BIOACTIVE METABOLITES DERIVED FROM SOIL ACTINOMYCETES AGAINST MULTI DRUG RESISTANT G+ ORGANISMS

A PROJECT REPORT

Submitted by

THIRIPURASUNDARI G R
(Reg. No.1120203016)

in partial fulfillment for the requirement of award of the degree

of

M.TECH. BIOTECHNOLOGY

FACULTY OF TECHNOLOGY

KUMARAGURU COLLEGE OF TECHNOLOGY, COIMBATORE 641 049
(An Autonomous Institution Affiliated to Anna University, Chennai)

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BONA FIDE CERTIFICATE

Certified that this project work entitled **BIOACTIVE METABOLITES DERIVED FROM SOIL ACTINOMYCETES AGAINST MULTI DRUG RESISTANT G+ ORGANISMS** is a bona fide work of Ms. THIRIPURASUNDARI, G. R. (Reg. No.1120203016) who carried out the research under my supervision. Certified further that to the best of my knowledge, the work reported herein does not form part of any other thesis or dissertation, on the basis of which, a degree or award was conferred on an earlier occasion on this or any other students.

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ABSTRACT

Pathogenic bacteria are becoming drug resistant recently and it leads to the production of new antibiotics. Among the naturally occurring antibiotic producers, actinomycetes are recognized to be rich sources of bioactive secondary metabolites with diverse group of gram positive, aerobic, mycelial bacteria and also play an important role in inhibiting the pathogens. In the present study, 19 different actinomycetes were isolated from soil samples collected from different places around Government hospital, Theni, using crowded plate technique. These isolates were screened for antimicrobial activity against various pathogens such as *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeuroginosa*, *Klebsiella pneumonia* and *candida albicans* in order to identify potential antibiotic producer. One promising strain, designed as HSSA 09 with strong antimicrobial activity against Gram positive organisms, was selected for further studies. This isolate was identified as *Streptomyces* sp. based on a great variety of morphological and microscopic characteristics. 16S rRNA sequencing was also done and found that the isolate may be *Streptomyces cinereoruber*. Solid state fermentation was carried out for the mass production of antibiotic compound. Its antimicrobial activity against Gram positive pathogens was again confirmed by agar well diffusion method using different strains of *Staphylococcus aureus* and *Bacillus subtilis* as test pathogens. The separation of the antimicrobial compound was carried out using thin layer chromatography with hexane/ ethyl acetate as mobile phase. Single spot with Rf value 0.55 was obtained. The fraction eluted from TLC was further used for spectral analysis such as FTIR and GC-MS. The results from the spectral analysis revealed that it was a partially purified compound with a mixture of compounds present in it.

Keywords: *Streptomyces*, crowded plate technique, antimicrobial activity, agar well diffusion method, 16S rRNA sequencing, TLC, FTIR, GC-MS

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THIRIPURASUNDARI G R

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LIST OF ABBREVIATIONS

min	Minutes
°C	Degree Celsius
mL	Millilitre
nm	Nanometre
mg	Milligram
g	Gram
mM	Millimolar
YPD	Yeast Extract Peptone Dextrose
DMSO	Dimethylsulfoxide
SCA	Starch Casein Agar
MHA	Muller Hinton Agar

CHAPTER 1

1.1 INTRODUCTION

Infections are very common and responsible for a large number of diseases that affect human health. Most of the infectious diseases are caused by microbes especially bacteria. It can be prevented and treated through a various group of anti-microbial compounds known as antibiotic. Antibiotic may be natural, synthetic or semi-synthetic.

The word antibiotic came from the word antibiosis a term coined in 1889 by Louis Pasteur's pupil Paul Vuillemin which means a process by which life could be used to destroy life. The ancient Egyptians, the Chinese, and Indians of Central America all used molds to treat infected wounds. But, they did not understand the mechanism behind the treatment of diseases. (Moyer *et al.*, 2000)

Selman Waksman first used the word “Antibiotic” as a noun in 1941 to describe any small molecule made by a microbe that antagonizes the growth of other microbes. This definition does not include substances that kill bacteria, but are not produced by microorganisms. (Clardy *et al*., 2009)

In 1928, Sir Alexander Fleming observed that colonies of the bacterium *Staphylococcus aureus* could be destroyed by the mold *Penicillium notatum*, proving that there was an antibacterial agent. His discovery has revolutionized the medical field and allowed to treat millions of people from bacterial infections. Major improvements in the early screening and the treatment of infectious diseases have resulted in a tremendous decrease in the mortality rate associated with these illnesses. Antibiotics had truly become the ‘‘cure-all’’ of medicine and were being

used to treat even the most common infections, many of these non-bacterial in nature. (Alanis, 2005)

Largely because of penicillin, World War II was the first war in history where more soldiers died from wounds than from disease. Penicillin was described as a wonder drug and it was widely believed that infectious disease would never again be a dominant issue for mankind. Since then numerous antibiotics have been found out against various pathogenic organisms from different sources and considered to be a boon to humans. . (Erik *et al*., 1956)

Sir Alexander Fleming, in an interview with The New York Times in 1945, warned that the inappropriate use of penicillin could lead to the selection of resistant ‘‘mutant forms’’ of *Staphylococcus aureus* that could cause more serious infections in the host or in other people that the host was in contact with and thus could pass the resistant microbe, based on his research. He was right and within one year of the widespread use of this drug a significant number of strains of *Staphylococcus aureus* had become resistant to penicillin. A few years later over 50% were no longer susceptible to this new drug. (Abdallah, 2011)

According to the World Health Organization (WHO), over-prescription and the improper use of antibiotics has led to the generation of antibiotic resistance in many bacterial pathogens. Nowadays, the drug resistant strains of pathogen emerge more quickly than the rate of discovery of new drugs and antibiotics. Because of this, many scientists and pharmaceutical industry have actively involved in isolation and screening of microbes from different habitats, for their production of antibiotics. Serious infections caused by bacteria have become resistant to commonly used antibiotics and become a major global healthcare problem in the 21st century. Hence

there is need to rediscover new drugs active against these drug resistance pathogens. (Hopwood, 2001)

According to the US Centers for Disease Control and Prevention, 1.7 million patients per year in the US acquire an infection while in hospital, resulting in 99,000 (5.8%) deaths. In 1992, deaths from hospital-acquired infections in the US were 13,300, showing a 67% increase over a decade, equivalent to around a 20% annual growth during that time. The CDC also reported that 70% of bacteria responsible for hospital-acquired infections are resistant to at least one of the antibiotics that were once used to treat them. (Nicolas, 2012)

All natural soils contain vast populations of microscopic plants and animals. It has been estimated that within the top one to three feet of soil as much as 17,000 lbs. fungi and 40 lbs. bacteria exist per acre. All the soil microorganisms compete with each other for food and space. Soil bacteria have long been potent antibiotics producers. This comes as no surprise due to their ability to live in a relatively heterogeneous environment: bacteria in soil have to withstand changing, sometimes rather extreme environmental conditions, and are also faced with a broad range of enemies and competitors. (Muntean, 1999)

Actinomycetes are threadlike bacteria that look like fungi. They also produce antibiotics to fight diseases of roots. Many of these same antibiotics are used to treat human diseases. Actinomycetes are responsible for the sweet, earthy smell noticed whenever a biologically active soil is tilled. Antibiotics are the best known products of Actinomycete. Over 5,000 antibiotics have been identified from the cultures of Gram positive and Gram negative organisms, and filamentous fungi, but only about 100 antibiotics have been commercially used to treat human, animal and plant diseases. One of the first antibiotics used is streptomycin produced by *Streptomyces*

grisius. On the whole, the last 55 years have seen the discovery of more than 12,000 antibiotics. The actinomycetes yielded about 70 % of these, and the remaining 30 % are products of filamentous fungi and non actinomycete bacteria. (Kumar *et al.*, 2012)

Considering the need to produce novel antibiotic compound, the present study was conducted to isolate and screen antibiotic producing actinomycetes from the soil sample. Identification of the isolate using 16s rDNA sequencing was done. Purification and characterization of the antimicrobial compound from actinomycetes was also done.

1.2 OBJECTIVES

The objectives of the present study are

1. To isolate and identify Actinomycetes from hospital soil sample.
2. To screen the antimicrobial activity of isolated Actinomycete against G+ organisms.
3. To purify and characterize the antimicrobial compounds

1.3 REVIEW OF LITERATURE

1.3.1 History of antibiotics

The search for antibiotics began in the late 1800s, considering the germ theory of disease. As a result, scientists began spending time for searching new drugs that would kill these disease-causing bacteria. The goal of such research was to find so-called “magic bullets” that would destroy microbes without affecting the person taking the drug. (Connor, 2005)

History of antibiotics can be described in two categories as under

1. Early history
2. Modern history

1.3.1.1 Early history (Hani, 2010)

1. Greeks and Indians used molds and other plants to treat infections.
2. In Greece and Serbia, moldy bread was traditionally used to treat wounds and infections.
3. Warm soil was used in Russia to cure infected wounds.
4. Sumerian doctors gave patients bear soup mixed with turtle shells and snake skins.
5. Babylonian doctors healed the eyes using a mixture of frog bile and sour milk.
6. Sri Lankan army used oil cake which serves both as desiccant and antibacterial.

1.3.1.2 Modern history (Hani, 2010)

YEAR	ORIGIN	DESCRIPTION
1640	England	John Parkington recommended using mold for treatment in his book on Pharmacology
1870	England	Sir John Scott Burdon- Sanderson observed that culture fluid covered with mould did not produce bacteria.
1871	England	Joseph Lister experimented with the antibacterial action on human tissue on what he called *Penicillium glaucium.*
1875	England	John Tyndall explained antibacterial action of *Penicilluim* fungus to the Royal Soceity
1877	France	Louis Pasteur postulated that bacteria could kill other bacteria(*Bacillus anthracis*)
1897	France	Ernest Duchensne healed infected guinea pigs from typhoid using mould (*Penicillium glaucium*)
1928	England	Sir Alexander Fleming discovered enzyme lysozyme and the antibiotic substance penicillin from fungus *Penicillium notatum*
1932	Germany	Gerhard Domagk discovered Sulfonamidochrysoidine(Prontosil)
During 1940’s and 1950’s streptomycin, chloramphenicol and tetracyclin were discovered and Selman Waksman used the term “antibiotics” to describe them (1942)		

Table 1.3.1 Early history of antibiotics

1.3.2 Mode of action

All the antibiotic compounds work in either of the following two ways (Clardy, 2010)

1) A Bactericidal antibiotic kills the bacteria generally by either interfering with the formation of the bacterium's cell wall or its cell contents. Some examples for bactericidal antibiotics are penicillin,

daptomycin, fluoroquinolones, metronidazole, nitrofurantoin and co-trimoxazole.

2) A Bacteriostatic antibiotic stops bacteria from multiplying by interfering with bacterial protein synthesis, DNA replication, or other aspects of the cellular metabolism of bacteria. Examples for bacteriostatic antibiotics are tetracyclines, sulphonamides, spectinomycin, trimethoprim, chloramphenicol, macrolides and lincosamides.

1.3.3 Classification of antibiotics (Moore, 2013)

Antibiotic Grouping by Mechanism	
Cell wall synthesis	Penicillins Cephalosporins Vancomycins Beta-lactamase Inhibitors Carbapenems Aztreonam Polymycin Bacitracin
Protein synthesis inhibitors	Inhibit 30s Subunit Aminoglycosides (Gentamycin) Tetracyclines Inhibit 50s Subunit Macrolides Chloramphenicol Clindamycin Linezolid Stretogrmins
DNA Synthesis Inhibitors	Fluoroquinolones

	Metronidazole
RNA Synthesis inhibitors	Rifampin
Mycolic Acis Synthesis Inhibitors	Isoniazid
Folic Acid Synthesis Inhibitors	Sulfonamides, Trimethoprim

Table 1.3.2 Classificaton of antibiotics

1.3.4 Resistance development of pathogens

Antibiotic resistance is the ability of a microorganism to withstand the effects of an antibiotic. More than 90 years ago, A. Fleming discovered penicillin which has revolutionized the medical field and allowed to treat millions of people against bacterial infections. Unfortunately, the intensive use and misuse of antibiotics have lead to the emergence of resistant strains. (Patricks, 2012).

Other factors contributing for the resistance include incorrect diagnosis, unnecessary prescriptions, improper use of antibiotics by patients, and the use of antibiotics as livestock food additives for growth promotion. Resistant pathogens *Staphylococcus aureus* is one of the major resistant pathogens. MRSA (Methicillin-Resistant *Staphylococcus aureus*) was first detected in Britain in 1961 and is now quite common. MRSA was responsible for 37% of fatal cases of blood poisoning in the UK in 1999, up from 4% in 1991. (Nicolas, 2012)

The molecular mechanisms by which bacteria acquired resistance to antibiotics are diverse and complex. Bacteria have developed resistance to all different classes of antibiotics discovered till date. The most frequent type of resistance is acquired and transmitted horizontally through the conjugation of a plasmid. In recent times new mechanisms of resistance have resulted in the simultaneous development of resistance to several antibiotic classes creating very dangerous multidrug-resistant (MDR) bacterial strains, some also known as ‘‘superbugs’’. (Alfonso, 2005).

Bacterial resistance to antibiotics may be broadly classified into two namely natural resistance and acquired resistance. The bacteria may be inherently resistant to an antibiotic in case of natural resistance. For example, some bacteria have an outer membrane that establishes a permeability barrier against the antibiotic; or an organism lacks a transport system for the antibiotic. In acquired resistance, bacteria can develop resistance to antibiotics in a course of time. Bacterial populations previously-sensitive to antibiotics become resistant are examples. This type of resistance results from changes in the bacterial genome. Acquired resistance is driven by two genetic processes in bacteria: (1) mutation and selection (sometimes referred to as vertical evolution); (2) exchange of genes between strains and species (sometimes called horizontal evolution). (Kenneth Todar, 2000)

1.3.5 Need for the production of new antibiotics

The rapid emergence of antimicrobial resistance among pathogenic organisms has lead to the search for new antimicrobial agents. Severe infections caused by bacteria that are resistant to commonly used antibiotics have become a major health care problem in 21st century. (Alanis, 2005)

Presently, drug resistant pathogens emerge more quickly than the rate of discovery of new drugs and antibiotics. Conditions emerged that it is essential that novel antibiotics to be developed so as to control the emerging drug resistant pathogens. (Projan, 2002)

To survive, bacteria developed antibiotic resistance mechanisms. So, it is not surprising that they have become resistant to most of the naturally occurring antimicrobial agents developed over past 50 years. (Hancock, 2007)

1.3.6 Actinomycetes – a potent antibiotic producer

Soil is the major reservoir of many antibiotic producing organisms. Clinically useful antibiotics have been isolated from several groups of soil microorganisms, including bacteria (*Streptomyces sp., Bacillus sp.*) and fungi (*Penicillium sp. Cephalosporium sp.*). (Ceylan, 2008). Actinomycetes is the most widely distributed group of microorganisms in nature which primarily inhabit the soil (Oskay *et al*, 2004).

Actinomycetes are the most economically and biotechnologically valuable prokaryotes that are responsible for the production of about 50% of the discovered secondary metabolites. Recently the rate of discovery of new compounds from terrestrial actinomycetes has decreased whereas the rate of re-isolation of compounds has increased. (Donia *et al.*, 2003)

Actinomycetes are best known for their ability to produce antibiotics and are gram positive bacteria which comprise a group of branching unicellular microorganisms. They produce branching mycelium which may be of two kinds *viz*., substrate mycelium and aerial mycelium. Among actinomycetes, the streptomycetes

are the dominant. The non‐streptomycetes are called rare actinomycetes, comprising approximately 100 genera. Members of the actinomycetes, which live in marine environment, are poorly understood and only few reports are available pertaining to actinomycetes from mangroves (Siva Kumar, 2001)

Actinomycetes are unparalleled sources of bio active metabolites including antibiotics, plant growth hormones and other substances. (Shahidi *et al.,* 2004). They are primarily recognized as a source for high value metabolites such as antibiotics, antivirals, anticancers, enzymes and recombinant products in which most of the antibiotics are of terrestrial origin. (Balagurunathan, 2010). Almost 80% of the world's antibiotics are known to come from *Actinomycetes*, mostly from the genera *Streptomyces* and *Micromonospora (*Pandey *et al*, 2004).

1.3.7 Solid state fermentation

Among existing technologies in the fermentation industry, Solid State Fermentation (SSF) shows many advantages over fermentation with submerged culture, such as lower cost and much higher reactor volume. The solid state fermentation has shown much promise in the development of several bioprocesses and products. (Pandey *et al.*, 2000)

The application of SSF process has a considerable economical potential in the food, feed, pharmaceutical, and agricultural industries. There are a great number of literatures reported to use the SSF process for producing antibiotics with industrial importance, such as penicillin, oxytetracycline, tetracycline, cephamycin C, cephalosporin C, meroparamycin, rifamycin B, and neomycin. (Vastrad *et al.*, 2011)

The use of SSF technology for the production of secondary metabolites should not be discounted. The mycelia morphology associated with the micro-organisms predominately used for secondary metabolite production was well suited to growth on a solid support. This can also have a detrimental effect on product formation in liquid media, because highly viscous liquid media are required for successful metabolite production and this can interfere with oxygen transfer. The filamentous morphology of these micro-organisms and the secretion of these metabolites into the growth media can increase viscosity further. Therefore, SSF technology can be exploited as an alternative, allowing better oxygen circulation. (Robinson *et al.,* 2001)

1.3.8 Screening for antimicrobial activity

There are a vast number of methodologies used for the determination of antimicrobial activity of natural products. The most popular methods employed among these are

1. Disc/Well diffusion method
2. Agar/Broth dilution method

The agar well diffusion method used was adapted from the punch plate assay for inhibitory substances described in the Microbiology Standard Methods Manual for the New Zealand Dairy Industry. (Hammer *et al.,* 1999)

CHAPTER 2

2.1 MATERIALS AND METHODS

2.1.1 Glassware

Good quality glassware was used for all tests. All the glassware were of brand Borosil. They were washed with good detergent, rinsed in tap water and soaked in chromic acid cleaning solution. (Appendix I)

2.1.2 Chemicals

Analytical grade chemicals supplied by Hi- Media, S.D. Fine Chemicals, Qualigens and Sigma Chemicals were used in this study.

2.1.3 Selection of source for actinomycetes isolation

Hospital soil was selected as sources for the isolation of new strains of actinomycetes since it has been believed that it is rich in microbial population with high competition of survival and drug resistance.

2.1.4 Isolation of Actinomycetes

Soil samples were collected from different areas surrounding the Government hospital, Theni district. Soil samples were air dried under shade conditions for about 2-3 days at room temperature and then they were used for serial dilution for screening of actinomycetes.

2.1.4.1 Serial dilution technique

1. 9ml of sterile saline was taken in a series of labeled test tubes for serial dilution. For serial dilution of soil sample, the first test tube alone contained 10ml of sterile saline.
2. 1g of the soil sample was added to the first tube and mixed well. This gave 10^{-1} dilution.
3. 1ml of 10^{-1} diluted sample was transferred with a fresh pipette to the second tube and mixed well to give 10^{-2} dilution.
4. The procedure was repeated till the desired dilution was obtained.

2.1.4.2 Spread Plate Technique

100 µl aliquots from serially diluted samples were spread aseptically onto the surface of Starch Casein Agar (SCA) (Appendix II) plates using an L –rod. Agar plates were incubated in inverted position. The plates were incubated at 28ºC for 5-6 days.

2.1.5 Isolation of Pure, Single Colony

Quadrants streaking of isolated actinomycetes cells were carried out on SCA plates to isolate single isolated colonies for pure cultures.

2.1.5.1 Quadrant Streaking Technique

1. The inoculating loop was heated to red hot conditions, allowed to cool and used to obtain one loopful of culture.

2. One edge of the petri plate cover was lifted and the first sector was streaked by making as many streaks as possible without overlapping previous streaks.
3. The loop was heated and allowed to cool, after which the plate was turned. Streaking was carried out through one area of the first sector, and then a few times away from the first sector. Similar steps were repeated accordingly to streak the third sector.
4. While streaking the fourth sector, care was taken not to make additional contact with any streaks in the previous sectors.
5. Pure cultures obtained were used for the determination of antimicrobial activity.

2.1.6 Preservation of Isolated Actinomycetes Cultures

The pure isolates were preserved to maintain their viability for further studies. They were preserved in 20% glycerol stocks, in SCA cultured slants overlaid with liquid paraffin and in Agar gel stab with cultures overlaid with liquid paraffin.

2.1.6.1 Glycerol stock

A loopful culture was taken and dispersed in 0.5ml sterile saline. 0.5ml of 80% glycerol was added to the suspension and stored at -20° C.

2.1.6.2 Slant culture

The isolates were streaked on SCA slants and incubated at room temperature for 4-5 days. The isolates were overlaid with liquid paraffin, sealed and stored at 4^{o}C

2.1.6.3 Agar stab

Agar gel (0.3%) was prepared and sterilized. A loopful of culture was inoculated (stabbed) up to 1/3rd of the agar gel. The agar stab was overlaid with liquid paraffin, sealed and stored at 4^{o}C.

2.1.7 Collection of test pathogenic organisms

Seven different test pathogens were collected from Government Medical College, Theni. The pathogens are as follows:

1. *Escherichia coli*
2. *Staphylococcus aureus*
3. *Bacillus subtilis*
4. *Proteus mirabilis*
5. *Pseudomonas aeroginosa*
6. *Klebseilla pneumoniae*
7. *Candida albicans.*

2.1.8 Primary screening for antimicrobial activity

1. McFarland solution (0.5M) was prepared and its turbidity was measured at 680nm and adjusted.

2. The test microbial suspensions were prepared by dispersing a loopful of culture in 2ml sterile saline and its turbidity was adjusted to that of McFarland standard solution.
3. The lawn cultures of test pathogens were prepared on Muller-Hinton agar (MHA) for bacterial cultures and Yeast Extract Peptone Dextrose (YPD) (Appendix- III) agar for yeast culture using sterile cotton swab from the prepared bacterial inoculum and from broth for yeast culture respectively.
4. This was allowed to dry for few minutes. Agar blocks were cut using well puncher and were placed on the lawn culture.
5. The MHA plates were incubated at 37°C overnight and the YPD plates were incubated at room temperature for 2 days.

2.1.9 Culture characterization

The Pure culture of actinomycetes isolates that showed good antimicrobial activity against two different test pathogens was selected and its morphological characteristics was examined in SCA plates and microscopic characteristics were also studied by Gram staining (Appendix- IV). The isolate was sent for 16S rRNA studies to Acme ProGen Biotech Pvt. Ltd., Salem for species identification.

2.1.10 Solid state fermentation for the production of antibiotics

For the better production of antibiotics, solid state fermentation was carried out.

1. Sterile SCA medium with agar percentage less than 10% was prepared and the pH was adjusted to 6.8.

2. Large petri plates (180X40mm) were heat sterilized at 120ºC for 2 hr and the SCA medium was poured on it and allowed to solidify.
3. Required amount of culture from the fresh culture plate was taken and dispersed in sterile distilled water for inoculum preparation.
4. 5ml of the suspension is poured on to the SCA plates and spread through out the plate using sterile L –rod.
5. The plates were then incubated at room temperature for 6 days. After the incubation period, the cells were harvested and extracted using ethyl acetate.

2.1.11 Cell disruption and Solvent extraction

1. 6 days old culture was harvested from the culture plates and it was macerated using mortar and pestle so that all the intracellular metabolites were released.
2. The metabolites were then extracted twice with ethyl acetate as solvent system in 250 ml separating funnel.
3. The organic phase was collected in sterile beaker which was already been weighed and noted.
4. The organic phase was allowed to evaporate in order to concentrate the metabolite present in it.
5. The weight of the beaker after the evaporation of organic phase was also noted so that the concentration of the metabolite can be easily obtained.
6. The metabolite was then dissolved in 100% DMSO and used for further studies.

2.1.12 Collection of different strains of Multi drug resistant *S.aureus*

Three different strains of each *S.aureus* were collected from Kovai Medical Centre Clinical laboratory, Coimbatore.

2.1.13 Secondary screening for antimicrobial activity of fractionated compounds

Secondary screening of antimicrobial activity was done by agar well diffusion method

1. 0.5M McFarland solution was prepared and its turbidity was measured and adjusted at 680nm.
2. The test bacterial suspensions were prepared by dispersing a loopful of culture in 2ml sterile saline and its turbidity was adjusted to McFarland standard solution.
3. The lawn cultures of test pathogens were made on Muller-Hinton agar (MHA) using sterile cotton swab with the prepared bacterial suspensions.
4. This was allowed to dry for few minutes. 9mm wells were cut on the MHA plates containing the test pathogens.
5. 25µl of extracted metabolite which was dissolved in DMSO was added to the wells and DMSO is used as control.
6. The MHA plates were incubated at 37°C overnight.

2.1.14 Thin layer chromatography

1. Thin layer chromatography was used for the separation and standardization of compounds from the ethyl acetate extract.

2. Glass plate (20X10cm) was taken. Silica gel dissolved in water in the ratio 2:1 was applied as a thin layer on to the glass plate with the help of applicator.
3. The plate was heated at 100ºC for 2 hrs to remove moisture so as to activate the plate.
4. The development tank containing the mobile phase (Table 2.1.1) was allowed saturate overnight with a filter paper in it aiding saturation.
5. 30µl of the concentrated sample which was dissolved in DMSO was loaded on the activated plate using micropipette.
6. The plate was immersed in the tank and allowed to develop. Note that the spotted area was not immersed in the mobile phase.
7. The plate was then dried and viewed under iodine vapor chamber.
8. The spot obtained was scraped off and eluted with methanol. The eluted fraction was subjected to further spectral analysis.

S.NO	MOBILE PHASE SYSTEM	RATIO
1	Ethyl acetate/ Methanol	6:4
2	n-Butanol/Acetic acid/Water	4:1:5
3	Methanol/ dichloromethane/ water	1:1:1
4	Chloroform/ Methanol	4:1
5	Hexane/Ethyl acetate	95:5

Table 2.1.1 Different mobile phase system used for the separation of bioactive compound using TLC

2.1.15 Fourier Transform Infra Red Spectroscopy (FTIR)

FTIR was carried out to identify the functional groups of the compounds present in the sample. Simultaneous measurement of various frequencies is possible in FTIR which is lack in conventional IR spectroscopy. It works based on Michelson interferometer. FTIR analysis was done in Central Research laboratory, PSG College of Arts and Science, Coimbatore.

2.1.16 Gas Chromatography and Mass spectroscopy (GC-MS)

The partially purified active compound was analyzed by gas chromatography- mass spectroscopy. The GC-MS analysis was done in central research laboratory, PSG College of arts and science, Coimbatore.

Equipment : Thermo GC-MS DSQ II
Column : DB 5 - MS Capillary Standard Non- Polar Column
Type of ionization : Electron impact ionization method
Carrier gas : He
Flow rate : 1.0 ml/min
Injection volume : 1µl.
Temperature : Oven temperature 70ºC rose to 260ºC at 6ºC per min.
Database : Trace Ultra Search

CHAPTER 3

3.1 RESULTS AND DISCUSSION

3.1.1 Isolation of Actinomycetes from soil sample

A variety of microbial colonies were observed on Starch Casein Agar plates after 5-6 days of incubation, of serially diluted hospital soil sample that was spread plated.

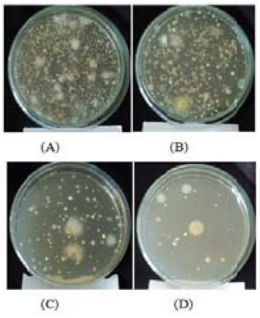


Figure 3.1.1 (A) Starch Casein Agar Plate displaying isolated microbes at 10^{-2} dilution
(B) Starch Casein Agar Plate displaying isolated microbes at 10^{-3} dilution
(C) Starch Casein Agar Plate displaying isolated microbes at 10^{-4} dilution
(D) Starch Casein Agar Plate displaying isolated microbes at 10^{-5} dilution

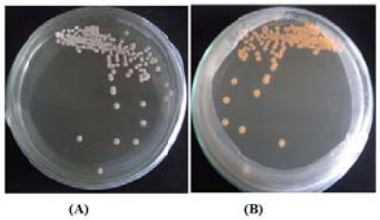
3.1.2 Primary screening for antimicrobial activity

Among 19 isolates that were isolated from soil sample, only one isolate showed good antimicrobial activity against the test pathogenic microbes indicated by a clear zone of inhibition. The isolate HSSA 09 showed higher inhibition towards two Gram positive test pathogens- *S.aureus* and *B.subtilis.*

Figure 3.1.2 Effect of HSSA 09 on the growth of *S.aureus* and *B.subtilis*

3.1.3 Identification of isolate HSSA 09

3.1.3.1 Macroscopic characterization (Colony Morphology)



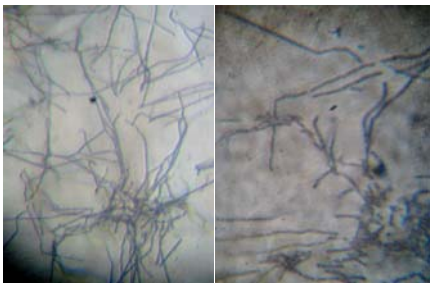
(A) (B)

Figure 3.1.3 (A) Isolated colonies of pure HSSA 09
(B) Reverse side appearance of HSSA 09

The macroscopic characterization the cells showed that the isolate is small circular, entire, opaque, crateriform, rough, brittle pinpoint colonies with white aerial mycelium and orange substrate mycelium.

3.1.3.2 Microscopic characterization

The isolate HSSA 09 was subjected to Gram staining and it reveals that the organism is a Gram positive organism. The unstained cells of the isolates HSSA 09 was observed under bright field microscopy. The cells showed mycelia growth.



(A) (B)

Figure 3.1.4 (A) cells at 40X magnification
(B) cells at 100X magnification

3.1.3.3. 16s rRNA sequencing for the identification of the isolate HSSA 09

The given sample sequence showed 99% sequence coverage and 99% homology with *Streptomyces cinereoruber* subsp, *cinereoruber* strain JCM 4205 (ACCESSION: NR_043345.1) in similarity search with BLAST programme. Therefore, this isolate may be *Streptomyces cinereoruber*

PCR primer set

5’AGAGTTTGATCTGGCTCAG 3’

5’ TACGGTACCTTGTTACGACTT 3’

Sequencing primer set

5’CCAGCAGCCGCGGTAATACG 3’

5’ TACCAGGGTATCTAATCC 3’

File: SSA09_518F.ab1 Signal G: 5516 A: 5514 C: 6433 T: 4592
Sample: SSA09_518F Lane: 40 Base spacing: 15.03375
842 bases in 10174 scans

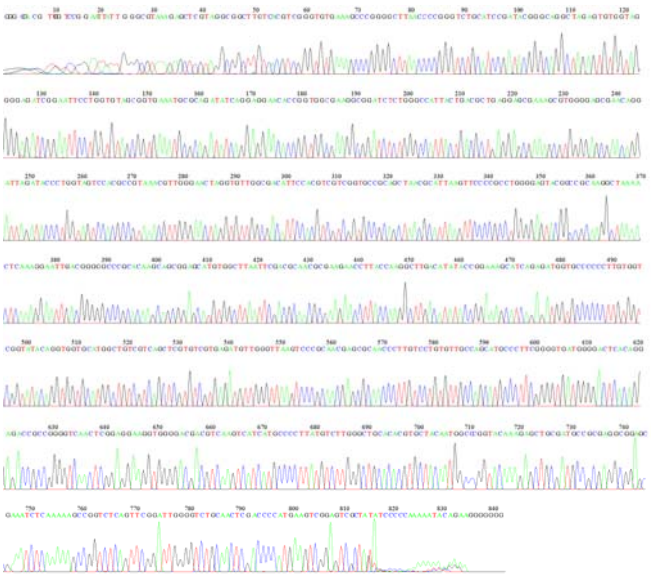


Figure 3.1.5 Sequence chromatogram of forward primer

File: SSA09_800R.ab1 Signal G: 6806 A: 6779 C: 13475 T: 7048

Sample: SSA09_800R Lane: 38 Base spacing: 15.226344

770 bases in 9316 scans

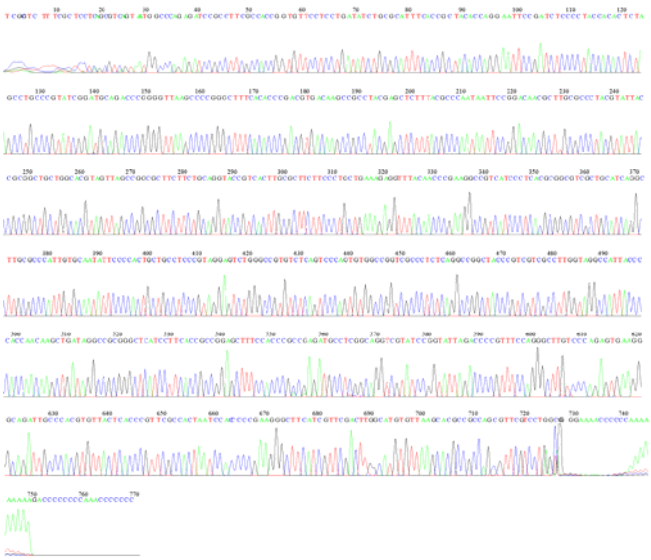


Figure 3.1.6 Sequence chromatogram of reverse primer

The genome of the actinomycete isolated from soil sample was subjected to BLAST analysis and the following reports were obtained, which showed that the isolated yeast was *Streptomyces cinereoruber*.

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Streptomyces cinereoruber subsp. cinereoruber strain JCM 4205 16S ribosomal RNA, partial sequence	2359	2359	99%	0.0	99%	NR_043345.1
Streptomyces violaceoruber strain NBRC 13102 16S ribosomal RNA, partial sequence	2342	2342	99%	0.0	99%	NR_041114.1
Streptomyces hirsutus strain NRRL B-2713 16S ribosomal RNA, partial sequence	2340	2340	100%	0.0	99%	NR_043819.1
Streptomyces viridobrunneus strain LMG 20317 16S ribosomal RNA, complete sequence	2326	2326	99%	0.0	99%	NR_042308.1
Streptomyces showdoensis strain NBRC 13417 16S ribosomal RNA, partial sequence	2313	2313	99%	0.0	99%	NR_041129.1
Streptomyces bikiniensis strain DSM 40581 16S ribosomal RNA, complete sequence	2311	2311	100%	0.0	99%	NR_026177.1
Streptomyces nashvilliensis strain NBRC 13064 16S ribosomal RNA, partial sequence	2309	2309	99%	0.0	99%	NR_041106.1
Streptomyces zaomyceticus strain NRRL B-2038 16S ribosomal RNA, partial sequence	2285	2285	100%	0.0	98%	NR_044144.1
Streptomyces lilmocidini strain NBRC 12792 16S ribosomal RNA, partial sequence	2285	2285	99%	0.0	99%	NR_041069.1
Streptomyces filamentosus strain NBRC 12767 16S ribosomal RNA, partial sequence	2285	2285	99%	0.0	98%	NR_041064.1
Streptomyces venezuelae strain JCM 4526 16S ribosomal RNA, partial sequence	2279	2279	100%	0.0	98%	NR_024764.1
Streptomyces omiyaensis strain NRRL B-1587 16S ribosomal RNA, partial sequence	2278	2278	99%	0.0	98%	NR_044150.1
Streptomyces werraensis strain NRRL B-5317 16S ribosomal RNA, partial sequence	2278	2278	99%	0.0	98%	NR_043846.1
Streptomyces termitum strain NBRC 13087 16S ribosomal RNA, partial sequence	2278	2278	99%	0.0	98%	NR_041112.1
Streptomyces laurentii strain LMG 19959 16S ribosomal RNA, complete sequence	2276	2276	99%	0.0	98%	NR_042299.1
Streptomyces roseolus strain NBRC 12816 16S ribosomal RNA, partial sequence	2270	2270	98%	0.0	99%	NR_041076.1
Streptomyces lateritius strain LMG 19372 16S ribosomal RNA, complete sequence	2270	2270	99%	0.0	98%	NR_042293.1
Streptomyces yerevanensis strain NRRL B-16943 16S ribosomal RNA, partial sequence	2268	2268	100%	0.0	98%	NR_044143.1
Streptomyces enissocaesilis strain NBRC 100763 16S ribosomal RNA, partial sequence	2265	2265	99%	0.0	98%	NR_041411.1
Streptomyces exfoliatus strain NBRC 13191 16S ribosomal RNA, partial sequence	2265	2265	99%	0.0	98%	NR_041117.1
Streptomyces roseofulvus strain NBRC 13194 16S ribosomal RNA, partial sequence	2263	2263	98%	0.0	98%	NR_041120.1
Streptomyces gultbargensis strain DAS131 16S ribosomal RNA, partial sequence	2263	2263	99%	0.0	98%	NR_043714.1
Streptomyces peucetius strain JCM 9920 16S ribosomal RNA, partial sequence	2242	2242	100%	0.0	98%	NR_024763.1
Streptomyces griseus subsp. griseus NBRC 13350 strain NBRC 13350 16S ribosomal RNA, complete sequence	2241	2241	100%	0.0	98%	NR_074787.1
Streptomyces xanthochromogenes strain NRRL B-5410 16S ribosomal RNA, partial sequence	2241	2241	100%	0.0	98%	NR_043847.1
Streptomyces spiroverticillatus strain NBRC 3931 16S ribosomal RNA, partial sequence	2241	2241	99%	0.0	98%	NR_041214.1
Streptomyces lavendofoliae strain LMG 19935 16S ribosomal RNA, complete sequence	2241	2241	99%	0.0	98%	NR_042297.1
Streptomyces luridiscabiei strain S63 16S ribosomal RNA, complete sequence	2241	2241	100%	0.0	98%	NR_025155.1
Streptomyces toxytricini strain NRRL B-5426 16S ribosomal RNA, partial sequence	2239	2239	99%	0.0	98%	NR_043839.1
Streptomyces gelaticus strain NRRL B-2928 16S ribosomal RNA, partial sequence	2239	2239	99%	0.0	98%	NR_043488.1
Streptomyces gobitricini strain NBRC 15419 16S ribosomal RNA, partial sequence	2237	2237	99%	0.0	98%	NR_041183.1
Streptomyces bacillaris strain NBRC 13487 16S ribosomal RNA, partial sequence	2237	2237	99%	0.0	98%	NR_041146.1
Streptomyces katrae strain NBRC 13447 16S ribosomal RNA, partial sequence	2237	2237	99%	0.0	98%	NR_041136.1
Streptomyces fulvorobeus strain NBRC 15897 16S ribosomal RNA, partial sequence	2235	2235	99%	0.0	98%	NR_041196.1
Streptomyces cinnamonensis strain NBRC 15873 16S ribosomal RNA, partial sequence	2235	2235	99%	0.0	98%	NR_041194.1
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Streptomyces michiganensis strain NBRC 12797 16S ribosomal RNA, partial sequence	2233	2233	99%	0.0	98%	NR_041071.1
Streptomyces drozdowiczii strain NBRC 101007 16S ribosomal RNA, partial sequence	2231	2231	99%	0.0	98%	NR_041424.1
Streptomyces sanglieri strain NBRC 100784 16S ribosomal RNA, partial sequence	2231	2231	99%	0.0	98%	NR_041417.1
Streptomyces spororaveus strain LMG 20313 16S ribosomal RNA, complete sequence	2231	2231	99%	0.0	98%	NR_042306.1
Streptomyces nojiriensis strain LMG 20094 16S ribosomal RNA, complete sequence	2231	2231	99%	0.0	98%	NR_042303.1
Streptomyces sp. SirexAA-E strain SirexAA-E 16S ribosomal RNA, complete sequence	2230	2230	100%	0.0	98%	NR_074561.1
Streptomyces clavifer strain NRRL B-2557 16S ribosomal RNA, partial sequence	2230	2230	100%	0.0	98%	NR_043507.1
Streptomyces atratus strain NRRL B-16927 16S ribosomal RNA, partial sequence	2230	2230	100%	0.0	98%	NR_043490.1
Streptomyces cinnamoneus strain NBRC 12852 16S ribosomal RNA, partial sequence	2230	2230	99%	0.0	98%	NR_041223.1
Streptomyces crystallinus strain NBRC 15401 16S ribosomal RNA, partial sequence	2230	2230	99%	0.0	98%	NR_041177.1
Streptomyces avidinii strain NBRC 13429 16S ribosomal RNA, partial sequence	2230	2230	99%	0.0	98%	NR_041132.1
Streptomyces melanogenes strain NBRC						

12890 16S ribosomal RNA, partial sequence	2230	2230	99%	0.0	98%	NR_041089.1
Streptomyces cirratus strain NRRL B-3250 16S ribosomal RNA, partial sequence	2230	2230	100%	0.0	98%	NR_043356.1
Streptomyces finlayi strain NRRL B-12114 16S ribosomal RNA, partial sequence	2230	2230	100%	0.0	98%	NR_043354.1
Streptomyces goshikiensis strain NRRL B-5428 16S ribosomal RNA, partial sequence	2228	2228	99%	0.0	98%	NR_044147.1
Streptomyces xanthophaeus strain NRRL B-5414 16S ribosomal RNA, partial sequence	2228	2228	99%	0.0	98%	NR_043848.1
Streptomyces virginiae strain NBRC 12827 16S ribosomal RNA, partial sequence	2228	2228	99%	0.0	98%	NR_041078.1
Streptomyces flavogriseus strain CBS 101.34 16S ribosomal RNA, partial sequence	2226	2226	99%	0.0	98%	NR_028988.1
Streptomyces mutomycini strain NBRC 100999 16S ribosomal RNA, partial sequence	2226	2226	99%	0.0	98%	NR_041421.1
Streptomyces noboritoensis strain NBRC 13065 16S ribosomal RNA, partial sequence	2226	2226	99%	0.0	98%	NR_041107.1
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Streptomyces tanashiensis strain IFO 12919 16S ribosomal RNA, partial sequence	2224	2224	98%	0.0	98%	NR_043369.1
Streptomyces sporoverrucosus strain NRRL B-16379 16S ribosomal RNA, partial sequence	2222	2222	100%	0.0	97%	NR_043837.1
Streptomyces nitrosporeus strain NRRL B-1316 16S ribosomal RNA, partial sequence	2222	2222	99%	0.0	97%	NR_044140.1
Streptomyces atroolivaceus strain :LMG 19306 16S ribosomal RNA, complete sequence	2220	2220	99%	0.0	97%	NR_042289.1
Streptomyces halstedii strain NRRL B-1238 16S ribosomal RNA, partial sequence	2218	2218	99%	0.0	97%	NR_044148.1
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Streptomyces eurocidicus strain NRRL B-1676 16S ribosomal RNA, partial sequence	2218	2218	100%	0.0	97%	NR_043355.1
Streptomyces cremeus strain JCM 4362 16S ribosomal RNA, partial sequence	2217	2217	99%	0.0	98%	NR_043340.1
Streptomyces subrutilus strain DSM 40445 16S ribosomal RNA, complete sequence	2217	2217	100%	0.0	97%	NR_026203.1
Streptomyces flavidovirens strain NBRC 13039 16S ribosomal RNA, partial sequence	2215	2215	99%	0.0	97%	NR_041099.1
Streptomyces flavovirens strain NRRL B-2685 16S ribosomal RNA, partial sequence	2215	2215	100%	0.0	97%	NR_043487.1
Streptomyces hiroshimensis strain NBRC 3720 16S ribosomal RNA, partial sequence	2215	2215	99%	0.0	98%	NR_041211.1
Streptomyces vinaceus strain NBRC 13425 16S ribosomal RNA, partial sequence	2215	2215	99%	0.0	98%	NR_041131.1
Streptomyces griseoplanus strain AS 4.1868 16S ribosomal RNA, partial sequence	2215	2215	98%	0.0	98%	NR_043377.1

Streptomyces cinereoruber subsp. cinereoruber strain JCM 4205 16S ribosomal RNA, partial sequence
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Range 1: 1 to 1290

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```


3.1.4 Secondary screening for antimicrobial activity by agar well diffusion method

The isolated *Streptomyces cinereoruber* exhibited good antimicrobial activity and it was subjected to solid state fermentation. After fermentation the cells were harvested and extracted using ethyl acetate. The extract was then concentrated by allowing it to dry. The dried secondary metabolite was then dissolved in DMSO and its activity against test pathogens was tested.

Figure 3.1.7 Activity of ethyl acetate extract dissolved in DMSO against *S.aureus* and *B.subtilis*

The ethyl acetate extract dissolved in DMSO showed good inhibitory effect against test pathogens except SSC 19. DMSO was used as negative control and Streptomycin disc was used as positive control. DMSO does not have any inhibitory effect on the test pathogens. Hence it was clear that the secondary metabolites extracted using ethyl acetate was responsible for the inhibitory action. The zone of inhibition against *S.aureus* by the secondary metabolites from *Streptomyces* sp. was found to be 19mm while Singh *et al.*, 2012 reported a zone of inhibition of 14 mm.

G+ Microbes	Zone of inhibition (mm)
S.aureus MTCC	19
MRSA	15
MLSB	18
Acquired penicillinase(*S.aureus*)	21
B.Subtilis MTCC	20
SSC 19	-
SSC 06	15

Table 3.1.1 Effect of *Streptomyces cinereoruber* against G+ microbes

4.4 Separation of crude extract by thin layer chromatography

The ethyl acetate extract of *Streptomyces cinereoruber* was taken for separation and characterization. Thin layer chromatography was carried out different solvent systems. The separation was good in the hexane/ ethyl acetate solvent system. The Rf value for the ethyl acetate extracted fraction dissolved in DMSO was found to be 0.55 whereas the Rf value for the antimicrobial compound from *Streptomyces sp*. reported by Matheiu *et al.,* 2011 was 0.44

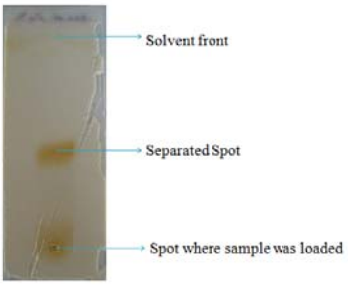


Figure 3.1.8 TLC plate showing the spot separated from the extract with Rf value 0.55

Fourier Transform Infra Red Spectrometry

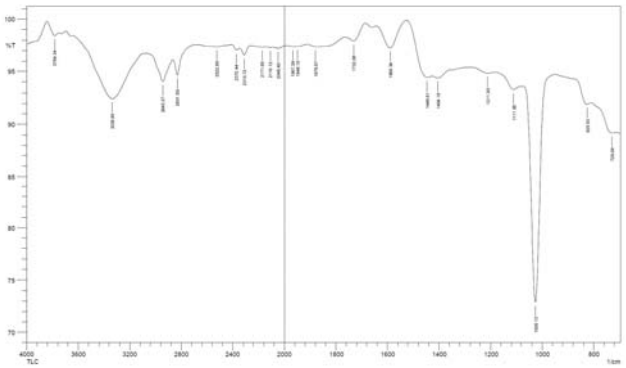


Figure 3.1.9 IR spectrum of sample separated from TLC

S.No	Major IR Frequency	Functional groups
1	3336.85	OH stretch
2	2943.37	OH stretch of carboxylic acid
3	2831.50	C-H stretch of C=O
4	1732.08	C=O stretch
5	1589.34	N-H bend
6	1026.13	C-O stretch

Table 3.1.2 Major peaks from IR spectrum

The FT-IR spectrum of the sample separated from *Sterptomyces cinereoruber* showed absorption at 3336.85 cm-1 which indicates the hydroxyl group, peaks at 2943.37 cm-1(OH stretch) and at 2831.50 cm-1(C-H stretch). It also showed absorption at 1732.08 cm-1(C=O stretch) and peaks at 1589.34 cm-1(N-H bend) and 1026.13 cm-1(C-O stretch).

Based on the IR spectrum pattern obtained, we conclude that the compound in the sample may be single amino acids, peptides or proteins.

4.5 Gas Chromatography Mass spectroscopy (GC-MS)

GC-MS is a sensitive technique used to purify the sample and to determine the chemical composition of the compound. Compounds which are volatile can be separated by GC and its chemical nature can be identified by Mass spectrometer coupled with GC.

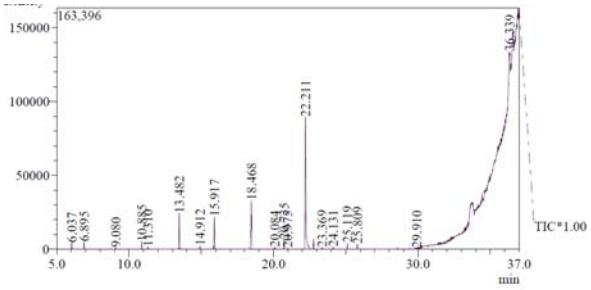


Figure 3.1.10 Gas chromatogram of the fraction obtained from TLC

Nineteen major peaks were obtained at various retention times. Hence the compound was not a pure compound. It was a mixture of various compounds with six major compounds based on the area percentage. They were represented in the following table.

S.No	Compound name	Retention time	Area percentage
1	Pentadecane	6.037	1.16
2	1- Methyl butyl nitrite	6.895	1.62
3	Oxirane, (bromomethyl)-	9.080	0.29
4	Undecane, 5- methyl-	10.885	1.37
5	Oxirane, 2,2'-oxybis(methylene)	11.310	0.49
6	Undecane	13.482	8.05
7	Disulfide, Dioctyl	14.912	0.56
8	Undecane, 4,7- dimethyl-	15.917	9.53
9	Octadecane	18.468	8.16
10	Isoxazolidine	20.084	0.93
11	(R,R)-3,8- dimethyldecane	20.735	2.50
12	Sulfurous acid, dibutyl ester	20.975	0.08
13	Benzoic acid, phenyl methyl ester	22.211	43.30
14	Isoxazolidine	23.369	0.39
15	2- propanone, 1-phenyl-, oxime	24.131	-0.37
16	Octanoic acid, methyl ester	25.119	0.91
17	2-(((carbobenzyloxy)amino)methane)	25.809	1.49
18	p- Mentha-6,8-dien-2-one, semicarbazone	29.910	0.42
19	Stigmasta-5, 22-dien-3-ol, acetate	36.339	19.12

Six major peaks with high area percentage were considered for the analysis. They were obtained at retention times 22.211, 29.910, 15.917, 18.468, 13.482 and 20.735 respectively corresponding to the area percentage.

Mass spectrum at R_t 22.211 minutes

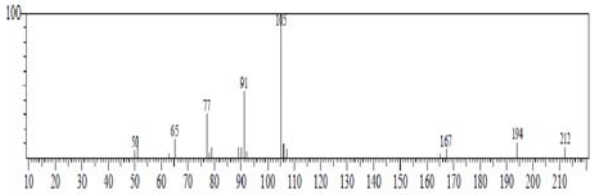
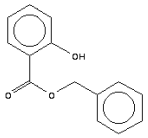


Figure3.1.11 Mass spectrum of unknown compound at R_t 22.211 minutes

m/z 212 corresponds to molecular ion or parent ion peak and 105 was the base peak. The pattern was matched with benzyl benzoate based on NIST library search. Benzyl benzoate, the simplest aromatic compounds. It is used as one of the principle antimicrobial preservative. Shankar *et al.*, 2012 reported that benzyl benzoate was active against Gram positive bacteria but not against Gram negative bacteria.

The structure of Benzyl benzoate



Mass spectrum at R_t 36.339 minutes

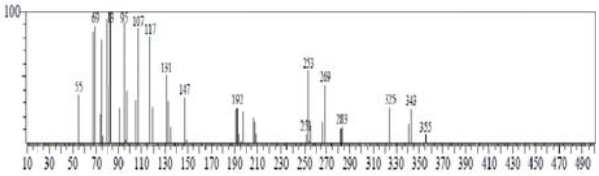
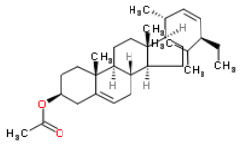


Figure3.1.12 Mass spectrum of unknown compound at R_t 36.339 minutes

m/z 355 corresponds to molecular ion or parent ion peak and 83 was the base peak. The pattern was matched with Stigmasta-5, 22-dien-3-ol acetate based on NIST library search. Stigmasterol is an unsaturated sterol which is used as a precursor in manufacturing semi synthetic progesterone. Sugumaran et al., 2012 reported that stigmasterol acetate possess some antimicrobial activity and found to be active against *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Escherischia coli.*

The structure of Stigmasta-5, 22-dien-3-ol acetate (Stigmasterol acetate)



Mass spectrum at R_t 15.917 minutes

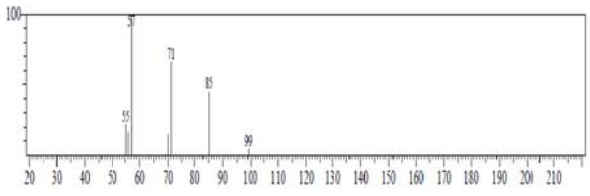
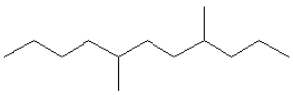


Figure3.1.13 Mass spectrum of unknown compound at R_t 15.917 minutes

m/z 99 corresponds to molecular ion or parent ion peak and 57 was the base peak. The pattern was matched with Undecane, 4, 7- dimethyl based on NIST library search.

The structure of Undecane, 4, 7- dimethyl



Mass spectrum at R_t 18.468 minutes

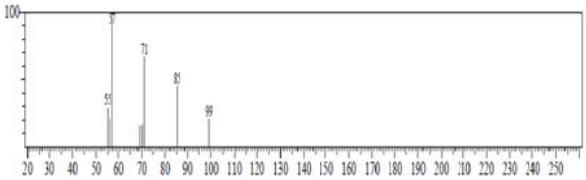


Figure3.1.14 Mass spectrum of unknown compound at R_t 18.468 minutes

m/z 99 corresponds to molecular ion or parent ion peak and 57 was the base peak. The pattern was matched with Octadecane based on NIST library search.

The structure of Octadecane

Mass spectrum at R_t 13.482 minutes

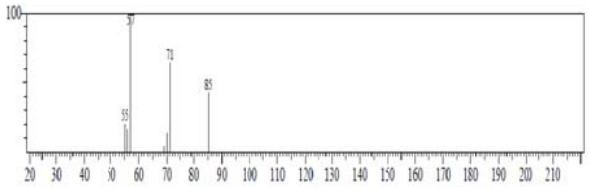


Figure3.1.15 Mass spectrum of unknown compound at R_t 13.482 minutes

m/z 85 corresponds to molecular ion or parent ion peak and 57 was the base peak. The pattern was matched with Undecane based on NIST library search.

The structure of Undecane

Mass spectrum at R_t 20.735 minutes

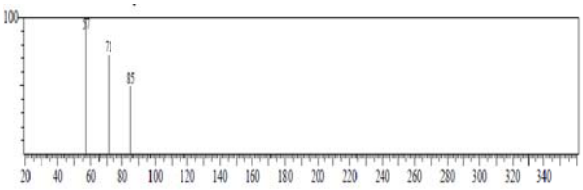
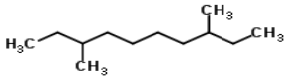


Figure3.1.16 Mass spectrum of unknown compound at R_t 20.735 minutes

m/z 85 corresponds to molecular ion or parent ion peak and 57 was the base peak. The pattern was matched with (R, R)-3, 8-dimethyldecane based on NIST library search.

The structure of (R, R)-3, 8-dimethyldecane



Hence the bioactive compound isolated from the *Streptomyces cinereoruber* was not a pure compound. The TLC eluted fraction which was further analyzed for the presence of functional groups and the chemical composition using GC –MS and FT –IR revealed that the compounds may be single amino acids, peptides are proteins. The GC –MS data obtained was slightly similar to that of the results obtained by Radulovi *et al*., 2010. Benzyl benzoate and stigmasterol are the two major compounds present in the sample and both the compounds are known to have good antimicrobial activity.

3.2 CONCLUSION

Novel antibiotic production from micro organisms is of great significance nowadays because of the existence of various drug resistant pathogens. Initially 19 different isolates were isolated from the hospital soil sample and screened for antimicrobial activity against seven different test pathogens. One promising isolate HSSA 09 showed maximum inhibition against two test pathogens *namely S.aureus and B.subtilis*.

The isolate was identified morphologically and microscopically and was found that it was Gram positive rod with mycelia growth. 16S rRNA gene sequencing was also done and the results revealed that the isolate HSSA 09 may be *Streptomyces cinereoruber*. Solid state fermentation was done for the mass production of antibiotic since submerge state fermentation was not suitable for the production. The cells were then harvested and extracted using ethyl acetate. The ethyl acetate extracted fraction dissolved in DMSO was used for the secondary screening by agar well diffusion method.

The bioactive compound was separated using thin layer chromatographic techniques and a single spot was obtained with Rf value 0.55. The TLC eluted fraction was subjected to GC –MS and FT –IR analysis. From the IR spectrum obtained, we may conclude that the compound may be single amino acid, peptide or protein. From the Gas chromatogram obtained, it is clear that the compound is not in pure form. It is a mixture of nineteen different compounds. The fragmentation pattern was matched with Benzyl benzoate and stigma sterol. Both the compounds are known to have good antimicrobial activity. Hence, it may be concluded that the partially purified compound was responsible for the inhibitory effect against Gram positive organisms. Isolation of pure metabolite must be done further in order to find out the exact compound responsible for the inhibitory effect.

3.3 APPENDICES

3.3.1 Appendix I

Acid Cleaning Solution

Potassium dichromate 400.0 g
Distilled water 4000.0 ml
Sulfuric acid 400.0 ml

Potassium dichromate was dissolved in water and was poured into crock. Sulphuric acid was slowly added in to the crock.

3.3.2 Appendix II

The composition of Starch Casein Agar Medium is as follows

For 1000ml of medium:

Soluble starch	- 10.0g
Potassium Ortho Phosphate	- 2.0g
Potassium Nitrate	- 2.0g
Sodium Chloride	- 2.0g
Casein	- 0.3g
Magnesium Sulphate	- 0.05g
Calcium Carbonate	- 0.02g
Ferrous Sulphate	- 0.01g
Agar	- 15.0g

3.3.3 Appendix III

The composition of Yeast Extract Peptone Dextrose Medium is as follows

For 1000ml of medium:

Peptic digest of animal tissue - 20g
Yeast extract - 10g
Dextrose - 20g
Agar - 20g

3.3.4 Appendix IV

Gram Staining

1. Placed a drop of saline on a microscope slide.
2. Immersed the specimen/material in the drop of saline.
3. Air dried and heat fixed. Added 2 drops of Crystal violet stain and left for 30 seconds.
4. Crystal Violet stain was rinsed off with distilled water.
5. Grams iodine was added on the smear and left for one minute, after which it was rinsed off with distilled water.
6. 10 to 15 drops of 95% ethanol was added and left for 10 to 15 seconds after which it was rinsed off with distilled water.
7. Saffranin stain was added on the slide and left for 45 seconds after which the slide was rinsed with distilled water.
8. The slide was air- dried and observed under the microscope.

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