Antibacterial and Anticancer property of bioactive secondary metabolites from Actinomycetes isolated from different regions of Himachal Pradesh

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Department of Biochemistry and Microbial Sciences

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For the degree of

M.Sc. Life Sciences with specialization in Microbial Sciences

Submitted by

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M.Sc. Life Sciences with Specialization in Microbial Sciences

Under the Supervision of

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DECLARATION

I declare that the project report entitled **"Antibacterial and Anticancer property of bioactive secondary metabolites from Actinomycetes isolated from different regions of Himachal Pradesh"** has been prepared by me under the supervision of Dr. Pramod K. Kushawaha, Assistant Professor, Department of Biochemistry and Microbial Sciences, School Of Basic and Applied Sciences, Central University of Punjab Bathinda. No part of this dissertation has formed the basis for the award of any degree or fellowship previously.

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CERTIFICATE

I certify that Gourav Chambiyal has prepared his Project entitled "**Antibacterial and Anticancer property of bioactive secondary metabolites from Actinomycetes isolated from different regions of Himachal Pradesh**", for the award of M.Sc Life Sciences with specialization in Microbial Sciences degree of Central University of Punjab, under my guidance. He carried out this work at the Department of Biochemistry and Microbial Sciences, School of Basic and Applied Sciences, Central University of Punjab.

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ABSTRACT

Antibacterial and Anticancer property of bioactive secondary metabolites from Actinomycetes isolated from different regions of Himachal Pradesh

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Keywords: Agar well diffusion method, antimicrobial activity, secondary screening, pathogenic microorganisms, antagonistic, bioactive secondary metabolites, starch casein agar

The prime objective of the present work is to isolate, characterize and purify soil microbes and to know about their antibacterial and anticancer properties. Soil samples were obtained from different locations in Dharamsala, Himachal Pradesh, India. Serial dilutions of the samples were made and plated on starch casein agar. Screening, purification and further tests were performed on the isolates to find the potential colonies. Biochemical and morphological characterization was done as well. Following characterization, isolates were used to extract bioactive secondary metabolites which can be anti-bacterial and anti-cancer. Secondary screening for antibacterial properties of isolates was evaluated by Agar well diffusion method. Further, the isolates were subjected to the GC-MS and MTT cytotoxic assay. Totally 7 isolates of actinomycetes were isolated and out of them only 4 were tested for antagonistic activity against 4 pathogenic microorganisms. Isolates M_1,and M_3 of dilution 10^{-1} were active, while M_2 and M_4 showed less activity against the pathogenic microorganisms. All actinomycetes isolates showed antibacterial activity against *S. enterica* and *P. putida* while they showed less activity against *S. aureus* and *E.coli*. These isolates showed antibacterial and anti-cancer activities and may be used for the growth of new antibiotics for pharmaceutical or agricultural purposes.

Gourav Chambiyal Dr. Pramod K. Kushawaha

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Gourav Chambiyal

Date

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LIST OF ABBREVIATIONS

Abbreviations	Name
+ve	Positive
µl	Micro liter
D/W	Distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxy Ribonucleic Acid
E.coli	*Escherichia coli*
e.g.	Example
G+C	Guanine + Cytosine
GC-MS	Gas-Chromatography Mass-Spectrophotometry
H_2O_2	Hydrogen Peroxide
Hrs.	Hours
IMViC	Indole; Methyl-Red; Voges-Proskauer; Citrate
min	Minute
Mm	Millimetre
Mm	Millimeter
mm	Milliliter
MR	Methyl-Red
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NB	Nutrient Broth
O_2	Oxygen
^oC	Centigrade
P.putida	*Pseudomonas putida*
RBF	Round Bottom Flask
RNA	Ribonucleic Acid
rpm	Rotations Per Minute
RPMI	Roswell Park Memorial Institute medium
S.aureus	*Staphylococcus aureus*
S.enterica	*Salmonella enterica*

SCA	Starch Casein Agar
Sec.	Seconds
v/v	Volume By Volume
-ve	Negative
VP	Voges-Proskauer

1. Introduction

Subsequently the antediluvian time the population, utilize diverse natural sources for the remedy of diseases. During such natural bases, microbial secondary metabolites have been measured one of the influential means for drug detection owing to their miscellaneous biological accomplishments. If we talk about diseases, cancer still holds the top-most position being one of the most severe human health complications (Cocco *et al*., 2003). These practices are exclusively beneficial in certain situations and when pooled, they offer an extra proficient treatment for tumor. Since microorganisms are almost a vast source of diverse substances with countless therapeutic applications, their selection for secondary metabolite and antibiotic production as well as drug lead molecules have supposed improved consideration in recent times. Numerous soil-living bacteria are renowned to yield secondary metabolites that can overcome microorganisms competing for the similar resources. Actinomycetes are a group of gram-positive bacteria, extensive in nature, playing a major role in the field of biotechnology, because they are the abundant producers of antibiotics, anti-tumor agents, immunosuppressive agents and enzymes. Streptomyces is the dominant among Actinomycetes. Approximately half of the bioactive secondary metabolites which were discovered have been produced by Actinomycetes i.e. more than 50% of the known antibiotics produced are from actinomycetes and as well, Actinomycetes are the most normally isolated microbes in nature, which largely occupies the soil environment. They form the chief and considerable group among the soil microbial community and comprise about 50% of the uncultivable soil microbes. Plentiful naturally occurring antibiotics have been found to be produced from actinomycetes. Consequently, the discovery of **Selman Waksman's** *streptomycin* from this group and relatively a few more studies specify their remarkable antibiotic production. However, studies on soil actinomycetes from diverse habitats including rice-paddies, grasslands, beach sands, underground caves and sub-glacial ice of Antarctica were reported. Only a small number of reports are available on high altitude soil actinomycetes communities. Although there are a numerous reports on division and traditional uses of medicinal plants from these early human inhabited areas easily found, data on their microbial resources inexact and

infamous. Due to distinctiveness, huge geographic variation and different soil types it is quite likely that there will be an immense availability of antibiotic and anti-cancerous properties producing actinomycetes in this environment.

1.1 Knowledge gap

Natural sources have been used for centuries as remedies against various diseases and ailments. Yet, there have been very few studies done on the targeting of secondary metabolites from actinomycetes as inhibitors of cancer cell proliferation for the treatment of cancer. Furthermore, identification of MIC and MFC can be done.

1.2 Hypothesis

The present study has been designed to identify and validate the anti-microbial and anti-cancerous activities of bioactive secondary metabolites from actinomycetes isolated from different hilly regions of Himachal Pradesh.

1.3 Objectives

1. Literature-based identification of actinomycetes producing anti-cancerous properties.
2. Primary and secondary screening of actinomycetes for antibacterial properties
3. Determination of secondary metabolites
4. *In vitro* cytotoxic assay (MTT assay)

2.1 Actinomycetes

Actinomycetes are a group of branching unicellular organism having high GC content. These are gram-positive bacteria having fungal morphology. They are rich source of secondary metabolites with varied biological activity. Their uniqueness among the prokaryotes lies in the wide variety of lifecycles that they exhibit. They have been positioned within the Actinobacteria phylum, subclass Actinobacteridae and Actinomycetales order. Being a huge group of microbial resources of extensive practical use and elevated commercial value, actinomycetes tend to add to around 70% of the source of antibiotics. They are also the producer of a numerous non-antibiotic bioactive metabolites, such as enzymes, anti-oxidation reagents, immunological regulators etc. Actinomycetes are broadly spread across natural habitats, especially soil and ocean. The marine species of microorganisms have been studied to play role in mineralization of complex organic matter, degradation of dead plankton, plants, animals, degradation of pollutants and toxicants. They posses a wide-ranging enzyme activity and can also catalyze diverse biochemical reactions. Actinomycetes are considered to be an important group among the marine microorganisms. They have an incredible potential to synthesize bioactive secondary metabolites. Numerous studies have been done in India and its shores for understanding the microbes and their behavior. Marine microbes producing bioactive products have been a part of interest recently for researchers worldwide. The major group studied in detail is the marine Streptomyces. Numerous species of Streptomyces are isolated from sea water, marine sediments including mangroves, marine mollusks and detritus. One of the important observations is that nearly 70% of Streptomyces sp. isolated from marine mollusks is antagonistic, whereas only 20-25% of cultures isolated from sediments show antagonism towards the test microorganism. During this period a novel marine *Vibrio sp.* was isolated from the marine sediments (east coast) which produced an antileukemic agent (L-asparaginase). This was found superior to commercial preparations at that time in the treatment of tumors.

2.2 Cancer

It is the sporadic growth of cells having the potential to spread to other body parts. It generally forms subdivision of tumors i.e. mass of unusually growing cells, which may be as follows:

- Benign tumors, which are, slow growing and do not undergo metastasis.
- Malignant tumor, which has rapid growth rate and can metastasize.

Normally, cells grow and divide in a well-regulated manner according to the requirements of the body and old cells are replenished continuously by new cells. But, when cancer develops the old and damaged cell instead of dying, start to grow and divide in uncontrolled fashion leads to initiation of cancer. These cancerous cells are generally malignant in nature i.e. having ability to spread to other body parts and can form secondary tumors. (Eckert *et. al.*, 2014)

Name of the cancer is generally based on its site of origin and affected organ. Cancer can be divided into following categories:

- ❖ Carcinoma: It is the most common type of cancer and derived from the epithelial cells.
- ❖ Sarcoma: It is cancer arising from connective tissue.
- ❖ Lymphoma/Leukaemia: These are two classes of cancer arising from hematopoietic cells (that give rise to other blood cells through the process of hematopoiesis).
- ❖ Germ Cell Tumor: It is derived from pluripotent cells (the cells having a tendency to differentiate into any cell type may be ectoderm, endoderm or mesoderm origin).

Blastomas: It is cancer derived from immature precursor cells (cells having a tendency to differentiate into a particular cell type)

Cancer has become the second largest cause of death after cardiovascular diseases. A report published in 2010 by the WHO showed that the most common diagnosis was lung cancer, breast cancer and colorectal cancer, but the highest mortality rate is the lung cancer, stomach cancer and liver cancer (Wang *et. al*., 2014).

2.3 Antibiotic production from actinomycetes:

Actinomycetes produce huge number of secondary metabolites with diverse biological activities such as antiparasitic, antibacterial, immunosuppressive actions, anticancerous as well antifungal (Demain, 1999). Except some species which may cause infections (isolated from the rhizosphere soil samples), actinomycetes are not particularly pathogenic (Mishra *et al*., 1980). According to one definition, Secondary metabolites produced from the microorganism are not actually important for the growth of the cell or in other vital (Vining, 1990). Microorganisms which reside in the soil mainly produced secondary metabolites and also experience some morphological changes e.g. bacilli, fungi and actinobacteria (Vining, 1990). Approximately 50%of the secondary metabolites are produced from actinomycetes. Streptomyces is one of the main species among all actinomycetes, which produce secondary metabolites.In Almost 10,000 known antibiotics are there among which around 55% is contributed by the Streptomyces (Lazzarini *et al*., 2000). Greater part of these components is used for medicinal purpose for fighting multi-drug resistance gram positive and gram-negative bacteria. The secondary metabolites produced by Streptomyces possess a broad range of biological activities such as antibacterial (tetracycline, chloramphenicol, streptomycin), immunosuppressive (rapamycin), antitumor (actinomycin, mitomycin C, anthracyclines), antifungal (nystatin), antiviral (tunicamycin), antiparasitic (avermectin) and diabetogenic (bafilomycin, streptozotocin). The key secondary metabolites produced by them are avermectin, anthracyclines, hygromycin, lincomycin, bafilomycin, chloramphenicol, mitomycin c, rapamycin, tetracyclines, valinomycin streptomycin and streptozotocin. Production of secondary metabolites is determined by the accessibility of nutrients. In fermentative experiments, the production of antibiotics is augmented by the presence of a non-favoured carbon source or by phosphate deprivation (Aharonowitz and Demain,1978). Nitrogen availability can also effect the production of secondary metabolites (Aharonowitz, 1980). The mechanism of the production of secondary metabolites has still been undiscovered. Advancement of drug-resistant pathogens predominantly in immunodeficient patients revealed the need for new and novel antibiotics (Jain and Jain, 2005.) In the course of screening for actinomycetes for potent antimicrobial agents, a promising isolate dominant and efficient in inhabiting

the growth of test organism was also isolated (Narayana and Vijayalakshmi, 2007). Actinomyces can be isolated from terrestrial soil, from rhizospheres of plant roots, from marine soil and recently as we have isolated from hilly region of Himachal Pradesh. This review describes the range of secondary metabolites produced from actinomycetes bacteria w.r.t their chemical structure, biological activities, anti-tumor, anti-bacterial activities.

2.4 Anti-tumor activity of Actinomycetes

Microbial metabolites production has been discussed since time immemorial, in which filamentous actinomycetes produced more than 10,000 bioactive compounds (Berdy, 2005). The notable production from *Streptomyces* spp. Origin were about 75% or 7600 whereas 2500 or 25% were from rare actinomycetes (*Actinomadura, Micromonospora* and *Streptoverticillium*). The rough amount of all actinomycetes products demonstrating antimicrobial activity to antitumor activity was in the ratio 70:30 respectively. Production of a great number of important drugs by actinomycetes is well known. Reports have revealed some significant drugs provided by actinomycetes to be tetracyclines, β-lactams, macrolides anthracyclines, chloramphenicol, and aminoglycosides (Okami and Hotta, 1988). *Streptomyces* contributed the maximum chemical diversity among the actinomycetes (Sanglier *et al.,* 1993). Studies have reported the obtaining of antifungal compounds from marine *Streptomyces* spp. These compounds have shown powerful antifungal activity against *E.coli, P. aeruginosa* and *C. albicans* (Cho *et al.*, 1999). Moreover, a wide-ranging antimicrobial activity of ten most potent marine *Streptomyces* spp. isolated from the Nahoon beach, a coastal shore of Indian Ocean in the Eastern Cape Province of South Africa was also studied (Ogunmwonyi*et al.,* 2010). Antitumor compounds are produced naturally principally by microorganisms. In fact, the key producer of a variety of natural products with different properties including antitumor activity is actinomycetes (Olano *et al.,* 2009). Isolation of *Streptomyces* was also made from marine sediments that were reported to produce two novel spiroaminals, marineosins A and B which hold substantial inhibition of human colon carcinoma (HCT-116) in an

in vitro assay and selective activities in diverse cancer cell types (Boonlarppradab *et al*., 2008).

3.1 Sample collection and pretreatment

The soil samples were collected from 3 different places at the same location of Dharamshala (Geographical coordinates: Latitude 32.2N and Longitude 76.3E, Height of 2788.92-2869.387m above sea level), Himachal Pradesh, India. Three different soil samples were collected from 5-7 cm depth of the surface from the ground.

3.2 Isolation of pure culture of Actinomycetes

The collected soil sample was initially heated for 30min. to 1 hour at 60˚C in order to reduce the number of other microorganisms in the soil. After this step, a serial dilution was done by suspending 1gm of dry soil in 9ml of distilled water in first test tube. 1ml of suspension from the first tube was added to 2^{nd} test tube which contained 9ml of D/W as well, and by this process dilution up to 10^{-9} was made. Each tube was vortexed in order to make suspensions uniform. A fraction of 10µl from 10^{-1}, 10^{-2}, 10^{-3}, 10^{-5} test tubes was spread in a uniform manner over the surface of "Starch Casein Agar" plates. The plates were incubated for 5-7 days at 30˚C. After 5-7 days, colonies/isolates were obtained.

Random isolates from 10^{-1} dilution i.e. M_1, M_2, M_3 and M_4 were selected in order to obtain pure culture by using "***streak plate***" method. Colonies obtained were streaked on a plate containing starch casein agar medium and incubated for 5-7 days at 28 ± 2˚C. After obtaining a pure culture, further characterization of the isolates was done.

3.3 CHARACTERIZATION OF ISOLATES OF ACTINOMYCETES:

All isolates obtained were further characterized morphologically and biochemically.

a) GRAM STAINING

Gram staining is a principle to distinguish between gram negative and gram-positive bacteria.

PROCEDURE:

1. A smear was made on a surface of the slide from the isolated colony and it was heat fixed.

2. After heat fixing, 2-3 drops of *crystal violet* were added to the smear as a primary stain for 1 min. and washed with tap water.

3. Following primary stain, Gram's iodine was added which acts as a mordant for 1min and further washed again.

4. Then smear was decolorized with Gram's decolorizer (95% Ethyl alcohol was used) until the blue color no longer flowed over the surface for 30 sec and washed with tap water.

5. After decolorizing, 2-3 drops of secondary stain, Safranin was added for 1 min. and gently washed with tap water.

b) MORPHOLOGICAL CHARACTERIZATION

Following Gram's staining, the colonies obtained were observed under a high-power microscope and morphology of each isolate was noted with respect to color, shape and branching nature of the colony.

c) PRELIMINARY SCREENING OF FOR ANTI-BACTERIAL ACTIVITY

10µl of the isolates M_1, M_2, M_3 and M_4 were cultured on nutrient agar plates by spread plate method and plates were incubated for 3 days at 28 ± 2˚C. 4 known strains of bacteria i.e. *Pseudomonas putida, Salmonella enterica, E.coli, Staphylococcus aureus* were used as test organisms. Test organisms were cultured in a nutrient broth for 18-24 hours at 37˚C. After incubation 100µl broth culture of test organism was spread over the already incubated plates of isolates and incubated for 37 ˚C for 18-24 hours. After incubation, the zone of inhibition was observed and recorded by measuring the diameter.

d) BIOCHEMICAL CHARACTERIZATION

After morphological characterization, biochemical tests were performed on the isolates having anti-bacterial activity (M_1, M_2, M_3, M_4). Catalase, Oxidase, Indole test, MR test, VP test, Citrate Utilization tests were some of the tests performed in the lab as a part of biochemical characterization for actinomycetes.

(i) INDOLE TEST:

Indole production in IMViC test is used to distinguish between bacteria. Tryptophan present in indole undergoes oxidation by the way of some enzymatic activities of some bacteria. Tryptophanase is an enzyme which converts tryptophan into some metabolic products. All microorganism cannot hydrolyze tryptophan with the production of indole and hence serves as a biochemical marker. Kovac's Reagent added to the solution contains amyl alcohol, HCL, and paradimehylaminobenzaldehye which reacts with indole and there is a formation of a RED ring which distinguishes between positive and negative results.

Procedure-

1. A loop full of bacterial culture was added in a 15ml test tube containing 5ml tryptone broth and tube was incubated for 24hrs at 37 ˚C.
2. After incubation, 2-3 drops of Kovac's Reagent was added.
3. The presence and absence of the ring were observed.

(ii) MR (METHYL-RED) TEST:

The MR test is used to detect the production of sufficient acid during fermentation of glucose. Methyl Red is a pH indicator, which remains red in color at a pH of 4.4 or less.

All enteric initially produce the pyruvic acid from glucose metabolism. Some bacteria subsequently use the mixed acid pathway to metabolize pyruvic acid to other acids, such as lactic acid, acetic acid, and formic acid. These bacteria are called methyl red positive. Other enteric subsequently use the butylenes glycol pathway to metabolize pyruvic acid to neutral end-products. These bacteria are called methyl-red-negative.

Procedure-

1. Bacterium to be tested was inoculated in test tubes containing MR-VP broth.
2. Test tubes were incubated at 37°C for 24 hours.
3. After incubation, five drops of Methyl Red reagent was added to test tubes.
4. The color change was observed.

(iii) VP (VOGES-PROSKAUER) TEST:

Voges-Proskauer test is used to demonstrate an organism ability to convert pyruvate to acetoin. Acetyl-methyl carbinol is an intermediate in the production of butylene glycol. In this test, two reagents, Barrit's A and Barrit's B reagent are added to test the broth after incubation and exposed to atmospheric oxygen. If acetoin is present, it is oxidized in the presence of alpha-naphthol to produce the red color. Alpha-naphthol acts not only as a catalyst but also as a color intensifier.

Procedure-

1. Bacterium to be tested was inoculated in test tubes containing MR-VP broth.
2. Test tubes were incubated at 37°C for 48 hours.
3. Few drops of Barrit's A and Barrit's B reagent were added.
4. The color change was observed.

(iv) CITRATE TEST:

The citrate utilization test helps to test the ability of an organism to utilize citrate as a source of energy. The medium contains inorganic ammonium salts ($NH_4H_2PO_4$) as the only nitrogen source and citrate as the only carbon source**.**

Bacteria that can grow on this medium produce an enzyme i.e. citrate-permease, which converts citrate to pyruvate**.** Pyruvate can then enter the organism's metabolic cycle for energy production. Growth indicates citrate utilization, which is an intermediate metabolite of Krebs cycle**.**

Procedure-

1. Slants of Simon Citrate Agar were prepared.
2. Slants were inoculated gently with a well-isolated colony in a zig-zag manner.
3. Slants were incubated aerobically at 37^0 C for up to 5 to 7 days.
4. The color change was observed.

(v) OXIDASE TEST:

The oxidase test is used for the identification of the bacteria that can produce cytochrome C oxidase, an enzyme of the bacterial electron transport chain (ETC). When present, this enzyme oxidizes the reagent to dark blue colored product. When the enzyme is absent, the reagent remains reduced and colorless.

Procedure-

1. The disc was taken and placed over the tissue paper.
2. Few drops of water were added on the disc.
3. A colony was picked up by a loop and made as a smear over the disc.
4. The color change was observed within 10-30 seconds.

(vi) CATALASE TEST:

It is a process of characterization of bacteria through which they are classified as aerobic and aerobic. Catalase is an enzyme which cleaves H_2O_2 on bacterial culture, if there is a production of O_2 during the test then bacteria is aerobic otherwise the bacteria is anaerobic.

Procedure-

1. A bacterial colony was transferred over a clean and dry glass slide using a loop.
2. Few drops of 3% H_2O_2 was added to the slide and mixed gently.
3. Formation of bubbles was observed.

3.4 Extraction of bioactive secondary metabolites from fermented broth

SOLVENT EXTRACTION METHOD

Fermentation carried out for the process of extraction of bioactive secondary metabolites was done in small scale. A loop full of culture isolates (M_1, M_2, M_3,) was suspended in four 500ml flasks, each containing 250ml of NUTRIENT BROTH. Flasks were shaken in a rotary incubator (200 rpm) at 28±2˚C for 5-7 days. After 5-7

days of incubation, the culture was filtered through sterile Whatman filter paper grade 1, aseptically. After filtration, the filtrate was added in a separating funnel and ethyl acetate was added to separate the organic phase and aqueous phase. The amount of ethyl acetate added in the filtrate was 1:1(v/v). The mixture was shaken vigorously for half to one hour. The ethyl acetate phase or organic phase was further separated in round bottom flask and was evaporated using rotary vacuum evaporator under reduced pressure at 40-45˚C until a solid mass was obtained. After evaporation, the weight of solid mass (crude extract) was taken. Further, from the obtained crude extract, secondary screening for antibacterial activities, GC-MS and anti-cancerous properties were done.

3.5 SECONDARY SCREENING FOR ANTIBACTERIAL ACTIVITIES OF ACTINOMYCETES

Secondary screening of actinomycetes was done by AGAR-WELL plate method. Test organism was cultured in nutrient broth for 18-24hrs at 37˚C. After incubation, 50µl culture broth of test organisms was spread over the nutrient agar plates and after that by using 1000µl sterile tips wells were bored on the plates. The 20µl crude extract obtained in methanol was suspended in each well carefully and plates were incubated for 37˚C. After overnight incubation, the zone of inhibition was observed by measuring the diameter.

3.6 DETERMINATION OF ANTI-CANCER ACTIVITIES

DESCRIPTION ABOUT THE CELL LINES CHOSEN FOR THE STUDY

a) K562 cells

Origin of K562 (suspension cells) cell line is Human Caucasian chronic myelogenous leukemia K562 cells. National Cancer Institute used K562 cells which are a part of the NCI-60 cancer cell line panel. These cells can easily be killed by natural killer cells and show less clumping when compared with other suspension cells. The cell was taken from Dr. Malkhey Verma’s lab, Department of Biochemistry and Microbial Sciences at Central University of Punjab, Bathinda. These cell lines were maintained in RPMI complete medium.

b) MDA-MB-231 CELL LINE

MDA-MB-231 is a breast cancer negative cell lines with an estrogen receptor. These cell lines are independent for growth and proliferation and were obtained from Animal tissue culture lab of Dr. Felix Bast at the Central University of Punjab Bathinda. These cell lines were maintained in DMEM medium with 10% FBS.

c) CELL LINE MAINTENANCE

Cell lines were taken out of -80˚C and thawed by placing in a water bath at 37˚C. After thawing, suspension of the cell was mixed in the 1ml complete medium. After mixing the cells were transferred to 15ml tube. The tube was centrifuged for 5 min. at 1000rpm. After centrifugation, the supernatant was removed, and the pellets were mixed with 1ml fresh media. Now, 25cm^2 tissue culture flask was taken and 4 ml of complete media was added in the flask. After this step, 1ml cell suspension was added to the tissue culture flask with 4ml media. The flask was incubated for 24 hours at 37˚C. Cells were observed at regular intervals of time under the light microscope.

3.7 *IN VITRO* CYTOTOXIC ASSAY (MTT ASSAY)

For suspension cell and adherent cell

MTT assay was done on both the cell lines to measure the cytotoxicity of the extracted bioactive secondary metabolites from actinomycetes. 96 wells plate was used to perform MTT assay. Cell suspension was mixed with media and after that 100µl of cells - media was seeded in 96 well plates. Triplicates of the plates were made with same cell suspension. Negative and positive control with blank was set up in the plate. Approximately 5×10^3 cells/well was seeded in each well except blank. The plates were incubated at 37˚C for different intervals of time i.e. 24hrs, 48hrs, 72hrs. After some time the treatment with bioactive secondary metabolite (crude extract) was given to each well excluding blank. The treatment was done in different concentration i.e. 0.6 µl was the lowest concentration and 0.27 µl was the highest concentration to be used. Plate was incubated for 24hrs. The 24hrs incubated plate was observed under inverted light microscope. Following microscopy, MTT solution was added in each well and the amount added was 10% of the solution i.e. approx.

10µl of 5mg/ml. Plate was incubated for 4-6 hours at 37˚C wrapped with aluminium foil because as suggested in the kit MTT was light sensitive. 100 µl of dissolving solution (DMSO) was added to the wells and the plate was shaken on gyratory rotor for half an hour. Following this, the plate was subjected to take OD in different wavelengths i.e. 570nm and 650nm. 650nm was used as a reference wavelength. Same procedure was followed for the rest of two plates which were incubated for 48hrs and 72hrs.

4.1 Isolation of potential colonies of actinomycetes

Isolation of actinomycetes was done after spreading the 10^{-1}, 10^{-2}, 10^{-3} and 10^{-5} dilutions over the selective media i.e. SCA (Starch Casein Agar). The results revealed that the colonies appeared in different numbers in each plate. The dilution 10^{-1} showed more dense growth compared to other two dilutions.

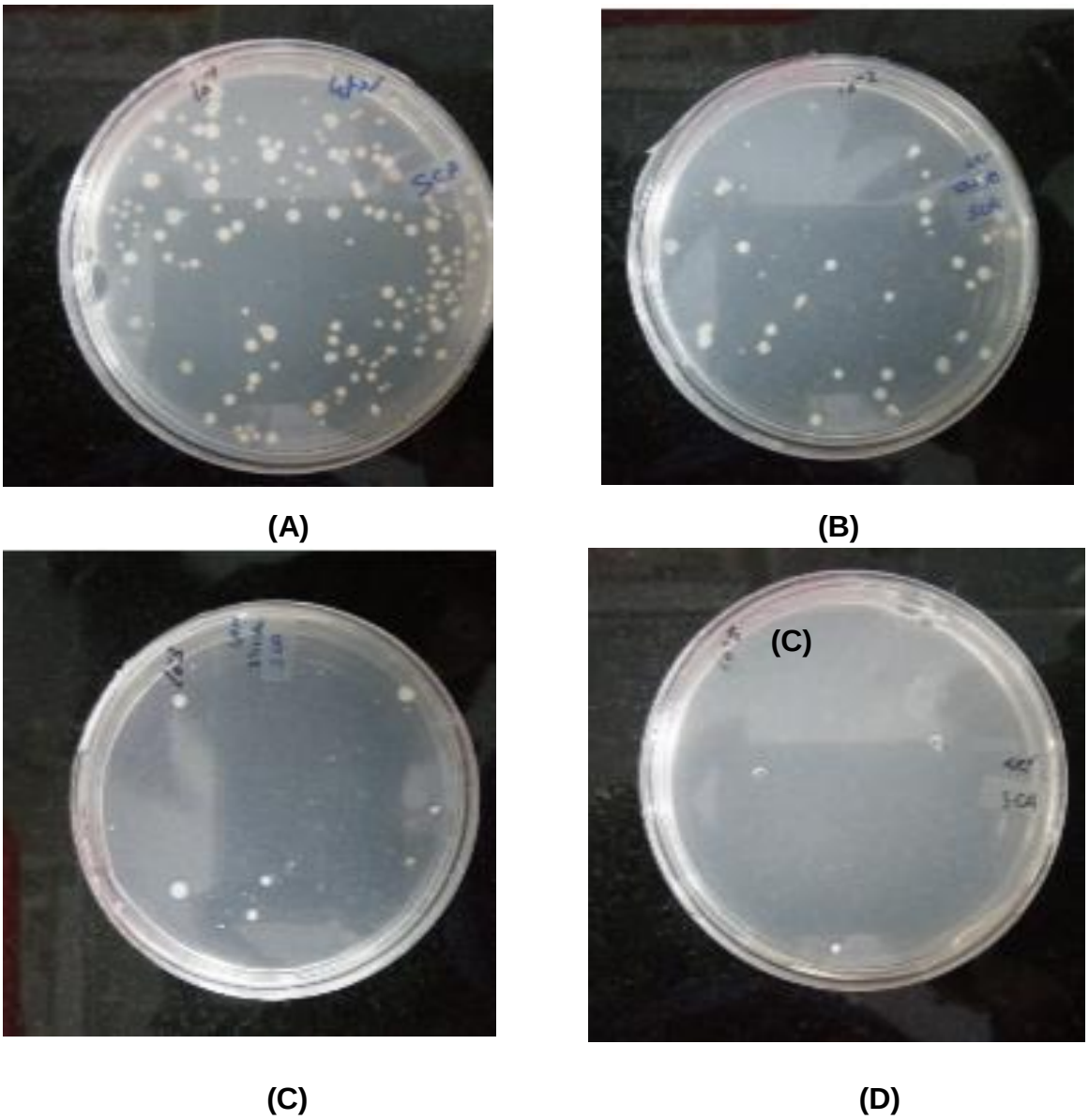


Figure 4.1: Colonies obtained after spreading dilutions. (A) Plate containing 10^{-1} dilution of soil sample **(B)** plate containing 10^{-2} dilution of soil sample **(C)** plate containing 10^{-3} dilution of soil sample **(D)** plate containing 10^{-5} dilution of soil sample

4.2 Pure cultures of actinomycetes by streak plate method

Isolates obtained after the “Streak-plate technique” of the isolated actinomycetes of 10^{-1} dilution over SCA plates for selective growth. The plates showed the growth of pure isolated colonies.

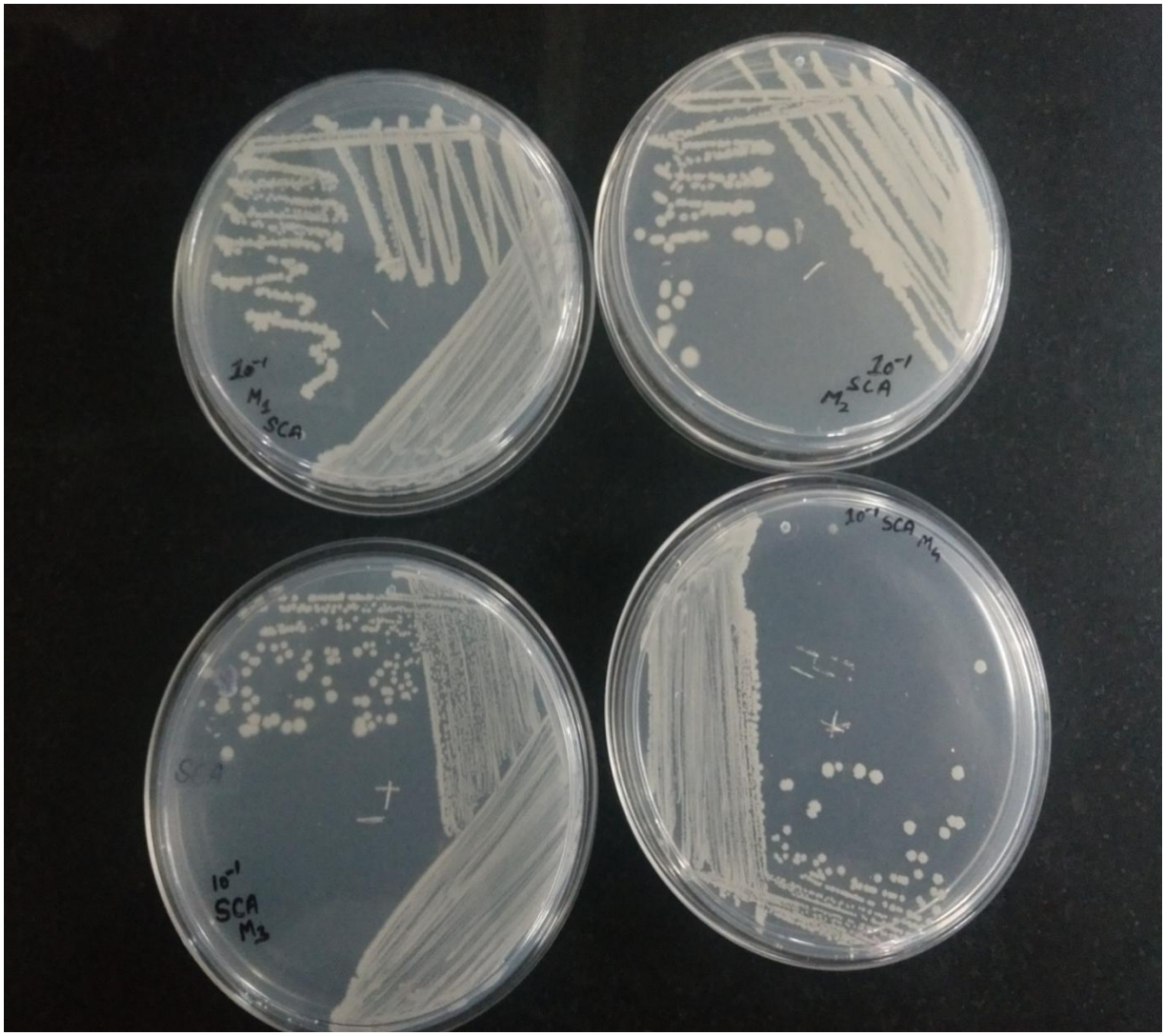


Figure 4.2: Four isolates of 10^{-1} dilution.

4.3 Pure isolates showing a zone of inhibition (ZOI)

Zone of inhibition was obtained after spreading known pathogenic strains over a well grown streaked plate of each isolate.

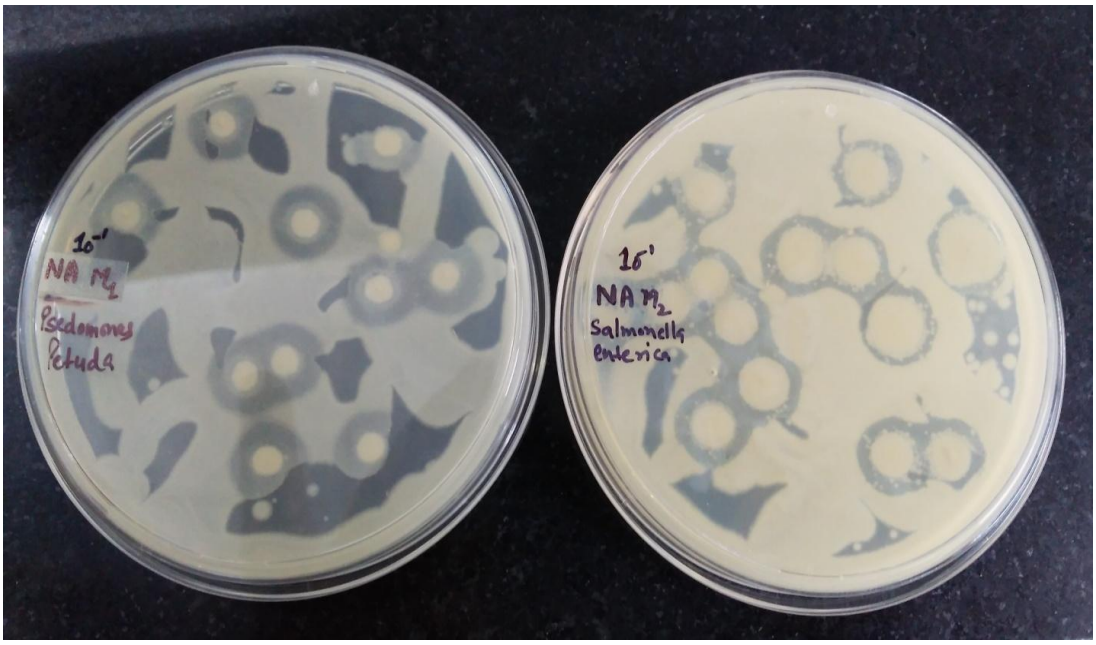


Figure 4.3: Zone of inhibition of M1 and M2 against *P. putida* and *S. enterica* respectively

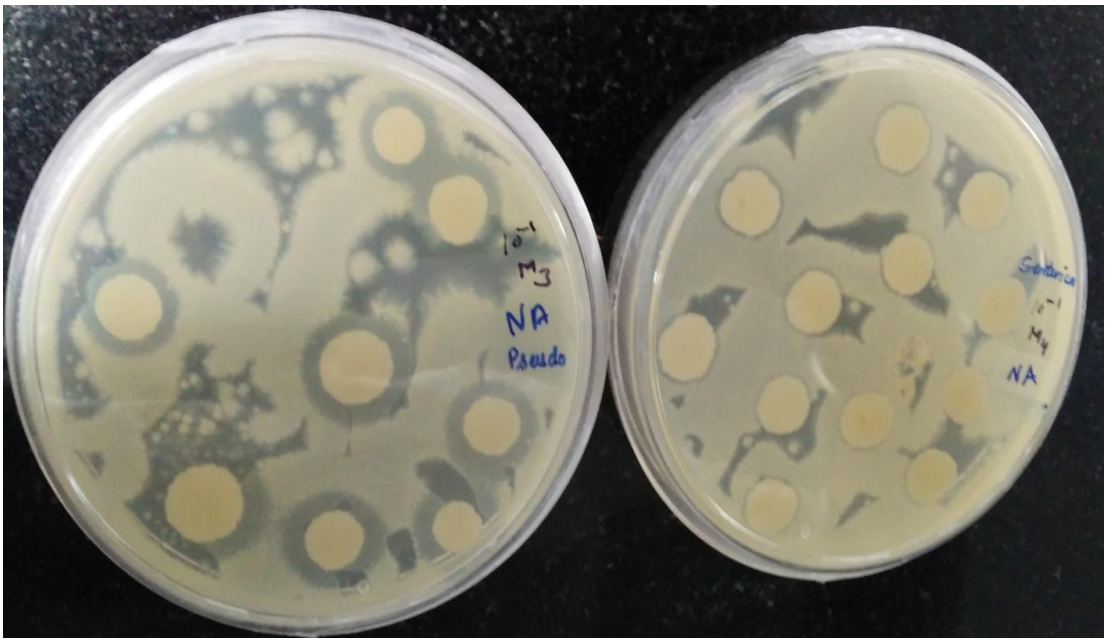


Figure 4.4: Zone of inhibition M3 and M4 samples against *P. putida* and *S. enterica* respectively.

TABLE OF SUSCEPTIBILITY TEST:

Isolates	Test organism	Zone of inhibition				
		Colony 1	Colony 2	Colony 3	Colony 4	Colony 5
Isolate M_1	*P.putida*	2.5mm	3mm	3mm	3mm	3mm
Isolate M_2	*S.enterica*	2.5mm	3mm	2.5mm	3mm	3mm
Isolate M_3	*P.putida*	2mm	2.5mm	2mm	2.5mm	2mm
Isolate M_4	*S.enterica*	1mm	2mm	1.5mm	1mm	1.5mm

Table 4.1: Isolates showing ZOI against the two test organisms

4.4 Solvent extraction method

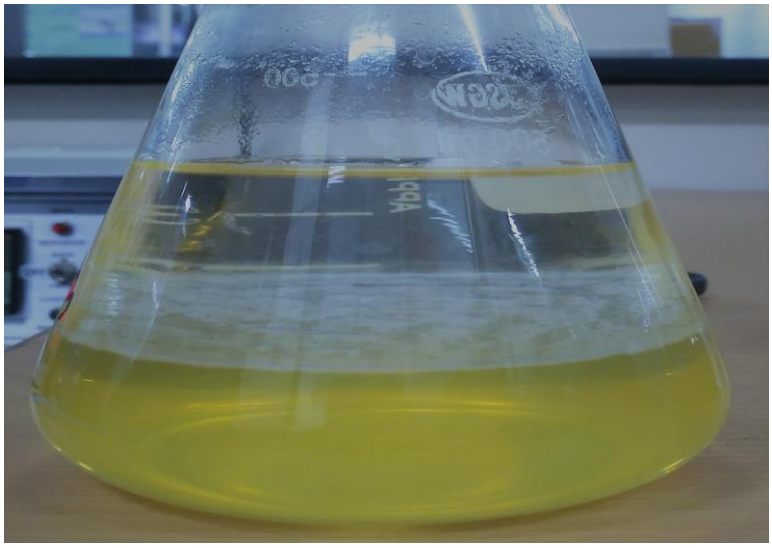


Figure 4.5: Flask containing two separate phase i.e. organic phase and aquous phase and secondary metabolites assumed to be in organic phase

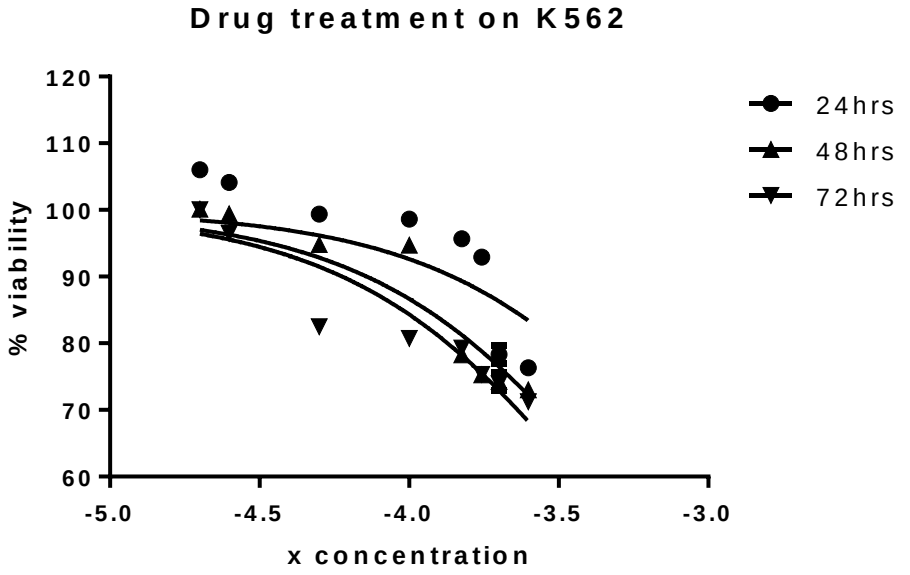
4.5. Results of Biochemical Tests (IMViC test, Oxidase test and catalase test) :

Name of the test	M1	M2	M3	M4
Indole test	+ve	-ve	+ve	+ve
Methyl-Red test	-ve	-ve	-ve	-ve
Voges-Proskauer test	-ve	-ve	-ve	-ve
Citrate utilization test	-ve	-ve	-ve	+ve
Oxidase test	+ve	+ve	+ve	-ve
Citrate test	+ve	+ve	+ve	+ve

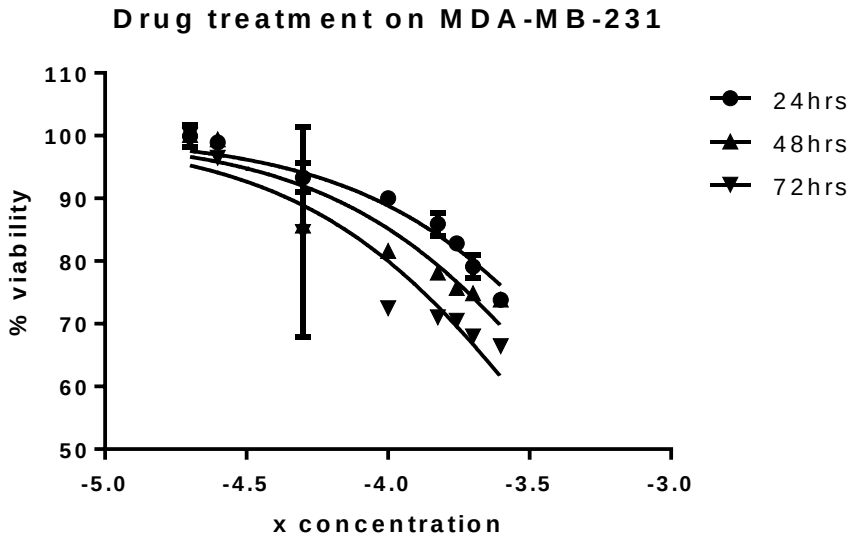
Table 4.2: Table containing biochemical analysis of isolated culture

4.6 *In vitro* anticancer potential of the crude extract by using MTT assay

MTT assay for cell viability was used to quantify the cytotoxic activity of crude extract produced from actinomycetes in k562 cells and MDM cells. Both the cell lines were exposed to different concentrations of crude extract (25µg-250µg).



4.6 (a) Graph showing the inhibition of drug concentration up to 25%.



4.6 (b) Graph showing the viability of cell against different concentrations

Figure 4.6: MTT assay showing anticancer potential of the crude extract

5. Discussion

Subsequently, the hilly region has been found to be a promising habitat for *Actinomycetes* species, and there is a dire need to discover novel secondary metabolites having potential antibacterial and anti-cancer properties for the treatment of cancer. Thus, our study focuses on the extraction, identification and subsequent anti-bacterial analysis of bioactive secondary metabolites produced by Actinomycetes as well as anti-cancer activity determination against cell lines.

In the present study, *Actinomycetes* were isolated from the sample and those with antimicrobial properties were identified. Four isolates were identified and each isolate showed antimicrobial property against 2 test strains i.e. *Pseudomonas putida* and *Salmonella enterica*.

Actinomycetes showed potential growth on the selective media i.e. Starch Casein Agar, and according to the serial dilution, variations were observed in the growth pattern. On selecting random isolates from 10^{-1}, 10^{-2}, 10^{-3} and 10^{-5} dilutions, their master plates were maintained for further reference. Considering the 4 isolates of 10^{-1}, antimicrobial screening was performed for them against 4 known pathogenic strains i.e. *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas putida* and *Salmonella enterica*. Isolates showed inhibition against *Pseudomonas putida* and *Salmonella enterica* only. And the amount of inhibition was clearly observed as a variation in diameter of different isolates against different test organisms. Gram staining gave positive result which is considered for actinomycetes. On performing biochemical tests, some samples showed expected results but some variations were observed. Even among different samples, result variations were recorded.

As a part of the qualitative study, many compounds have already been reported in previous studies (Khattab *et al.*, 2016). Some reported groups of antimicrobial compounds are; β-lactams, aminoglycosides, glycopeptides, anthracyclines, macrolides, tetracyclines and polyenes (Sharma *et al.*, 2014). Secondary screening of the isolates from crude extract was also done by using Agar Well diffusion method. Following GC-MS, *in-vitro* cytotoxic MTT assay was performed using two cell lines i.e. K562 (human immortalized myelogenous leukemia line) and MDA-MB-231 cell line (breast cancer cell line) for determining the anti-cancer activity of the isolates. The

result showed the inhibition range of drug used for treatment and viability of the cell present in the sample.

Thus, the present study identified the secondary metabolites produced by the soil *Actinomycetes* of hilly region. Besides, a potential anticancer and anti-bacterial activity of the sample was also observed through the MTT assay performed. Thus, this data can be analyzed for studying individual metabolite, their derivatives along with their therapeutic properties.

6. Conclusion

Over the years, the search for potential anti-cancer therapeutic approaches from natural sources has remained an active area of research for the scientists worldwide. Thus this study focuses on the antibacterial and anti-cancer activities of bioactive secondary metabolites produced by randomly isolated strains of Actinomycetes from unknown sample. In the above detailed work, isolation of actinomycetes was performed using selective media. Further, the antibiotic producing strains, showing antimicrobial activity during screening were isolated and grown further. The GC-MS results gave us the qualitative record of secondary metabolites present in the sample, their expected structures and possible derivatives. Following GC-MS, the samples were subjected to perform *in-vitro* cytotoxic MTT assay for anti-cancer activities determination which showed the inhibition of drug (crude extract) and viability of the cells present in the sample. Thus, the present study depicted the anti-cancer and anti-bacterial potential of the bioactive secondary metabolites from Actinomycetes against cell lines.

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