to 1000ml

2.1.5.5: Antibiotic production medium (Leach *et al*., 1986)

Glucose	25g
Yeast extracts	2.5g
$CaCO_3$	8g
KCl	4g
KH_2PO_4	0.4g
Soya bean meal	25g
Tap water	Up to 1000ml

2.1.5.6: Starch-Yeast agar (Shrilling, 1960)

Starch	1g
$K_2HPO_4.7H_2O$	1g
NaCl	3g
Agar	20g
D.W	Up to 1000ml
pH	7.4

2.1.5.7: Czpeck- medium (Oxoid)

It was prepared by dissolving 48g of this medium in D.W. then complete the volume to1000ml, then sterilized by autoclaving, and used for bacterial growth.

2.1.5.8: Hydrolysis of Urea (Kuster, 1976)

Basal medium

Na_2HPO_4	2 g
Casien- peptone	1 g
KH_2PO_4	1.5 g
$MgSO_4.7H_2O$	0.5 g
NaCl	5 g
Phenol red	0.012 g
Agar	15 g
D.W	Up to 900 ml

Basal medium was supplemented with urea solution at 20% as final concentration and glucose at 1 %.

2.1.5.9: Blood agar medium (Oxiod)

Blood medium was prepared as instructed by the manufacture

2.1.5.10: R2YE medium (Hopwood *et al*., 1985)

Sucrose	103g
K_2SO_4	0.25g
Mg $Cl_2.6H_2O$	10.12g
Glucose	10g
Casamino acids	0.1g
D.W	Up to 800ml

After dissolving the ingredients in the D.W, solution was distributed in portions of 80 ml in each 250 ml flask, and then 2.2g of agar was added to each flask. At the time of use, the media were heated and 5ml volume of yeast extract (10%) was added to each flask

KH_2PO_4 (0.5 %)	1 ml
$CaCl_2.{}_2O$ (3.68 %)	8 ml
L- proline (20 %)	1.5 ml
TES buffer (5.73 % pH 7.2)	10 ml
Trace element solution	0.2 ml
NaOH (1N)	0.5 ml

2.1.5.11: **Sugar utilization medium (**Kustr, 1976**)**

Basal medium

$(NH_4)_2SO_4$	2.46g
KH_2PO_4	2.38g
K_2HPO_4	5.56g
$MgSO_4.7H_2O$	1g
$CuSO_4.5H_2O$	6.4g
$FeSO_4.7H_2O$	1.1g
$MnCl_2.4H_2O$	7.9g
$ZnSO_4.7H_2O$	1.5g
H_2O	Up to 1000ml
Agar	15g

The pH was adjusted to (6.8-7).
Carbone source was prepared using the following sugars

D-glucose

L- arabinose

D- Xylose

Rhaminose

Sucrose

Raffinose

D-mannitol

L- inositol

Each sugar was sterilized by filtration and supplemented to the basal medium to a final concentration 1%.

2.1.5.12: Tissue cultures medium (RPMI-1640)

It was prepared according to the method described by Freshney (2000) by dissolving the following ingredients in quantity of D.D.W completing the medium to 1000ml.

Crystalline Pencillin	100000 i.µ
RPMI- 1640 with hopes buffer, L-glutamine	10.4
Streptomycin	0.1 g
Bovin Calf Serum	10 %
Sodium bicarbonate	2 %

Well mixed, sterilized by filtration, dispensed into well sealed 20 ml aliquots &stored at (-20˚C) until used.

2.1.5.13: Cells Cultures

The following steps were carried out under aspect conditions

2 ml of trypsin- versene solution was added to the falcons of 25cm² that contain cells of (Hep-2) confidentially after discarding the culture medium and washing with PBS, then falcons were shake lightly and incubated at 37 ˚C for 15 min. to dispersed adherent cells and transfer cells with container to get as single cell as far as possible.

- A quantity of 20 ml of new growth medium was added to falcons containing suspension of dispersed cell with well stirring followed by transferring the contents of each falcon into another in away that each falcon contained equal volume of broth culture medium and cells, and this is called subculture.
- These containers were incubated at 37℃ for 48 h. During the period of incubation, the cells were daily watched to check whether there is contamination or not and examine their growth by inverted microscope.
- **2.1.6: Antibiotic Discs**

Antibiotic	**Concentration of Discs (µg/ des)**
Cephlexin	25
Trimethopirm	25
Benzathinpenicilin	25
Chlorompheniколbase	25
Ampiclox	25
Lincomycin	25
Amoxycilin	25
Clidamycin	25
Kanamycin	25
Erythromycin	25
Streptomycin	25
Tetracyclin	25
Belomycin	250

Doxorubicin	250

2.2: Methods

2.2.1: Preparation of Solution and Reagents

2.2.1.1 Catalase reagent (Atlas *et al*., 1995)

This reagent is composed of 3% hydrogen peroxide.

2.2.1.2- EDTA Stock solution (0.25) M

Stock solution of EDTA was prepared by dissolving 0.93g of EDTA in 10ml D.W, and used in cell fractionation at pH 7.3.

2.2.1.3- Phosphate Buffer Saline (PBS) (Cruikshank *et al*., 1975)

This solution was prepared by dissolving 8 g of NaCl, 0.2 g KCl, 0.2 g of KH_2PO_4 and 1.15 g of Na_2HPO_4 in 1000 ml of distilled water; pH was adjusted to 7.3 and sterilized by autoclaving.

2.2.1.4- Trypsin

It was prepared by dissolving 1g of Trypsin powder in 100 ml PBS.

2.2.1.5- Versene

It was prepared by dissolving 1g of EDTA powder in 100 ml of D.W, mixed until complete dissolved.

2.2.1.6- Trypsine-Versene Solution

It was prepared by mixing of each:

PBS	370 ml
Trypsine-solution	20ml
Versene solution	10ml

It had mixed all these solution, filtration by filter paper with 0.22µm under sterile conditions, stored at 4˚C.

2.2.1.7- Crystal violet Stain

It was prepared by dissolving 5g of methyl-violet in 200 ml of methanol and filtered it by Whatman paper, then 50 ml of formalin was added at concentration (37%), then the volume was completed by D.W to 1000 ml, then kept it in dark places until time of using (Mether and Roberts, 1998).

2.2.1.8- Bovine Calf Serum (BCS)

It was get from Iraqi Centre for Cancer and Medical Genetic research, filtrated using Millipore filter 0.22 µ under sterile conditions , then mixed of (10%) from sterile BCS with sterile tissue culture RPMI-1640, and stored it at -20˚C in well sealed tubes or falcons until using.

2.2. 2: Sterilization Methods

Three techniques were used for sterilization:

2.2.2.1: By heat method (Oven)

The temperature was about (180˚C for 2hrs), used for sterilizing glassware.

2.2.2.2- By autoclave

This used for sterilization of culture media (Liquid and Solid) and solutions, at 121˚C, 15 pounds/in for15 min.

2.2.2.3- By filtration (Membrane sterilization**)**

This method was used for sterilization of antibiotic solution and Trypsin- Versene solution, tissues culture media (RPMI-1640), dye solution, and Lysozyme were sterile by using Millipore filter with 0.45 and 0.22µm of diameter.

2.2.3: Preparation of bacterial spore suspension (Hopwood, D.A.; Kieser *et al*., (1986).

a- 9ml of sterile distilled water or Ringer solution was added to the mature slant or plate.

b- The surface of culture was scrapped with an inoculation loop to suspend.

c- The cured suspension was poured back into the container that held the sterile water, and the liquid was agitated on a vortex mixer for one minute.

d- The suspension was filtered through cotton wool.

e- The filtered suspension was poured into a centrifuge tube and spind for 5-10 min. at 3000rpm to pellet the spores.

f- The supernatant was removed.

g- Spores were resuspended with sterile distilled water or Ringer solution.

h- Absence of mycelia was checked by testing a drop under light microscope.

2.2.4: Maintenance of Cell Line

After obtaining of this type of cell line from Iraqi Center for Cancer and Medical Genetic Researchers/Al-Mustansiyra University, under sterile conditions. It had made all these steps for tissue culture.

Tumor cell line was monitored to form confluent monolayer. Subculture was established by discarding the old medium followed by washing the cells with sterile PBS under aseptic conditions, and then a 3 ml of trypsin- versene solution was added with gentle shaking of the flask, the solution was discarded.

The cells were incubated at 37ºC until be separated from the flask wall, then trypsin- versene solution was discarded by washing using growth medium followed by the addition of new growth medium, redistributed in special falcons and incubated at 37ºC (Freshney, 2000).

2.2.5: Storage of Tumor Cell Lines

This preservation procedure was done according to the method demonstrated by Freshney (2000). Long term storage was accomplished by freezing down in liquid nitrogen. Cell suspension freezing medium (0.4 ml RPMI-1640, 0.5 ml BCS and 0.1 ml Dimethyl sulphoxide) were added drop by drop to the cell suspension with continuous shaking. Then the cells suspension was dispensed into 1 ml in sterile plastic ampoules. The ampoules were packed in an insulated, expanded a cooling rate of less than 1 ˚C/ min. After this time, ampoules were rapidly transferred to holders and immersion in liquid nitrogen at -196˚C.

For using the stored cells, cells were recovered by placing the ampoules into water bath at 37˚C until the suspension has thawed. The

contents of the ampoules were transferred to a sterile plastic centrifuge tube, and then an 8 ml of prewared growth medium was added, well mixed and spun with 1000 rpm for 8 min to get rid of DMSO. The cell pellet was reconstituted in prewared growth medium and seeded into 25cm² flasks.

2.2.6- Cytotoxicity of the locally isolated *Streptomyces* crude filtrate on tumor cell line:

- Each extract was sterilized by 0.22µ g pore filtering unit and diluted with serum free medium starting with (10, 20, 30, 40, 50, and 60 µ g/ ml) under aseptic conditions, these concentrations were used directly.
- Cell suspension was prepared by treating the container of (25cm²) with trypsin-versene solution followed by the addition of 20 ml of growth medium supplemented with 10%bovine calf serum .Cell suspension was mixed well followed by transferring 200µl/well into 96 well flat bottom microtiter plate using automatic micropipette in some how that each well had contained (1x10 cell/ well) by counting the viable cell using neutral red dye.
- The plate was incubated at 37 ˚C for 48h for Hep-2 cell line until the adhesion of the cell to the flat internal surface of the wells , then the used medium was discarded followed by the addition of 200 µl/well from each concentration that were previously prepared

for each extract as much as three replicates, also six replicates were made for control which contained only the cells with 200µl/well of serum free medium (SFM), then plates were incubated at 37˚C in an incubator supplemented with (5%) CO_2.

- After elapsing the incubation period 48hr., 50 µl/well of neutral red dye was added and incubated again for 2h. The contents of the plates were removed by washing the cells with PBS to remove the excess dye followed by the addition of 20 µl/well of extraction dye solution that draw out the dye from the viable cells that had stained. The results were read using ELISA reader at wave length 492 nm.
- The previous steps were applied for Hep-2 cell line using the two extracts for the period of incubation which are (48h).

2.2.7: Preservation of *Streptomyces* Isolates (Maniatis *et al*., 1982),

A-Preservation f short term

Slants were preserved at 4˚C in refrigerator.

B-Preservation for long term

(a): In soil

A suitable garden soil was prepared for preserving the spores. The soil was cleaned dried and sieved (0.05mm in diameter), dispensed in small Bijo bottles which have cotton plugs. Then sterilized by autoclaving and dried at 105˚C for 3hr.the steriled soil was then

inoculated with 1ml of dense spore suspension, placed in desiccators and left at room temperature (for a week till drying) then preserved at 4˚C.

(b): In 40% glycerol

Small Bijou bottles were prepared, each contained 5ml of 40%glycerol, sterilized in autoclave, inoculated with 5ml of spore suspension, mixed and then preserved at -20˚C.

2.2.8: Antibiotic Sensitivity Test (Okami *et al*., 1960)

The sensitivity of *Steptomyces* species was determined against the antibiotic discs.

a- *Sterptomyces* isolates were grown in TSB for 48hour.

b- The bacterial species were streaked on Muller Hinton agar medium.

c- Antibiotic disks were placed on the culture plate and incubated for 24 hours at 37˚C.

Inhibition zone were observed and measured in (mm).

2.2.9: Determination of biological activity on agar medium: (Rudd and Hopwood, 1979)

A -*Streptomyces* isolates were cultivated in antibiotic production medium agar for different period of time (5, 10, and 14 days).

B - Plugs of 7mm were cut and embedded with the indicator organism streaked on the medium in plates.

C- The plates were incubated over night at 37ºC and inhibition zones of the indicator organism were observed and measured in (mm).

2.2.10: Determination the effect of pH on antibiotic production (Rancourt and Lechevalier, 1963)

- Spores were inoculated onto antibiotic production medium with following pH values of 5, 7, and 9.
- Colonies were observed for good growth.
- Plugs from well grown colony were transferred onto medium contains the test organism, each plug from different pH value.
- Inhibition zones were observed and recorded in (mm).

2.2.11: **Determination of the effect of incubation period on antibiotic production (**Jakimowiecz *et al*., 1988**)**

a- Spores were inoculated onto antibiotic production medium and incubated for 5, 10, and 14 days.

b- Colonies were observed for good growth.

c- Plugs from well grown colony were transferred onto medium containing the test organism, each plug from each incubation time.

d- Inhibition zones were observed and recorded in (mm).

2.2.12: **Determination the effect of temperature on antibiotic production** (Hopwood, 1980**)**

- Spores were inoculated onto antibiotic production medium and incubated at 28, 37, and 45Cº for 5 days.
- Colonies were observed for good growth.
- Plugs from well grown colony were transferred onto medium containing the test organism, each plug was transferred from each temperature.
- Inhibition zones were observed and measured in (mm).

2.2.13: Screening of NaCl-tolerant *Streptomyces* (Edward and Ball, 1987)

0.1ml of spore suspension of each isolates was cultured on Starch-Yeast extract agar; this medium contains various concentrations of NaCl (1-9) %. The culture plates were incubated at 37˚C for (7-21) days, NaCl-tolerance of each isolate was determined by its growth and viable count.

2.2. 14: Growth on Czapeck medium (Cruishank *et al*., 1975)

Spores were inoculated onto Czapeck medium and incubated at 37˚C for 10 days. Positive results were observed after bacteria were able to grow on the medium.

2.2.15: Urea Utilization (Stolp and Gadkari, 1981)

Spores were inoculated into the urea medium and incubated at 37˚C; observation of the change of medium color was made after 2 and 5 days to indicate fast and strong, medium and weak urea hydrolysis.

2.2.16: Blood hemolysis (Harrely and Prescott, 1996)

Spores were inoculated into the medium at 37˚C. Blood hemolysis was observed after 5 days of incubation.

2.2.17: Catalyses test (Maza *et al*., 1997)

Bacterial cells from slant were inoculated on R_2YE agar medium, were left to grow at 37 ºC for 48 hours, and catalase reaction was made by dropping H_2O_2 of 20 % (v/v) on bacterial cells and foaming was observed for catalase positive colonies.

2.3: Production of antitumor agents on Broth medium (Wagman and Wienstien, 1973)

1-Doxorubicin

a- Paper chromatography: Watman No.1

Solvent System:

Propanol	7ml
Ethyl acetate	1ml
Water	2ml

R_f value 0.25

b- Thin layer chromatography

Medium: Kieser gel G layer buffered with 1% oxalic acid in water.

Solvent System:

n-butanol	4ml
Water	1ml
Acetic acid	5ml

R_f value 0.33

2-Bleomycin

Thin layer chromatography

Medium: Silica gel G

Solvent System:

10% ammonium acetate	1ml
Methanol	1ml

R_f value 0.25

3- Streptomycin

Paper chromatography

Solvent System:

n-butanol	78.4ml
Water	18.6ml
Piperdine	2ml

R_f value 0.25

4-Tetracycline

a- Paper chromatography

Solvent System:

n-butanol	4ml
Water	1ml
Acetic acid	5ml

R_f value 0.55

Or

Detection by U.V

B-Thin layer chromatography

Medium: Silica gel G

Solvent System:

n-butanol	100ml
Tartaric acid	6g
Water	1000 ml

Detection by spraying with 5% methanolic solution of ferric chloride.

R_f value 0.26 for tetracycline

R_f value 0.31 for oxytetracycline

R_f value 0.36 for chlortetracycline

2.4: Statistical Analysis

Regarding tumor cell line, the results were the average of three replicates, while the control (control I&II) the results were the mean of six replicates. The results were subjected to statistical analysis for determining the significance effect among the rates of the concentrations of crude extracts and their effect on tumor cell lines .The comparison between groups has based on analysis of variance test (ANOVA), while the significance differences based on Denkin test. (AL-Mohammed *et al*., 1986).

Chapter Three

Results and Discussions

3-Results and Discussions

In this study the antitumor agent that produced by the *Streptomyces* was studied. The used bacteria had isolated by the collection of soil samples from different regions of Baghdad governate.

Isolates were primarily identified depending on growth of these on selective media (Gauza agar media) and some of them form clear zones around the colonies of *Streptomyces* due to the utilization of soluble starch in media , then the isolates were further identified depending on cultural, morphological and biochemical tests.

3-1: Identification of *Streptomyces* Bacteria

3.1.1: Cultural Characteristics

Results showed that the main characteristics of these isolates were gram positive, can grow under aerobic conditions, forming two types of mycelia (substrate and aerial). Colonies of these isolates were appeared white, yellow- green, and they gray on Gauza agar medium. Spores of these isolates appeared to be straight or wavy and long spiral. These results were came according to Williams and Cross, 1971; Singh and Agrawal, 2003).

Depending on the results of cultural and microscopic tests, five isolates belonging to the genus *Streptomyces* referred as ERM1, ERM2, ERM3, ERM4, &ERM5 were studied for further work.

The abundance of the genus *Streptomyces* in soil special agriculturist is reasonable because they posses extra cellular enzyme system that encourage them to grow in soil (Williams and Cross, 1971).

3.1.2: Biochemical Tests

Biochemical tests shown in the table (3-1), demonstrated that each of the two isolates were catalase positive since bubbles were observed after addition of H_2O_2, positive test for sugars utilization, positive urea hydrolysis with change color of medium due to reduction of phenol red, finally able to grow on czapeck medium , blood hemolysis which appeared after 5 days of incubation and antibiotics resistance test for these isolates it which found that only two isolates (ERM1&ERM2) were able to resist the bleomycin.

3.1.3: Temperature tolerance test

It was noticed that *Streptomyces* used for studying differ in their ability on growth in various temperatures (28, 37, 40, and 50˚C) for 5-days and the result showed very good growth for the two isolates was at 28˚C, less growth was at 37˚C and no growth was at 40- 50˚C. From this result the ideal growth temperature was 28˚C for two isolates as shown in table (3-2). Our results showed that only one strain of Streptomyces was able to grow at 40- 50 ºC.

In a study of **الراشدي** (1999) on a large number of *Streptomyces* isolate showed for temperature degree different effect on phenotypes of isolates deal with color substrate and aerial and production of pigments it could change through the change of incubation temperatures.

Table (3-2): Characterization of *Streptomyces* isolates grown on Gauza medium at different Temperature for 7 days.

Isolates	Temperature	Growth	Color of aerial	Color of substrate
ERM1	28 ºC	++	Grey	Grey
	37 ºC	++	Brown	Grey
	40 ºC	+	Grey	Grey
	50 ºC	_	_	_
ERM2	28 ºC	+++	Grey	Brown
	37 ºC	+++	Grey	Brown
	40 ºC	+	Grey	Brown
	50 ºC	_	_	_

_: No Growth

+: Good Growth

++: Very good Growth

3.1.4: Sodium chloride tolerance

Starch- Yeast culture medium with various NaCl concentrations (0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 %) was used in this experiment to determine the NaCl tolerance of two *Streptomyces* isolates. The result in table (3-3) showed that ERM1 has tolerant up 4% and was slight growth at 5% and no growth at 6% while ERM2 has NaCl tolerance to 6%, slight growth at 7% and no growth was seen at 8 – 10 % for both isolates.

Table (3-3): NaCl tolerance of two isolates grown on SY- medium for 5-days incubated at 30C.

	NaCl%									
Streptomyces isolates	1	2	3	4	5	6	7	8	9	10
ERM1	+	+	++	+	±	-	-	-	-	-
ERM2	+	++	++	++	++	++	±	-	-	-

+: Good growth

++: Very good growth.

±: Slight growth.

-: No growth.

The above results showed that preliminary results might indicate halotolerance is genetically controlled property of the microorganisms. The halosensitive strains can only be adapted to low NaCl concentration; they cannot protect their cytoplasm well against NaCl. In contrast, halotolerance *Streptomyces* protect and transform their cytoplasm to a higher degree of NaCl (Neumeier and Kuster, 1981).

3-2: Antibiotics production by *Streptomyces*

Production of antibiotics in *Streptomyces* is common feature and clearly observed in most species. It can be prescreened by examining the biological activity against certain bacteria and microorganisms that are ceased to grow showing an inhibition zone surrounding the *Streptomyces* colony with various diameters depending on the quantity, type, and response of test organism to that product.

Detection and identification of antibiotics produced by the local isolates under study took several phases using the most convenient methods recommended for this purpose.

3-2-1: Detection and identification of antibiotics produced by *Streptomyces* isolates

3-2-1-1: Antibiotic Sensitivity

Hopwood (1994), and Chater and Bibb (1997) suggested after extensive genetic analysis of antibiotic producing Streptomycetes has ability to resist their own antibiotic, else they will be killed by their product.

This observation may indicate in one way or another that using antibiotic sensitivity procedure may indicate presence of the same antibiotic
produced by the local isolates if they resist it.

So, the antibiotic sensitivity was determined against thirteen antibiotics by spreading the spore suspension of ERM1 and ERM2 on Muller-Hinton agar medium and the antibiotic discs were placed on the seeded plates. Antibiotic sensitivity results are shown in table (3-4).

Table (3-4): Antibiotic sensitivity of *Streptomyces* ERM1 and ERM2 on Muller-Hinton agar medium, for 24hr at 37°C. Numbers indicates diameter of inhibition zone in millimeter (mm).

Antibiotic	**Concentration of discs(µg/disc)**	**Diameter of inhibition zone (mm)**	
		ERM1	**ERM2**
Cephalexin	25	30	25
Trimethopirm	25	R	10
Benzathinpenicilin	25	R	R
Chloromphenicolbase	25	20	R
Ampiclox	25	R	R
Lincomycin	25	20	R
Amoxycilin	25	R	R
Clindamycin	25	30	15
Kanamycin	25	15	15
Erythromycin	25	30	25
Streptomycin	25	20	R
Tetracycline	25	R	R
Belomycin	250	R	R
Doxorubicin	250	R	25

R= Resistance

Results in table (3-4) showed that ERM1 was resistant to the following antibiotics: Trimethoprim, Bleomycin, Benzathin penicillin, Ampiclox, Amoxycillin, Tetracycline and Doxorubicin while it was sensitive to Clindamycin, Kanamycin, Erythromycin, Streptomycin, Cephalexin, Chloramphenicol base and Lincomycin.

Also the table showed that the ERM2 was resistant to Benzathin penicillin, Chloramphenicol base, Ampiclox, Amoxycillin, Tetracycline, Lincomycin, Streptomycin and Bleomycin while sensitive to Cephalexin, Trimethoprim, Clindamycin, Kanamycin, Erythromycin, and Doxorubicin.

The results showed the differences in pattern of antibiotic sensitivity of these two isolates. The difference in the rate of resistance of these antibiotics which are used in this study may be attributed to the (1): the bacterial species and cell wall prevents uptake the antibiotic into the cell. (2): the organism may be producer of the same antibiotic that means it has the ability to produce enzymes which confirm resistance to that type of antibiotics, also the diameter of inhibition zone of Trimathoprim wide in ERM2, while ERM1 was had large resistance to this antibiotic, this may be explained to permeability of the membrane which forbid admitancey of this antibiotic since it is a synthetic not normal antibiotic (Galae. 1972; and العيبيدي, 1996)

3-2- 1-2: Antibacterial activity

The most important product of secondary metabolisms in *Streptomyces* is antibiotic which is produce during the production phase (Idio-phase) after complete the cellular growth for this bacterium and reached to stationary phase (Bulock *et al*., 1975; Martin and Demain, 1989).These antibiotics that are produced by *Streptomyces* differ in their formation and effect on microorganisms (Kaewawski *et al*., 1989).

For this purpose antibacterial activity was determined by classical diffusion method (Agar plug technique) on Muller-Hinton agar at 37˚C for 24hr (after the *Streptomyces* isolates were grown on antibiotic production medium for 5-days at 37˚C).

This method based on observation of inhibition zone of the test microorganisms around the plug. Only from five isolates, two isolates were selected for their ability to inhibit the growth of the test microorganisms. However, the antibacterial activity procedure may indicate the ability of the isolates to produce antimicrobial agents that may resemble antibiotics. Results obtained are shown in table (3-5). These differences can be categorized into two types:

(a) Both *Streptomyces* local isolates had showed wide range of effect on test organism.

(b) One of *Streptomyces* local isolates practiced severs inhibition zone like ERM2.

Table (3-5): Antibacterial activity of *Streptomyces* local isolates against different test organisms.

Test organism	REM1	ERM2
Proteus mirabilis	_	25mm
Esherchiae coli	_	15mm
Pseudomonas aeuroginosa	_	40mm
Candida albicans	20mm	_
Staphylococcus aureus	20mm	20mm
Bacillus thurengensis	15mm	15mm
Bacillus spahericus	15mm	15mm

.

These results may suggest the following explanation that the *Streptomyces* local isolates may produced more than one type of antibiotics that may affect different test organisms depending on the type of the antibiotic and test organisms, the *Streptomyces* local isolates may produce high quantity of antibiotics that causes wide diameter inhibition zone, in both cases, *Streptomyces* local isolates showed clear biological activity indicating presence of more than one type of antibiotics needs to be detected among them.

3-3: Detection of antibiotic produced by *Streptomyces* local isolates

3-3-1: Thin layer Chromatography (TLC)

TLC method is considered fast and recommended for the preidentification of antibiotic type (s) produced by the microorganism. This method mainly depends on the mobility of the compounds in relation to the stationary phase with different R_f values.

The experiment was made by cultivating the bacteria in a broth media for antibiotic production, then after filtering the broth, products were extracted by organic solvents and subjected to TLC. Each isolate extract was determined by comparing the R_f values and observing the compound in relation to the standard antibiotic that was moving on the same plate. Results showed in the table (3-6).

Table (3-6): Types of antibiotics and R_f values detecting using TLC analysis of isolates products. The (+) sign means presence of an antibiotic with R_f value similar to that given to the standard antibiotic.

***Streptomyces* isolates**	**PRESENCE OF ANTIBIOTIC**				
	Neomycin R_f=0.33	**Tetracyclin R_f= 0.6**	**Bleomycin R_f= 0.5**	**Doxorubicin R_f= 0.3**	**Streptomycin R_f= 0.9**
ERM1	+	+	+	+	+
ERM2	+	+	+	+	+

As shown in the table, result obtained may give an indication that the local isolates may produce the same antibiotic used as standard due to the similarities in position and R_f values.

3-3-2: Factors affecting antibiotic production by *Streptomyces* isolates

Production of antibiotics is a complicated process in *Streptomyces.* This process is not concerned with competing for food and space, but it interferes with the complex process of differentiation of the bacterium (Chater and Bibb, 1997).

Production of these compounds may be affected by various factors that affect growth of *Streptomyces sp*. Some of these factors were studied and their effect on antibiotic production was determined.

3-3-2-1: Effect of Incubation period on antibiotic production

To establish complete maturation of *Streptomyces*, the bacterium need about 14 days of incubation on solid medium .During this time, the bacteria may produce various types of secondary metabolites, including antibiotics.

Bacterial isolates were cultivated for different periods of time (2, 5, and 14 days). At each period a plug was removed and placed on a plate containing test organism to measure the inhibition zone that may be produced due to antibiotic effect. Results obtained are shown in the table (3-7).

Table (3-7): Effect of Incubation period on antibiotic production of *Streptomyces* isolates.

Isolates	Test organisms	2- Days	5- Days	14- Days
ERM1	*Staphylococcus aureus*	_	30 mm	15 mm
	Bacillus thurengensis	_	20 mm	_
	Bacillus sphaericus	_	_	_
	Candida albican	_	12 mm	_
	E. coli	_	10 mm	_
	Proteus mirabilis	_	10 mm	30 mm
	P. aeroginosa	_	15 mm	30 mm
ERM2	*Staphylococcus aureus*	_	15 mm	15 mm
	Bacillus thurengensis	_	10 mm	_
	Bacillus sphaericus	_	15 mm	_
	Candida albican	_	_	_
	E. coli	_	15 mm	_
	Proteusmirabilis	_	_	_
	P. aerginosa	_	20 mm	20 mm

Results obtained from this experiment showed that cultivation period for 5-days gave the highest production of antibiotics in two isolates, while some required longer time for complete maturation and production of antibiotics.

The reason for this may be:

***At 2-** days of incubation, bacterial isolates are still in log phase of growth, and genes responsible for antibiotic production are not yet activated.

***At 5**-days of incubation, antibiotic genes are activated and production of antibiotic had begun during projection of aerial mycelia.

***At 14-** days of incubation, isolates had complete maturation, but production of antibiotic was ceased due to production of degrading enzymes that destroy their activity and transforming them to inactive compounds (Chater and Bibb, 1997).

*- Isolates that gave the highest biological activity after 14 days of incubation may not be able to produce antibiotic destructive enzymes, and may still require antibiotics for their flourishing. This may be completely physiological feature.

However, the period of 5 days is the best period for obtaining high production of antibiotics, and was employed for further tests.

3-3-2-2: Effect of growth temperature

Streptomyces isolates may live in wide range of growth temperature.

Some may be psycrophilic, mostly mesophilic, or thermophilic.

Experiment made on the local isolates under study showed that they are of mesophilic type that can grow at 28-37˚C.

Effect of growth temperature on antibiotic production was determined and listed in table (3-8).

Table (3-8): Effect of growth temperature on antibiotic production of *Streptomyces* isolates.

Isolates	Test organisms	28ºC	37ºC	45ºC	50ºC
ERM1	*Staphylococcus aureus*	25 mm	15 mm	_	_
	Bacillus thurengensis	10 mm	_	_	_
	Bacillus sphaericus	10 mm	5 mm	_	_
	Candida albican	20 mm	_	_	_
	E. coli	_	10 mm	_	_
	Proteus mirabilis	12 mm	10 mm	_	_
	P. aeroginosa	15 mm	10 mm	_	_
ERM2	*Staphylococcus aureus*	30 mm	15 mm	_	_
	B. thurengensis	20 mm	15 mm	_	_
	Bacillus sphaericus	_	_	_	_
	Candida albican	25 mm	_	_	
	E. coli	15 mm	_	_	
	Proteusmirabilis	20 mm	10mm	_	__
	P. aerginosa	12 mm	10 mm	_	__

This table shows that both *Streptomyces* local isolates preferred growing at 28˚C, while low growth at 37˚C, and no growth at (45-50) ˚C.

The reason for this can be explained on physiological bases and the nature of the organism; however, the best temperature for antibiotic production was found to be 28C that allows full activation of enzymes responsible for secondary metabolism pathways leading for high production of antibiotics.

3-3-2-3 Effect of pH on antibiotic production

The pH of growth may show an adverse effect on *Streptomyces* growth and secondary metabolism.

The local isolates were grown on different pH values media to determine the effect of this factor on antibiotic production. Results obtained are showed in table (3-9).

Table (3-9): Effect of pH on antibiotic production in *Streptomyces* isolates on antibiotic production.

Isolates	Test organisms	pH 4	pH 7	pH 9
ERM1	*Staphylococcus aureus*	_	15 mm	10 mm
	Bacillus thurengensis	_	20 mm	_
	Bacillus sphaericus	_	15 mm	_
	Candida albican	_	15 mm	15 mm
	E. coli	_	10 mm	10 mm

	Proteus mirabilis	_	10 mm	_
	P. aeroginosa	_	25 mm	_
ERM2	*Staphylococcus aureus*	_	15 mm	15 mm
	Bacillus thurengensis	_	_	_
	B. sphaericus	_	18 mm	_
	Candida albican	_	20 mm	25 mm
	E. coli	_	25 mm	_
	Proteusmirabilis	_	_	_
	P. aerginosa	_	30 mm	_

The table shows that both *Streptomyces* local isolates preferred grow on pH7and gave the highest biological activity, while less production was detected at alkaline pH and no growth at acidic pH.

3.4: The Effect of Crude Extract of Streptomyces on Hep- 2 Cell Line

Cytotoxic effect is shown in table (3-10) and (3-11); this crude extract has cytotoxic effect on the growth of Hep-2 cell line starting at the concentration 10 µg/ ml with high significance difference ($p \leq 0.017$). With increasing extract concentration it more inhibit cytotoxic effect toward the higher concentrations when compared with the control (Hep- 2 cell line treated with SFM) during the incubation period 48h. and the higher effect was enclosed to the concentration 60 µg/ ml with inhibition growth 42.41% when compared with the other concentrations during the same incubation period. This could be attributed to the effect of the bleomycin which refers to a family of structurally related compounds that

when used as anticancer agents, used in treatment of Hopkin lymphoma, sequamous cell carcinoma, and testicular cancer (Shen *et al*., 2001).

Table (3-10): The inhibitory effect of various concentrations of the ERM1 and ERM2 on the growth of Hep- 2 cell line.

Concentration µg/ml	**Growth inhibitory %**	
	ERM1	ERM2
10	9.49	9.49
20	25.95	17.09
30	39.71	23.42
40	36.71	40.51
50	39.87	39.87
60	42.41	53.80

Table (3-11): The effect of ERM1 and ERM2 crude extracts on Hep-2 after 48 hr.

Concentration mg/ml	**ERM1 Mean ± (SE)**	**ERM2 Mean ± (SE)**
10	0.143 ± 0.017 ab	**0.143** ± 0.00 8a
20	0.117± 0.004 bc	**0.131** ± 0.006 ab
30	0.100 ± 0.004 c	**0.121**± 0.003 ab
40	0.100± 0.003 c	**0.094**± 0.008 Bc

50	0.095± 0.005 c	**0.095**± 0.002 Bc
60	0.091± 0.005 c	**0.073**± 0.006 c
Control	0.158± 0.015 c	**0.158**± 0.015 a

Different letters in the same column: significant difference (P 0.05) between means.

Conclusions & Recommendations

Conclusions

- The two isolates subjected to study were found to be of genus *Streptomyces*.
- Two isolates were found to be high stability and less variation on culture media which used in this study.
- These two isolates were found to enhance biological activity in different spectrums. This led to find all of them are antibiotic procedure. These antibiotics were differing in type and concentration, and some of them are clinical use.
- The crude extracts of two isolates possess conspicuous cytotoxic effect in growth inhibition of tumor cell line Hep- 2 in vitro depending on concentration.

Recommendations

- Supporting the studies that goal to the purification and identification of substances produced by local isolates and making application experiments on them in treating some tumors in laboratory animals.

- Trying to get local substitutes to imported substances (Getting isolates which have antitumor activity instead of chemicals).

- Making efforts and studies on the chromosome of selected isolate to know gene- spots responsible for the production of antitumors to improve the productivity of these isolates

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الخلاصة

تضمنت هذه الدراسة فعالية مركبات الايض الثانوي المنتجة بواسطة بكتريا الستربتومايسس بشكلها الخام وتأثيرها على نمو الخلايا السرطانية خارج الجسم الكائن الحي.

جمعت عشرون عينة من التربة من أنحاء متفرقة من محافظة بغداد ,وعزلت منها خمس عزلات تعود لجنس *Streptomyces* تم تنميتها بأستخدام الوسط الزرعي Gauza ثم أخذت العزلات للفحوصات المجهرية والزرعية فضلا عن الأختبارات الكيموحيوية المعتمدة والنمو بوجود تراكيز مختلفة من الملح في الوسط الزرعي SY-Media , النمو بدرجات الحضن المختلفة (وأظهرت النتائج أنه لم يكن أيا من هذه العزلات متحمل لدرجات الحرارة العليا والمنخفضة وأيضا لوحظ ان هذه العزلات تفضل الرقم الهيدروجيني المعتدل الذي يتراوح بين 7-8 لأنجاز النمو).

تم معرفة قابلية العزلات على أنتاج مركبات مضادة للبكتريا ,وهذا تم من خلال دراسة الفعالية الحيوية ضد الكائنات البكتيرية السالبة و الموجبة لصبغة غرام وضد الكائنات حقيقية النواة أذ أظهرت النتائج قابلية عزلتين فقط من هذه العزلات الخمسة القدرة على وقف نمو هذه الكائنات المجهريةمما قد يعطي الدليل على قابليتها لأنتاج مركبات شبيهة بمضادات الحياة . وعززت هذه النتائج الستحصلة من كروماتوغرافيا الطبقة الرقيقة , كروماتوغرافيا الورق, اطياف ألأشعة فوق البنفسجية فقد وجد أن العزلتين منتجة ل Doxorubicin, Streptomycin, Neomycin, Tetracyclin and Bleomycin.

ولدى تحديد العوامل المؤثرة على انتاجية هذه العزلات وجد أن فترة نمو لخمسة أيام بدرجة حرارة 28ºC عند الأس الهيدروجيني 7 هي أفضل الظروف لأنتاج المضادات الحيوية.

تم أختبار الفعالية السمية الخلوية للتراكيز المجضرة لكل مستخلص خام من المستخلصات وهي (10و20و30و40و50و60) مايكرو غرام/ مل على الخط الخلوي السرطاني وبفترة حضن 28 ساعة وكانت النتيجة وجود تأثير سمي واضح وبمعنوية عالية في تلك المستخلصات على النمو الخلايا لسرطانية ، مما يدل على وجود بعض التخصص في التأثير السمي على الخلايا السرطانية.

الإهداء

إلى...منير ظلمات البشرية الحبيب المصطفى محمد (صلى الله عليه وسلم).

إلى... الشمعة التي لولاها لما أمسكت أناملي قلما..لما قرأت عيناي سطرا..
جوهرتي الثمينة التي أضأت مسيرة حياتي...

أمي الغالية... إجلالا وإكراما...

إلى... من ناجى ربه وصلى في سبيل مواصلة مسيرتي...
الذي أخذ بيدي في طريق المعرفة وسلك بي دروب الحياة...

أبي الغالي... أجلالا وإكراما...

إلى... من طال ترقبه هذا اليوم "ولاء"ا لذي غرس في نفسي الأمل...
والنجاح حفظه الله من كل سوء .

زوجي الحبيب...

الى... مهجة عيني وبهجة قلبي وسندي في الحياة أخي الكبير...

"عمار"
امتنانا وعرفانا...
إلى... خيمة الحب و ألأمان...من ارتويت منها الحب و الحنان حفظها الله...

"حنين"
إلى... القناديل المنيرة و المشرقة ..
" أخوتي ألأعزاء "

أليهم جميعا أهدي ثمرة جهدي المتواضع...

أيفين

بِسْمِ اللهِ ألَّرحمَنِ ألرَّحِيم

نَرفَعُ دَرَجاتٍ مَن نَشَاءُ

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صَدَقَ الله ألعَظِيم

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وزارة التعليم العالي و البحث العلمي
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كلية العلوم
قسم التقانة الأحيائية

أنتاج المواد المضادة للخلايا السرطانية من قبل بكتريا الستبربتومايسس

رسالة

مقدمة إلى كلية العلوم / جامعة النهرين

وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الاحيائية

من قبل

أيفين سعدون عبد الوهاب

بكالوريوس تقانة احيائية- جامعة النهرين(2005)

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