EXPLOITATION OF SOIL AND RHIZOSPHERE INHABITING FUNGAL SPECIES FOR BIOLOGICALLY ACTIVE SECONDARY METABOLITES

Ph. D Thesis

By
Saeed Ullah Khattak

CENTRE OF BIOTECHNOLOGY AND MICROBIOLOGY UNIVERSITY OF PESHAWAR
2015

IN THE NAME OF ALLAH, THE MOST GRACIOUS, THE MOST MERCIFUL

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A thesis submitted to the University of Peshawar for the fulfillment of the requirements for the degree of

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Saeed Ullah Khattak

AUTHOR'S DECLARATION

It is declared that the overall research work presented in this thesis is the original work conducted by the author in accordance with University of Peshawar's regulations for the requirements of Research Degree Program. This is the author's original work and has never been previously submitted elsewhere for any kind of academic award. The views expressed in the thesis, belongs to the author.

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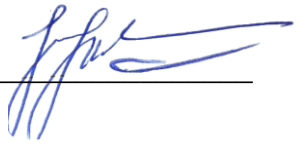
Signature 

DEDICATION

THIS HUMBLE EFFORT IS DEDICATED TO

MY PARENTS (LATE) AND MY FAMILY

AS

WITHOUT THEIR SUPPORT IT WOULD

NEVER BE POSSIBLE FOR ME

TO COMPLETE THIS TASK

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

µg	=	Microgram
µl	=	Microlitre
^{o}C	=	Degree Celsius
ACP	=	Acyl Carrier Protein
ACP	=	Acyl Carrier Proteins
ACV	=	Aminoadipate-L-Cys-D-Val synthase
AM toxin	=	Toxin produced by *Alternaria* species originally isolated from *A. mali*
AT	=	Acyl transferase
CAMRSA	=	Community Acquired Methicillin Resistant *Staphylococcus aureus*
CCT	=	Column Chromatography Technique
CDA	=	Czapec Dox Agar
CFU	=	Colony Forming Unit
CGTB	=	Crown Gal Tumor Bioassay
Cm	=	Centimeter
COSY	=	Correlation Spectroscopy
CRD	=	Completely Randomized Design
CYA	=	Czapec Yeast Agar
CYB	=	Czapec Yeast Broth
CYC	=	Cyclase
Da	=	Dalton
DEPT	=	Distortion less Enhancement by Polarization Transfer
DH	=	Dehydratase

DMSO	=	Di-Methyl Sulfa Oxide
EI	=	Electron Ionization
ER	=	Enoyl Reductase
EU	=	European Union
FASs	=	Fatty Acid Synthases
FDA	=	United States Food and Drug Administration
FID	=	Free Induction Decay
FTIR	=	Fourier Transform Infrared
GYA	=	Glycerol Yeast Agar
HC toxin	=	Toxin produced by *Cochliobolus carbonum*
HCC	=	Human Colon Carcinoma
HCl	=	Hydrochloric Acid
HLC	=	Human Lung Carcinoma
HMBC	=	Hetronuclear Multiple Quantum Coherence
HPLC	=	High Performance Liquid Chromatography
HPTLC	=	High Performance Thin Layer Chromatography
Hrs	=	Hours
HSQC	=	Hetronuclear Single Quantum Coherence
IEC	=	Ion Exchange Chromatography
IR	=	Infrared Spectroscopy
Kg	=	Kilogram
KR	=	Keto reductase
KS	=	ketoacyl CoA synthase

LCMS	=	Liquid Chromatography Mass Spectrometry
LFH	=	Laminar Flow Hood
m	=	Meter
M	=	Molar
MALDI	=	Matrix Assisted Laser Desorption Ionization
MIC	=	Minimum Inhibitory Concentration
mL	=	Milliliter
Mm	=	Millimeter
MRSA	=	Methicillin Resistant *Staphylococcus aureus*
MS	=	Mass Spectrometry
MT	=	Methyl transferase
NA	=	Nutrient Agar
NMR	=	Nuclear Magnetic Resonance
NOE	=	Nuclear Overhauser Effect
NPC	=	Normal Phase Chromatography
NP-HPLC	=	Normal Phase High Performance Liquid Chromatography
NRPs	=	Non Ribosomal Peptides
NRPSs	=	Non Ribosomal Peptide Synthases
PCR	=	Polymerase Chain Reaction
PDA	=	Potato Dextrose Agar
PKSs	=	Polyketide Synthases
Rf	=	Radio Frequency
RNA	=	Ribonucleic Acid

RPC	=	Reverse Phase Chromatography
RPM	=	Rotations Per Minute
SDA	=	Saboraud Dextrose Agar
SEC	=	Size Exclusion Chromatography
SFB	=	Star Fish Bioassay
SUB	=	Sea Urchin Bioassay
TE	=	Thioesterase
TLC	=	Thin Layer Chromatography
US	=	United States
UTIs	=	Urinary Tract Infections
UV	=	Ultra Violet
VA	=	Vesicular Arbuscular
WA PKS	=	A napthopyrone Polyketide Synthase of *Aspergillus nidulans*

ABSTRACT

In quest for new bioactive compounds the present study deals with isolation, identification and bioassay screenings of fungi and purification and structural elucidation of pure compounds isolated from fungal strains under study. For production of fungal bioactive secondary metabolites fungal strains were isolated from soil and rhizosphere of *Mentha piperita* using Potato Dextrose Agar (PDA) and Sabouraud Dextrose Agar (SDA) media. After preliminary antimicrobial testing, two fungal strains were selected for metabolites production, their bioassays and subsequent structural characterization. The rhizospheric strain was identified as *Aspergillus* specie while soil isolate was identified to be *Penicillium* specie. For secondary metabolites production both fungal strains were cultured in Czapec Yeast Broth (CYB) medium at 28 ^{o}C for 14 days at 150 rpm. Crude secondary metabolites produced by fungal strains were then extracted and tested for their respective bioassays. In our study fungal metabolites were tested for five different activities i.e. antibacterial, antifungal, cytotoxic, phytotoxic and herbicidal activities.

In antibacterial bioassay crude extracts of *Aspergillus* and *Penicillium* species were tested against eight pathogenic bacterial strains i.e. *Xanthomonas oryzae, Klebsiella pneumonia, Bacillus subtilus, Proteus vulgaris, Staphylococcus aureus, Escherichia coli, Salmonella typhi* and *Shigella flexeneri*. A dose concentration from 1 to 500 µL mL^{-1} of the crude extracts was tested against pathogenic bacterial strains and zone of inhibition was recorded in millimeters (mm). A dose concentration of 500 µL mL^{-1} of ethyl acetate extract of *Aspergillus* sp. inhibited growth of all test bacterial strains but the highest zones of inhibition were recorded against *B. subtilus* (47.5 mm) and *S. flexeneri* (45 mm) while lowest zone of inhibition was recorded against *X. oryzae* (9 mm). At 500 µg/mL of ethyl acetate extract of *Penicillium* sp growth of *P.*

vulgaris, *E. coli, S. aureus, B. subtilus* and *S. flexeneri* was inhibited by forming zones of 34, 28, 24, 23 and 21 mm respectively. Lowest zone of inhibition was recorded against *S. typhi* (9.5 mm) while considerable inhibition was noted against *K. pneumoniae* (16 mm) and *X. oryzae* (13 mm) at 500 µL mL^{-1} of *Penicillium* sp. extract.

Antifungal activities were tested against six different pathogenic fungal strains i.e. *Fusarium solani, Aspergillus flavus, Microsporium canis, Candida albicans, Candida glabrata, and Trichophyton longifusus.* Different dose concentrations i.e. from 1 to 1000 µL mL^{-1} of both fungal strains were prepared and tested against pathogenic fungal species. 1000 µL mL^{-1} of *Aspergillus* extract inhibited the growth of *M. canis, C. glabrata* and *A. flavus* by 60.5%, 52% and 44.5% respectively. *C. albicans* and *F. solani* showed 35 and 38.5% growth inhibition while lowest inhibition was recorded against *T. longifusus* (27.5%). A dose of 1000 µL mL^{-1} inhibited the growth of all test pathogenic fungal species, however, comparatively *T. longifusus, M. canis* and *C. glabrata* showed highest inhibition i.e. 70%, 63.5% and 52% respectively. In cytotoxicity assay three dose concentrations i.e. 10, 100 and 1000 µL mL^{-1} of crude extract of both fungal strains were used to test the toxicity of fungal metabolites against *A. salina*. At µL mL^{-1} highest mortality percentage for *Aspergillus* metabolites was recorded as 98.33 while at this concentration highest mortality percentage for *Penicillium* metabolites was 63.33 after 24 hrs.

Crude metabolites of both fungal strains were tested against *Lemna minor* for phytotoxicity. At a dose of 1000 µL mL^{-1}, 85% mortality was recorded for *Aspergillus* extract while 56.66% mortality was recorded for *Penicillium* extract against the *Lemna minor.* Extracts of both fungal strains showed strong herbicidal activities against seeds of *Silybum marianum* L. by completely inhibiting seed germination.

In light of above mentioned activities isolation of compounds was performed using column chromatography and high performance liquid chromatography (HPLC) techniques. Structure elucidation of isolated compounds was performed using 1D and 2D nuclear magnetic resonance (NMR) spectroscopy techniques. From the organic extracts a total of 5 compounds were isolated among which 3 were known compounds while 2 were new compounds. The two new compounds were 3-allyl-6-(hydroxy (phenyl) methyl) piperazine-2, 5-dione and (2*R*, 4*S*) -2, 4-dimethyl-4-((*E)*-2-((3*S*, 4*S*)-2, 4, 5-trihydroxy-3-methoxy-4-phenyl-1, 2, 3, 4-tetrahydroquinolin-6-yl) vinyl) cyclohexanone while the three known compounds were (*E*)-3-((3*S*,4*R*)-8-hydroxy-3,4-dimethyl-1-oxoisochroman-7-yl) acrylic acid, 2-(1, 4-dihydroxybutan-2-yl)-1,3-dihydroxy-6, 8-dimethoxyanthracene-9, 10 (4a*H*, 9a*H*)- dione and 5-hydroxy-2-(hydroxymethyl)- 4*H*-pyran-4-one.

This thesis presents a research work on isolation and structure elucidation of secondary metabolites from soil and rhizosphere inhabiting fungi that may have potent biological molecules.

CHAPTER 1

INTRODUCTION

CHAPTER 1 INTRODUCTION

Fungus is a Latin word which means mushroom. Fungi are eukaryotic, spore producing, achlorophyllous organisms which can reproduce both sexually and asexually showing absorptive mode of nutrition. Fungi can be microscopic or macroscopic having fleshy bodies. The study of fungi is called mycology and those who study fungi are called mycologists [1]. Fungi are mostly found in terrestrial environments but fungi can be found in diverse environments. For example some fungi are aquatic and can be found in fresh water and marine habitats. Many fungal species cause diseases in plants, animals and humans but fungi are also beneficial as they form beneficial relationships with other organisms e.g. Mycorrhiza (fungal association with roots of higher plants) and lichens (association of fungi with algae particularly with members of chlorophyta and cyanophyta) while some live as endophytes in internal tissues of different plant organs like root, stem and leaves [2-5].

According to the five kingdom system fungi is placed in separate kingdom Fungi which constitute a monophyletic group called Eumycota which is a Greek word meaning 'true fungi' [1, 6]. It is believed that kingdom fungi have more than 100,000 described species and more than 1.5 million species still awaits description [7]. The kingdom fungi is classified into many groups but some of the important groups of fungi are *Oomycetes, Chytiridiomycetes, Zygomycetes, Basidiomycetes, Ascomycetes* and Mitosporic fungi also known as *Deuteromycetes* [8].

Fungi have numerous applications in food industry, genetic engineering, environmental technologies [9-11] and in agriculture e.g. in biological control of insects [12-13]. In modern times fungi are being exploited for production of bioactive secondary metabolites of immense pharmacological importance [14]. For example, production of antibiotics like fusidic acid and Penicillins from *Fusidium coccineumis* and *Penicillium chrysogenum* and antifungal agents like

griseofulvin (*Penicillium griseofulvum*) have been obtained from fungi [15-17]. An interesting feature of fungi is that depending upon the nutrients availability and environment, the same fungal strain will produce different secondary metabolites [18].

1.1 Fungi as chemical industries

Fungal Kingdom is extremely diverse with so many organisms having unique and complex biochemical pathways. These pathways results in the formation of wide range of metabolites which are termed as natural products. Some of these products includes antibiotics for example penicillin and cyclosporine etc. some are toxins like trichothecenes and aflatoxin while some metabolites have both properties of antibiotics and toxins for example ergot alkaloids. Most of these natural products are secondary metabolites which are produced when the fungal development is completed [19]. These secondary metabolites usually have low molecular weight and show biological activities [20, 21]. The research area of natural products is of considerable interest as many of secondary metabolites are pharmaceutically, industrially and agriculturally important [22]. There are several classes of fungal secondary metabolites but some important classes includes polyketides, which are the most abundantly occurring secondary metabolites of fungi, non ribosomal peptides derived from both proteinogenic and non proteinogenic amino acids, terpenes and indole alkaloids [19].

With the discovery of penicillin in 1928 by Alexander Fleming, the importance of fungal secondary metabolites as antibiotics was realized and search for new fungal metabolites with bioactive properties geared up [23]. In modern times, fungi proved to be the sources of different therapeutically important compounds with complex structures [24]. Scientists are discovering new sources for isolation of fungi with bioactive secondary metabolites and endophytic fungi

[25], marine derived fungi [26] and soil borne fungi [27] have been shown to be the best sources of secondary metabolites. A large number of fungal secondary metabolites have been reported to be pharmacologically very important with antibacterial [28], antifungal [29], cytotoxic [30], phytotoxic [31], anticancer [32] and antiviral properties [33].

1.1.1 *Aspergillus*

The word *Aspergillus* was first used by Micheli in Nova Plantarum Genera of 1729 to describe those fungi which had erect stalks and conidia at their tips. However, in the middle of nineteenth century these fungi were recognized as active decomposers and the agents to cause human and animal diseases [34]. Today the genus *Aspergillus* consists of about 180 known species and most of them are producers of biologically important secondary metabolites [35]. Most of the members of the genus *Aspergillus* are filamentous living as saprobes in the soil and belong to order *Eurotiales* of the class *Ascomycota* [36].

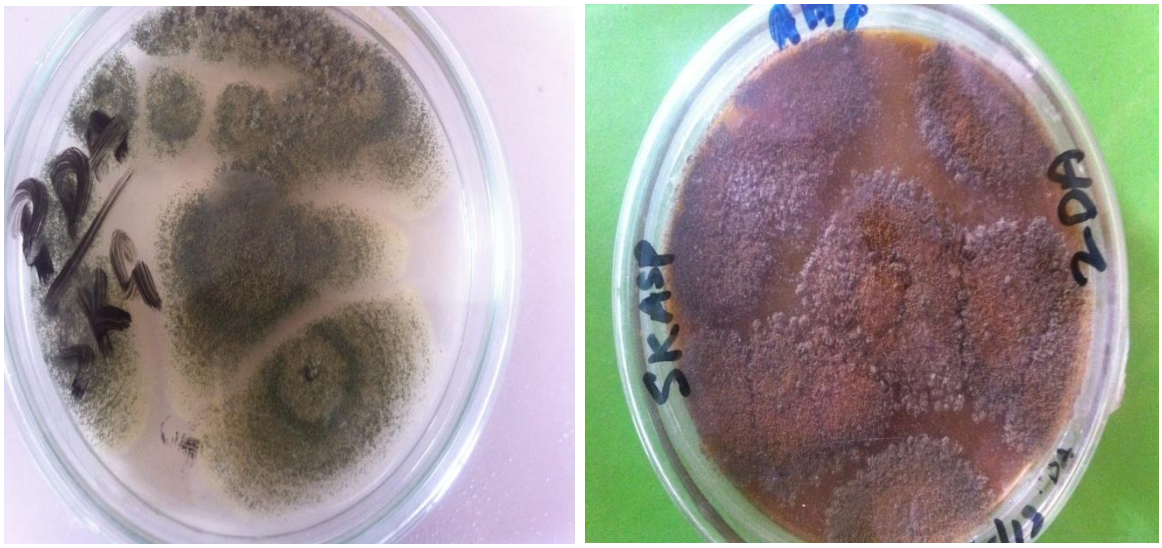


Figure 1.1 *Aspergillus* species growing on PDA medium (left) and on CDA medium (right)

Although members of *Aspergillus* genus are generally considered non pathogenic but some species of this genus can cause life threatening infections in immune compromised individuals.

For example aspergillosis is a serious pulmonary disease that can occur in patients of tuberculosis [37-38]. *Aspergillus fumigatus* is found to be the most frequent cause of aspergillosis and this fungus has the ability to survive in extremely harsh environmental conditions. It is estimated that around the world aspergillosis accounts for more than 600,000 deaths annually [39-40]. Some other common species of the genus *Aspergillus* are *A. flavus, A. ochraceus, A. niger, A. alliaceus, A. carbonarius* and *A. parasiticus* etc.

As fungi are decomposers, they contaminate agricultural and food products by rotting, pigmentation, producing bad odor, taste and discoloration. The main reason for contamination of food is that they release mycotoxins in food and also in feeds of animals [41]. Several members of this genus produce different types of mycotoxins, but two types of toxins are most important i.e. ochratocin A and aflatoxins [42]. Aflatoxins are further divided into different types and some of them are extremely toxic and carcinogenic and considered as the major cause of hepatocarcinogenecity [43-44]. The most threatening aspect of aflatoxins to human health is that they can contaminate cereal crops for example, corn, soybean, peanuts and tree nuts during pre harvest stages. Also as they can contaminate animal feeds and sometimes this contamination will appear in milk. The adverse effects will vary from species but risk of contamination is also high in dairy products and processed meat. *A. flavus* and *A. parasiticus* are important aflatoxin producing species of the genus *Aspergillus* [45].

Ochratoxin A (OTA) is a nephrotoxin that can contaminate a wide range of food and feeds for example beer, wine, grains, coffee, legumes, meat and dry fruits. This toxin has been proved to be carcinogenic in rats and possibility has been shown that it could be carcinogenic to humans [46]. The most important ochratoxin producing organisms belongs to *Circumdati* and *Nigri* section of the genus *Aspergillus* [47-48].

However, a more fascinating aspect of studying *Aspergillus* species is production of secondary metabolites that are pharmaceutically, industrially or agriculturally important. Different species of this genus produces a wide range of useful compounds which have shown antibacterial, antifungal, anticancer, antiviral, phytotoxic and cytotoxic activities [49]. For example *Aspergillus niger* has been used industrially for fermentation processes [50], for production of citric acid [51], as producer of antioxidants and for chemical biotransformation [52-53]. Recently cytotoxic Polyphenols have been reported from *Aspergillus versicolor* associated with a sponge in marine environment [54], an endophytic *Aspergillus fumigatus* have been reported to produce growth hormones gibberellins [55] and anticancer metabolites have been recovered from a marine derived *Aspergillus protuberus* [56].

1.1.2 *Penicillium*

The word *Penicillium* is derived from Latin word "Penicillus" which means an artist's brush. This is because members of the genus *Penicillium* have branches of conidiophores on which conidia are borne in chains [8]. Johann Heinrich first describes *Penicillium* as genus in his work in 1809 *Observationes in ordines plantarum naturals* [57]. This genus contains more than 300 species which consists of profusely branched, septate and colorless hyphae [7]. This genus is included in class *Ascomycota* and order *Eurotiales* [58].

Figure 1.2 *Penicillium* species growing on Czapec Dox Agar (left) and on PDA medium (right)

In moderate climatic conditions with low humidity many species of *Penicillium* are saprophytic and perform decomposition of dead organic matter. Some species are plant pathogens for example *P. digitatum* attacks citrus fruits and *P. expansum* cause storage rot of apples [8]. Many species of this genus cause spoilage of food and some produce potent toxins [59-60]. However, some species of this genus are useful for humans for example *Penicillium* species produce a wide range of secondary metabolites with antibacterial [61-62], antifungal [63], immunosuppressant and cholesterol lowering properties [64]. Since the discovery of Penicillin in 1928 thousands of *Penicillium* species have been screened and they continue to be the sources of novel bioactive compounds which reflect their importance as sources of important secondary metabolites [65-67].

1.2 Aims and objectives

Multidrug resistant pathogens have become a serious threat to mankind. Although efforts are being made to synthesize and discover new antibiotics but there are no enough new drugs in

pharmaceutical pipeline to compete with the pace at which drug resistant pathogens are evolving. According to the Infectious Disease Society of America more than 70% bacteria are resistant to at least one commonly used antibiotics. Pharmaceutical companies have also cut back investing on antibiotic production as their use is for short term. Rather they are focusing on chronic drugs which are used for longer time. Health professionals warn that a lack of interest in antibiotic development field can cost humans their health.

Already some species of the genus *Acinetobacter* and *Pseudomonas* are resistant to all good antibiotics and many members of *Enterobacteriaceae* are resistant to all penicillins except carbaphenems. New antibiotics are also needed for community acquired infections like tuberculosis and urinary tract infections (UTIs). The problem of antibiotic resistance is more serious in developing countries like Pakistan because of abundant and misuse of antibiotics. If the research area regarding antibiotic production and development is not strengthened, we will be short of antibiotics. There is a serious threat that because of growing population of antibiotic resistant pathogens, many infections will be left untreated.

So it is the demand of the modern era to explore new sources for the production of antibiotics. In this regard, the main aim of this study is to explore biologically active fugal strains and exploit them for production of antibiotics and other bioactive secondary metabolites.

The main objectives of this study are:

1. To isolate the fungal strains of interest from the top soil and rhizosphere of *Mentha Piperita* ;
2. To extract crude metabolites from target fungal strains

3. To perform bioassay screenings of crude extract by performing antibacterial, antifungal, cytotoxic, phytotoxic and herbicidal activities;
4. To purify and isolate biologically active metabolites from crude fungal extract;
5. To determine the structure of the isolated metabolites/compounds using different spectroscopic techniques;

1.3 Socioeconomic benefits

The discovery of penicillin in 1928 opened a new gateway for the discovery of therapeutically important secondary metabolites. Since then fungi are treated like reservoirs which have been used by the scientists as natural industries for production of bioactive secondary metabolites. The discovery of new metabolites will lead to the synthesis and discovery of new compounds or their derivatives in the laboratory by determining the skeleton of the compounds. As in this work different fungal strains will be tested for the production of biologically active metabolites, this work will act as a bridge to correlate a useless microbe to be the producer of new antibiotics. The newly discovered drugs will play vital role in improving the socioeconomic condition of the country as imports of these drugs will lead to improvements in both health and economy.

Also developing countries of the world including Pakistan is facing a major problem of antibiotic resistant pathogens and the problem is progressing with time as new strains of multidrug resistant pathogens are emerging. In developed world still second generation of cephalosporin are effectively used but in some areas of Pakistan the use of fourth generation of cephalosporin have been reported. So there is an urgent need to discover new antibiotics otherwise a time will come as many infectious diseases will be left untreated because of

unavailability of antibiotics. The discovery of new antibiotics and therapeutic agents will not only improve the health of individuals but will also play a vital role in enhancing the pharmaceutical imports of the country.

CHAPTER 2

REVIEW OF RELATED LITERATURE

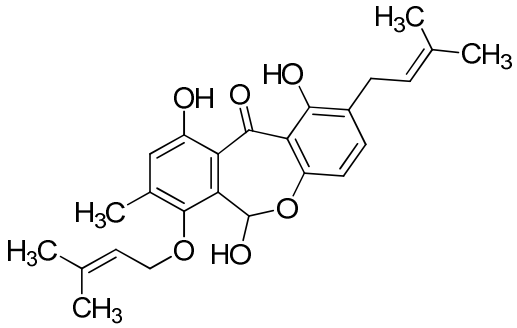
CHAPTER 2 REVIEW OF RELATED LITERATURE

2.1 Natural products and their importance

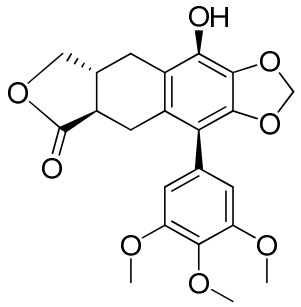
Secondary metabolites produced by living organisms which are biologically important are termed as natural products [68]. Natural products are organic compounds which are being used by humans since ancient times [69] but true understanding and their systematic characterization started in 20th century. In recent times natural products are considered to be persistent sources of novel antibiotics and other therapeutic agents [70]. According to a recent survey conducted on fungal secondary metabolites, 1500 compounds were isolated and studied between the year 1993 and 2001. Among these more than 50% compounds showed antibacterial, antifungal and cytotoxic activities [71]. These secondary metabolites are generally of low molecular weight and mostly bacteria and fungi are responsible for synthesis of these compounds. Bioactive metabolite producing microorganisms live in harsh environments where they have to compete with a wide range of other organisms for nutrients and space and as a result new metabolites are synthesized for survival [72].

Microorganisms can produce a diverse array of secondary metabolites and this evolution became possible over millions of years because these compounds are themselves used by microorganisms to inhibit the growth of competitor organisms, to protect their habitat and also for communication with other organisms. It is a fact that a large number of secondary metabolites have been discovered in the last decades but a bulk of new metabolites are still waiting to be discovered as a large number of microorganisms dwelling in wide range of environments needs to be cultured [73]. It has often been observed that successful culturing of fungal strains from a new environment results in the discovery of new natural products. For

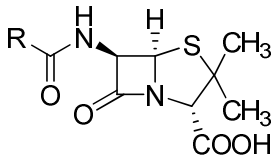
example, culturing marine derived fungus *Emericella nidulans var acristata* resulted in isolation of arugosin **(1)** [74] and isolation of an anticancer drug podophyllotoxin **(2)** from the endophytic *Aspergillus fumigatus Fresenius* [75].



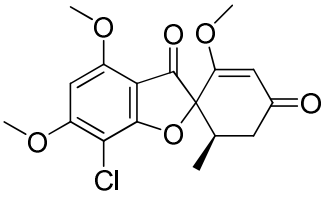
Arugosin A (1)



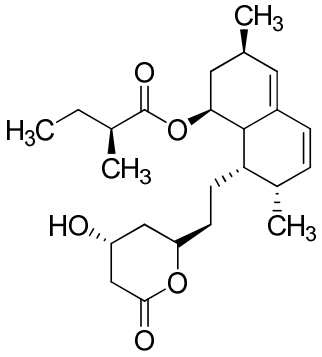
Podophyllotoxin (2)



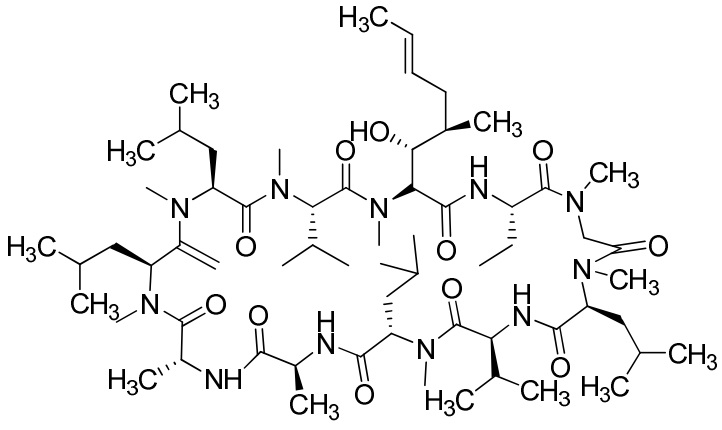
Penicillin (3)



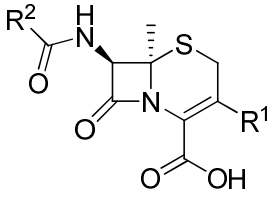
Griseofulvin (4)



Lovastatin (5)



Cyclosporine A (6)



Cephalosporin (7)

Figure 2.1 Structures of some well known natural products (1-7)

Natural products play an important role in development of new drugs for all disease types but particularly in case of cancer and infectious diseases natural products have laid an excellent platform for new drug development. Of the total new drugs approved between 1983 and 1994, 78% antibacterial and 61% anticancer drugs have originated from natural products [76]. In survey conducted in 2003, these numbers are even higher, according to which 78% antimicrobials and 74% anticancer drugs from have originated from natural sources [77]. More than 100 compounds obtained from natural sources are currently under clinical trials and most of them have been derived from plants and microorganisms [78]. In case of microorganisms the main sources for isolation of natural products to date are fungi and soil actinomycetes but recently actinomycetes of marine origin have also been reported as sources of bioactive metabolites [79].

Apart from their role in drug discovery and development natural products have a number of other applications. Fungi produce variety of colored pigments [80] and fungal natural pigments like carboxylic acids, anthraquinone and pre-anthraquinone are used industrially for dyeing of garments made of silk, wool and cotton [81-82]. Recently filamentous fungi have been reported to produce potent pigments that can be used industrially for dyeing of leather without altering the properties of the leather samples [83]. The demand for natural dyes increases because most of the available dyes contain ingredients that are carcinogenic particularly colon carcinogens when exposed chronically [84]. Hence there is growing demand for coloring agents that are environment friendly and non toxic for coloring of textile and food products, particularly for sensitive individuals like children, old age people and patients.

One of the recent applications of fungi is their exploitation for the production of water soluble pigments to be used as food colorants. Food colors are used as additives in food industry

which play an important role in acceptability of the food items [85]. But a major problem is that only a limited number of colorants have been authorized to be used as food additives. European Union (EU) has authorized 43 and United States has approved only 30 colorants to be used as food additives [86, 87]. Also currently available food colorants have a number of drawbacks e.g. most are heat, light and oxygen labile and are sensitive to oxidation, bleaching and changes in pH. This limits their application to be used as food additives in different types of food items. However, natural pigments produced by fungi can be efficiently used as food colorants which can be extracted easily and in large amounts by the exploitation of fungi [88].

Postharvest fungal diseases are mostly controlled by using chemical fungicides and according to an estimate more than 23 million Kg fungicides are used annually for control of various fruits and vegetable diseases [89]. Continuous use of chemical fungicides have posed different problems as they can cause carcinogenicity and teratogenicity, some are highly toxic and some are difficult to be degraded causing environmental problems [90]. Recently biologically active natural products particularly of fungal origin have been reported to have the potential to be used as biofungicides and as an alternative to chemical fungicides. It has been shown that different natural products like acetic acid, flavor compounds, jasmonates, chitosan and essential oils play very important role in the control and management of fruit and vegetable rots caused by fungi and increase their shelf life [91].

Natural products have been successfully evaluated for agrochemical applications by testing their phytotoxic and herbicidal capabilities. Studies have shown that novel compounds have been isolated from fungi with phytotoxic and herbicidal effects on plants of monocots, dicot families and weeds [92]. *Helminthosporium* species isolated from grasses are reported to produce phytotoxic compounds called ophiobolins which along with antibacterial and antifungal

properties also showed strong phytotoxic activities by inhibiting the growth of eight plant species in leaf wounding assays [31]. Nakajima and coworkers (1991) isolated a novel compound with herbicidal properties from a newly reported fungus *Paecilomyces variotii* SANK 21086. After the physiochemical analysis compound was identified as Cornexistin belonging to nonaride group. This compound showed herbicidal activities against young annual and perennial plants of both monocot and dicot families with selective toxicity for corn plants [93].

Many natural products are used for defense mechanisms by fungi against fungivorous insects and there are evidences for such fungal chemical defense mechanisms. It has been shown that sclerotia of many fungi have antiinsectant compounds which prevent them from predation. *Carpophilus hemipterus* is a dried fruit beetle and an insect that feeds on different parts of *Aspergillus fumigatus* despite of the fact that it produces aflatoxins. But this insect cannot feed on sclerotia of *Aspergillus fumigatus* as it produces antiinsectant chemicals [94].

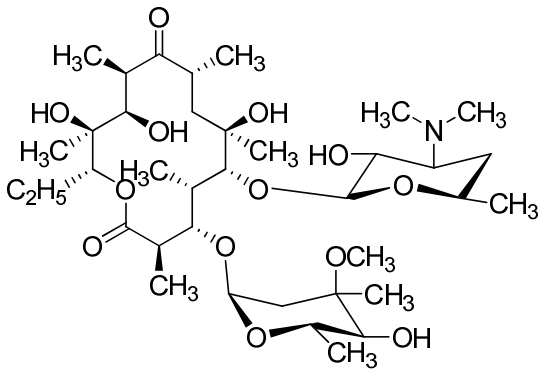
2.2 Natural products classification

Secondary metabolites are placed in different classes which differ from one another in their structure and mechanism of action. Some of the important classes of fungal natural products include polyketides, terpenoids, alkaloids and non-ribosomal peptides. Detailed description of important classes of secondary metabolites is given as under.

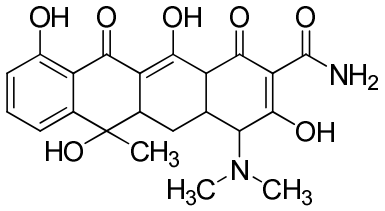
2.2.1 Polyketides

Among the fungal secondary metabolites Polyketides are the most widely occurring class. These are natural organic molecules which have complex structures and show wide range of

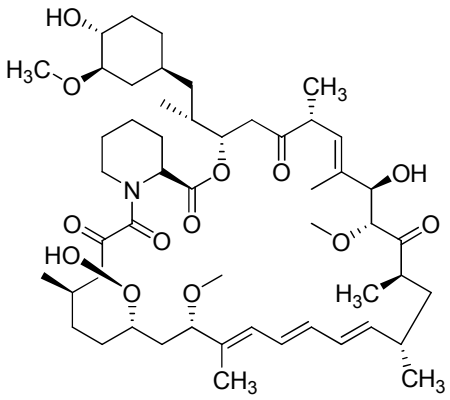
biological activities e.g. antibacterial, antifungal, anticancer and immunosuppressant activities [95]. Polyketides are actually a large group of organic compounds which are produced not only by fungi but also by other organisms like bacteria and plants. They show a great deal of structural and functional diversity and many polyketides acts as important classes of different clinical drugs being used today e.g. Erythromycin **(8)**, Tetracycline **(9)**, Rapamycin **(10)**, Daunorubicin **(11)** and Lovastatin **(5)** etc [96].



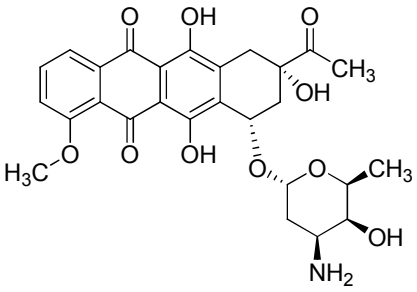
Erythromycin (8)



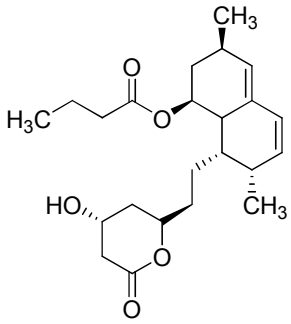
Tetracycline (9)



Rapamycin or Sirolimus (10)



Daunorubicin (11)



Lovastatin (5)

Figure 2.2 Structures of some important polyketide compounds (5, 8-11)

Among all polyketides the best genetically studied polyketides include naphthopyrone which is a yellow colored pigment present in the spores of *Aspergillus nidulans*, aflatoxin which is a potent carcinogen that cause cancer and lovastatin, a commercially used cholesterol lowering

agent [97-98]. Polyketides are synthesized by living organisms e.g. by bacteria and fungi from the precursors of acyl CoA by action of a class of enzymes called polyketide synthases (PKSs). There are three classes of PKSs which are termed as type I, type II and type III PKSs but recent research have proved that polyketides may also be produced beyond these classes of PKSs paradigm [96, 99].

PKSs share similarities with fatty acid synthases (FASs) both in their structure and mechanism of action because both PKSs and FASs requires short chain carboxylic acids e.g. acetyl CoA or malonyl CoA to construct polymers of β-ketoacetyl. In case of fatty acid biosynthesis this condensation step is followed by reduction and dehydration steps to finally synthesize unsaturated fatty acids. While in polyketide biosynthesis these reductions and dehydration steps either do not occur or they are optional. PKSs are classified into different types on the basis of their structure (i.e. whether it is made of one subunit or multiple subunits) and the mechanism involved in its synthesis (i.e. linear in which active site of an enzyme is used only once or iterative which involves repeated use of active site). A total of eleven domains have been identified so far in PKSs [100]. In case of fungi type I PKSs are responsible for the synthesis of polyketides which have at least three functional domains designated as KS (ketoacyl CoA synthase), AT (acyltransferase) and ACP (acyl carrier protein) domains which contribute to the minimal structure of PKS and other optional domains designated as DH (dehydratase), MT (methyltransferase), ER (enoyl reductase), KR (ketoreductase), CYC (cyclase) and TE (thioesterase) domains as shown in the figure 2.3.

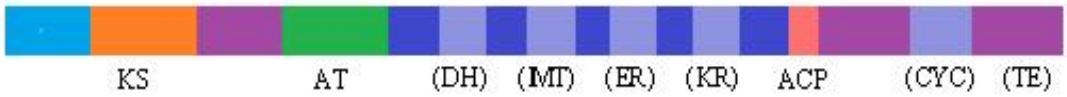


Figure 2.3 Domain structure of fungal polyketide synthase. Optional domains are written in brackets

In PKS the domain is a region of conserved amino acids which has a specific function. The domains in PKS are responsible for addition of ketide units to growing polyketide chain. The set of domains in PKS and also in non ribosomal peptide synthase that is required for polypeptide chain elongation and modification is called a module [19]. Fungal PKS type I enzymes are different from bacterial PKS type I enzymes as in bacteria for incorporation of every methylmalonyl CoA a different module is used but in fungal PKS type I enzymes a single module is repeatedly used to add ketide units to biosynthesize polyketides. That's why fungal PKS type I enzymes are termed as iterative PKS. For example in WA PKS enzyme (a napthopyrone synthase of *Aspergillus nidulans*) the minimal domain that consists of KS–AT–ACP, is repeatedly use to perform condensation reactions to form a chain of heptaketide. In the C-terminal region of WA PKS enzyme, a thioesterase (TE) domain is present that is responsible of the formation of naphthopyrone, an aromatic polyketide [100].

2.2.1.1 Types of polyketides

Among the natural products polyketides are one of the most diverse groups in which carbon atoms extracted from acyl building blocks are used to form complex carbon skeletons with the help of enzymes. Combinatorial chemical, biochemical and genetics based approaches to study the structure of polyketides have provided new insights into structure and assembly of these natural products. On the basis of their activities and structural analysis these compounds or

their derivatives have been classified into important drugs e.g. Polyenes, Polyethers, Polyphenols, Enediynes and Macrolides while some are classified as important toxins and virulence factors. Recently discovered biosynthetic pathways have provided an insight into these complex and structurally diverse molecules to understand their structure and types [101]. Some of the important types of polyketides are briefly discussed here.

2.2.1.1.1 Linear polyketides

This is a small group with limited number of polyketides that do not have macrolide or carbocyclic rings in their structures.

2.2.1.1.2 Halogenated acetogenins

These polyketides have been isolated from marine organisms particularly from *Laurencia* species which is a type of red algae. The characteristic feature of these polyketides is that they contain halogen atoms in their structures which may be bromine or chlorine. A typical example of halogenated acetogenins is Bermudenynol **(12)** [102].

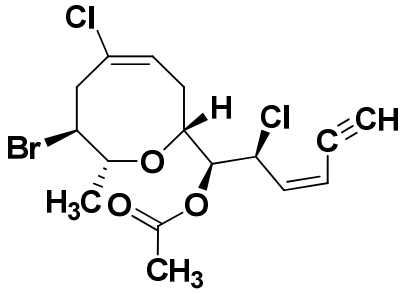


Figure 2.4 Structure of Bermudenynol (12)

2.2.1.1.3 Annonaceae acetogenins

The Annonaceae is a family of large flowering trees and shrubs which includes about 130 genera and more than 2300 species [103]. Annonaceae acetogenins are compounds of polyketide origin which were first isolated from the members of this family. Uvaricin **(13)** is a typical example of these types of polyketides. These compounds contain about 35 to 38 carbon atoms and various oxygen functional groups. Usually in the structure one or two and rarely three tetrahydrofuran rings and a γ-lactone is also present. As these compounds are waxy and amorphous they are unsuitable for X-ray analysis and hence difficult to perform their structural elucidation [104-105].

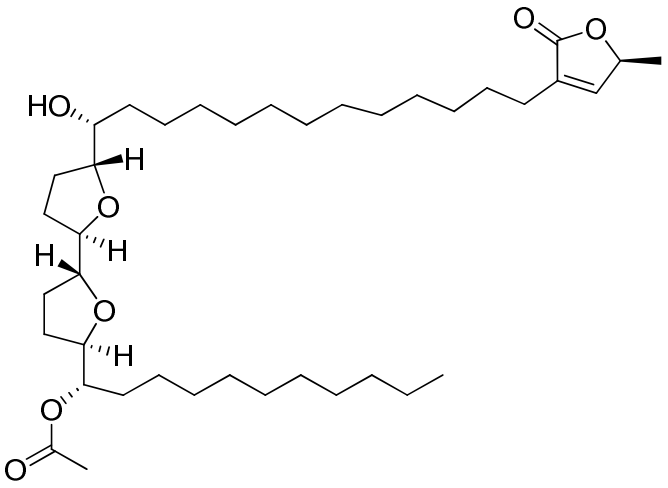


Figure 2.5 Structure of Uvaricin (13)

2.2.1.1.4 Macrolides and lactone polyketides

These types of polyketides have been isolated from the species of *Streptomyces* and *Micromonospora.* Antibiotics derived from these types of compounds are classified as macrolides. These compounds have complex structures as they have 12-16 membered macro

cyclic ring and one to three amino sugar residues which are attached to aglycone through ether linkages. A typical example of macrolides antibiotic is Erythromycin **(8)**.

Examples of lactone polyketides are milbemycins and avermectins. These are 16 membered macro cyclic lactones which contains a heterocyclic oxygen ring attached to lactones [106, 107].

2.2.1.1.5 Ansamycins and related polyketides

Ansamycins are aromatic compounds that contain benzene or naphthalene rings in their structures. In these compounds non adjacent positions are joined with one another through an aliphatic chain which results in the formation of cyclic structure. At one of the aromatic-aliphatic junction there is always an amide bond present. These polyketides are further classified on the basis of aromatic moiety and nature of aliphatic chain attached Ansamycins not only show differences in their structure but also in their biological activities. Most members of this group inhibit RNA polymerase and show antibacterial and antitumor activities.

This type of polyketides have mostly been isolated from microbial sources e.g. *Streptomyces, Micromonospora* and *Nocardia* species but few of these compounds have also been reported from plant sources. Rifamycin **(14)** is a well known member of this group [108-109].

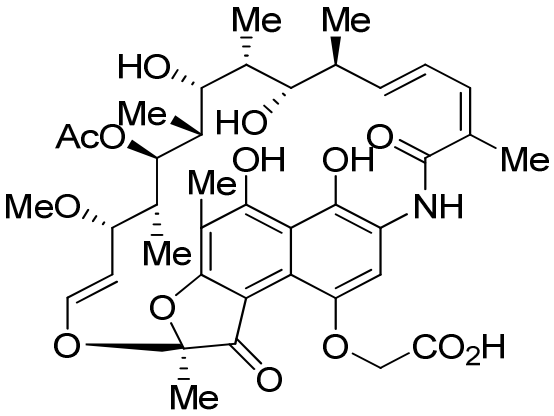


Figure 2.6 Structure of Rifamycin (14)

2.2.1.1.6 Polyenes

Polyenes are antibiotics which are structurally identified by the presence of lactone ring with a series of double bonds. On the basis of double bonds polyenes are further subdivided into trienes and tetraenes etc. Polyenes are produced by the members of *Streptomyces* and show antifungal activities. One of the important members of this group is nystatin **(15)** which shows strong antifungal properties [107, 110].

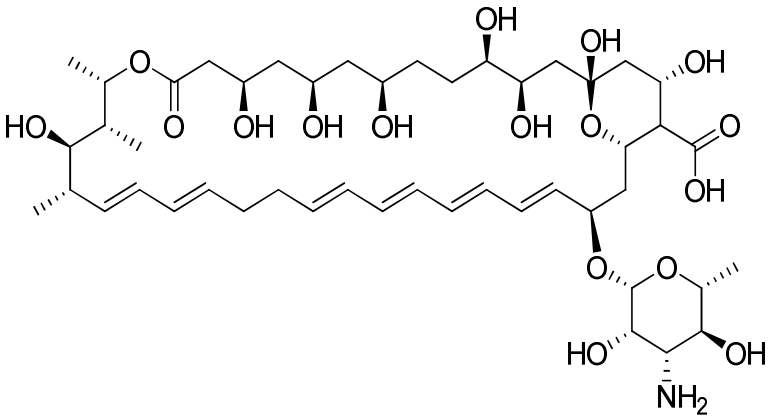


Figure 2.7 Structure of Nystatin (15)

2.2.1.1.7 Tetracyclines

Tetracyclines **(9)** are produced by members of the genus *Streptomyces* and since their discovery in 1940s they are clinically used as broad spectrum antibiotics. Tetracyclines show

broad spectrum activities against both Gram positive and Gram negative bacteria, mycoplasmas, spirochetes, amoeba and also some large size viruses. They also have poultry applications as they promote growth and increase feed efficiency. Although it is a small group of antibiotics but after β-lactams these are the most widely used clinically as they have broad spectrum of activities, show low toxicity and better oral absorption . Their mechanism of action is different from β-lactams as β-lactams inhibit cell wall synthesis but tetracyclins inhibit protein synthesis [111, 112].

2.2.1.1.8 Angucyclines

This group is considered as a new group of antibiotics which are characterized by the presence of benzanthracene ring. These antibiotics are more or less similar to tetracyclines as far as their structure is concerned but in angucyclines e.g. in Urdamycin A **(16)**, the tetracycline ring structure show angular arrangement. These antibiotics were originally derived from the members of *Streptomyces* and have been reported to show interesting biological properties like antibacterial, antifungal, antiviral and anticancer activities [113].

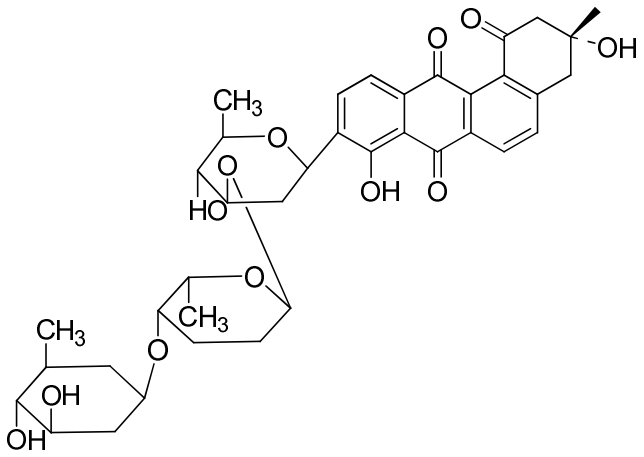


Figure 2.8 Structure of Urdamycin A (16)

2.2.1.1.9 Polyether polyketides

Polyether polyketides are natural compounds which are characterized by the presence of tetrahydrofuran and tetrahydropyran rings in their structure. It is a large group of antibiotics and more than thousand polyether compounds have been identified so far which are mostly of bacterial origin. Important producer organisms are *Streptomyces* species, but some other organisms like *Nocardia*, *Actinomadura* and *Dactylosporangium* species have also been reported for the production of polyether antibiotics.

Polyether antibiotics have the ability to bind to metal ions and they show broad spectrum activities against both Gram positive and Gram negative bacteria, mycobacteria and fungi. But the fact that they are toxic has greatly reduced their application [114]. Monensin **(17)** is an important member of this group which is mostly used in animal feed and dairy products to prevent bacterial contamination [115].

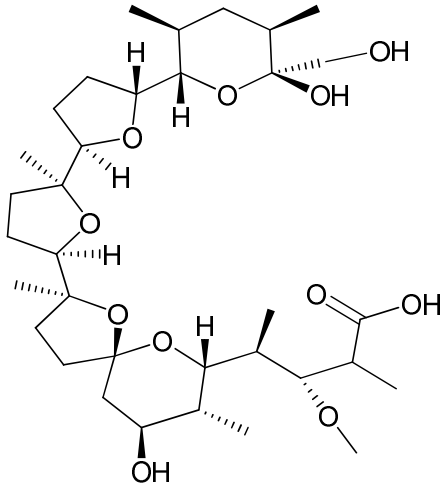


Figure 2.9 Structure of Monensin (17)

2.2.1.1.10 Aflatoxins

Aflatoxins **(18)** are fungal secondary metabolites and potent mycotoxins. Aflatoxins are produced by species of *Aspergillus*, particularly by *Aspergillus flavus*. These toxins were

discovered around 1960 as they were considered to be the cause of turkey X disease [116]. Naturally occurring aflatoxins are extremely carcinogenic and mutagenic. Structurally, aflatoxins are difuranocomarins. These toxins after metabolism can give rise to a number of other potent aflatoxin derivatives [117]. The synthesis and structure of aflatoxin B1 has been studied in detail as it is formed from a single decaketide chain through a series of intermediates [118].

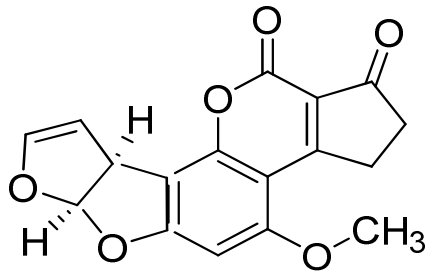


Figure 2.10 Structure of Aflatoxin (18)

2.2.2 Terpenoids

Terpenoids are considered to be the most diverse class of natural products because they show great structural diversity. In this family of natural products more than twenty two thousand compounds have been identified and the number is still increasing [119]. Terpenoids have a number of vital roles in the cell as they act as hormones (gibberellins), as photosynthetic pigments (carotenoids) and as part of electron carrier systems (plastoquinones). Terpenoids also acts as structural components of the cellular constituents e.g. polysterols are terpenoids that act as important structural components of membranes of living organisms. Apart from these physiological and structural roles terpenoids have other vital roles e.g. terpenoids of C_{10}, C_{15} and C_{20} families acts as toxins and antibiotics [120]. In addition to these, terpenoids have industrial, pharmaceutical, agrochemical and other diverse range of applications [121].

All terpenoids are derived from five carbon monomeric units called isoprene (C_5) units **(19)** which are repeatedly joined to one another in linear fashion (head to tail manner) to form a polymer. This polymer is later on use to derive different terpenoid skeletons. As isoprene units are mainly present in the structure of terpenoids, they are sometimes termed as isoprenoids, although it was known for more than 100 years that isoprene units are not the precursors of terpenoids [122]. The principle that different terpenoids can be hypothetically formed by linear joining of isoprene units was presented by Wallach in 1914 which is called "isoprene rule". This rule initially provided immense help in performing early structural analysis and to find out structural relationships among different terpenoids. This concept was later on refined by Ruzicka (1953) into "biogenetic isoprene rule" which ignores the role of biological precursor in terpenoids but focus mainly on fact that isoprene units are involved formation of these compounds as they perform electrophilic interactions, structural rearrangements and cyclization [121].

Classification of terpenoids is based on two things i.e. the number of isoprene or C_5 units they contain and they way these C_5 units are joined because in most of the cases isoprene units are joined in head to tail fashion but head to head and head to middle fusion is also known. On the basis of five carbon isoprene units terpenoids are classified into different classes. These classes include:

2.2.2.1 Monoterpenoids or C_{10} terpenoids

Although there are two isoprene **(19)** units but C_{10} terpenoids are called monoterpenoids because the first terpenoid which was isolated from turpentine in 1850s is regarded as base unit of terpenoid nomenclature and from this rest of terpenoid nomenclature was formed. In some

plants monoterpenoids are present as five percent of total plant weight [122]. In plants terpenoids are present as aromas and as volatile essential oils. They have a number of industrial applications e.g. in perfume and flavor industry. Some monoterpenoids have been isolated from marine organisms which are halogenated compounds. These halogenated monoterpenoids are used for defense purposes to kill other organisms and also as pheromones in some marine insects [123, 124]. On the basis of arrangement of isoprene units, monoterpenoids are further classified into different subclasses e.g. acyclic, irregular cyclic, cyclohexane and cycloheptane monoterpenoids etc [125, 126].

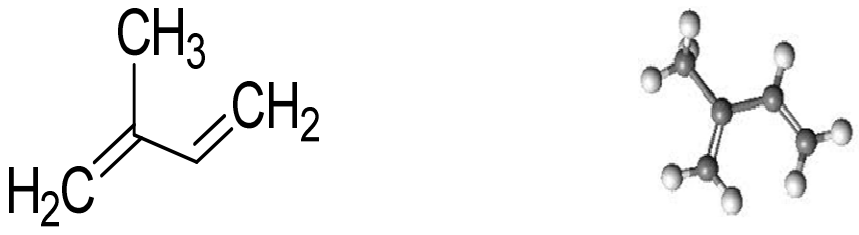


Figure 2.11 Structure and conformation of isoprene (19)

2.2.2.2 Sesquiterpenes or C_{15} terpenoids

Sesquiterpenes are terpenoids that contain three isoprene units in their structure. They are also known as one and half terpenes. They are found in many organisms but particularly they are present in higher plants. There exists a great variety of Sesquiterpenes skeletons but they are all formed by the process of cyclization and structural rearrangements from a common precursor called farnesyl pyrophosphate [127]. Like monoterpenoids, Sesquiterpenes are also present in essential oils of plants. Important Sesquiterpenes includes phytoalexins (e.g. Capsidiol **(20)**) which are used as antibiotic compounds by plants against microorganisms and also as antifeedants to repel plant feeding insects [122].

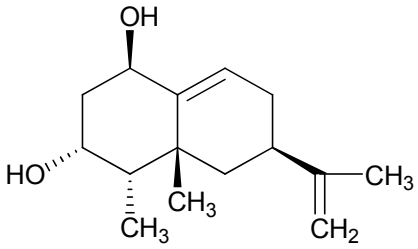
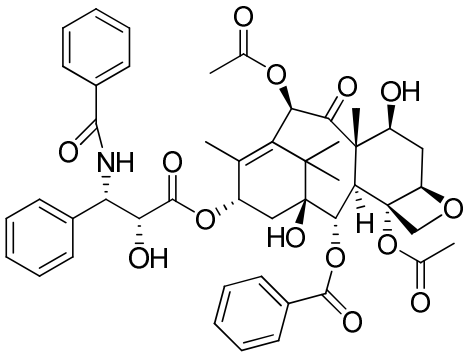


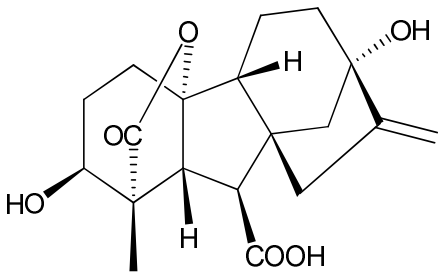
Figure 2.12 Structure of Capsidiol (20)

2.2.2.3 Diterpenoids or C_{20} terpenoids

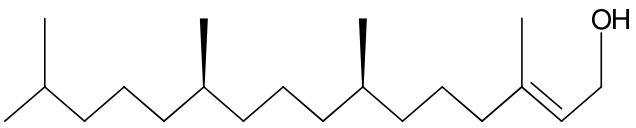
Diterpenoids or C_{20} terpenoids contain four isoprene units and they contain twenty carbon atoms in their structure. These are large group of compounds which are mostly present in plants particularly in resins of coniferous plants but they have also been reported from a number of other organisms including fungi, marine organisms, primitive bacteria and different types of insects [128-129]. Important examples of diterpenoids include an anticancer drug taxol (Paclitaxel) **(21)**, gibberellins hormones **(22)** and phytols **(23)** present in the side chain of chlorophyll [122].



(21) Paclitaxel



(22) Gibberellins

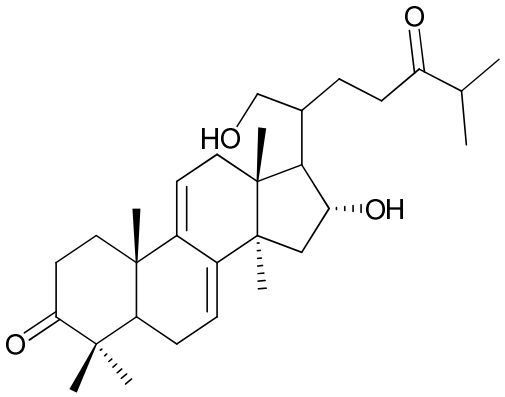


(23) Phytol

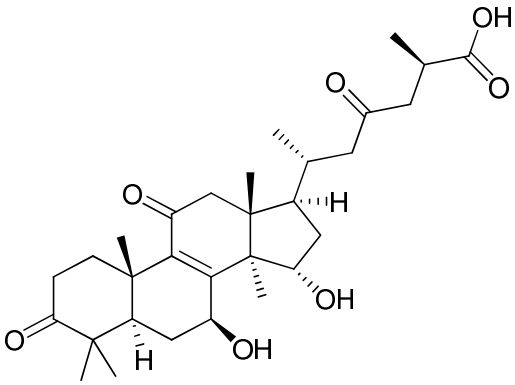
Figure 2.13 Structures of diterpenoids (21-23)

2.2.2.4 Triterpenoids, Tetraterpenoids, Polyterpenoids and Meroterpenoids

Two C_{15} units containing three isoprene units each are joined in a head to head fashion to form a triterpenoids. In each triterpenoid molecule consists of thirty carbon atoms and it is a large class of compounds with a number of important metabolites. Squalene is the simplest triterpene which was originally isolated as fat of fish liver and later on it was also isolated from the resin oil of plants. The presence of triterpenes has been confirmed in fruiting bodies of fungi. For example polyporenic **(24)** and ganoderic acids **(25)** have been isolated from the members of *Basidiomyces* i.e. *Polyporus* and *Ganoderma* species [130-131].



Polyporenic acid (24)



Ganoderic acid (25)

Figure 2.14 Structures of Polyporenic (24) and Ganoderic acid (25)

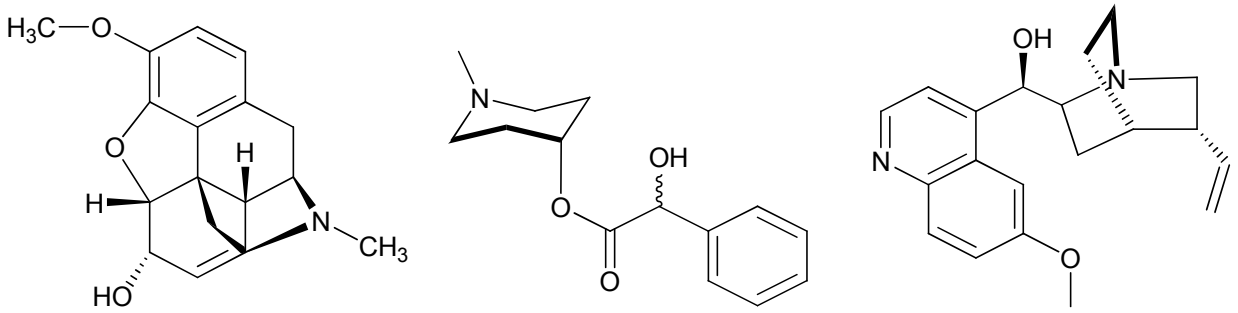
Tetraterpenes are formed by the head to head joining of eight isoprene units and consist of forty carbon atoms [132]. Important example of tetraterpenoids is carotenoid also termed as accessory pigments because during photosynthesis it helps the chlorophyll in trapping of light.

Some times terpenoids exists as long polymers consisting of more than 40 carbon atoms and more than eight C_5 units. Such complex terpenoids are called polyterpenoids. Examples of Polyterpenoids include electron carriers (ubiquinone and plastoquinone) and long polymers for example rubber which have a molecular mass of more than 10^6 Da [122, 133]. Some natural compounds are of mixed biosynthetic origin i.e. partly they are made of terpenoids and partly they are made of non terpenoid fragments. Such compounds are called meroterpenoids. For example tocopherol and some anticancer drugs like vincristine and vinblastine have terpenoid part in their structure [134].

2.2.3 Alkaloids

Alkaloids are organic natural products of low molecular weight and have nitrogen atoms in their structure. They mostly occur as colorless crystals but may also occur as liquids. Although alkaloids mostly have been isolated from plants i.e. 21000 out of 27000 alkaloids have been reported from plants but the presence of these compounds have also been confirmed in other organisms e.g. in microorganisms and animals. Alkaloids usually contain one, two or more nitrogen atoms in their structure and they primarily exist as primary, secondary or tertiary amines. Because of their ability that they can make salts and form complexes with metal ions, alkaloids are of basic or alkaloid nature and hence the name alkaloid is derived from alkali [135]. The term alkaloid was initially used for biologically active natural products of plant origin but later on this term was broadened and with exception of amino acids, proteins and polyketide antibiotics such as amino glycosides that contain nitrogen atoms, all the nitrogen containing naturally occurring compounds are included [136].

For about 3000 years alkaloids from plants have been used by humans as both medicines and poisons. For example the use of poppy plant (*Papaver somniferum*) can be traced back from 1400 to 1200 B.C. In fact plants with alkaloid compounds were human beings original source of medicines termed as "materia medica". Even today many alkaloid compounds are in use in modern times as commercially available drugs. For example codeine **(26)** isolated from poppy plant is used as analgesic and antitussive. Plant derived alkaloids acts as models for the synthesis of modern medicines such as atropine **(27)** used in synthesis of commercial drug tropicamide is used during eye examinations to dilate pupils. The famous anti malarial drug quinine **(28)** used in synthesis of chloroquine is also an indole alkaloid [122].

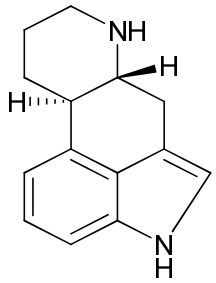


Codeine or 3-methylmorphine (26) **Atropine (27)** **Quinine (28)**

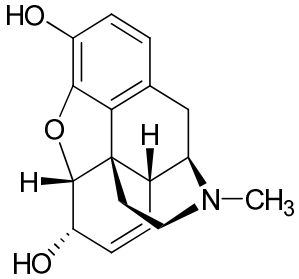
Figure 2.15 Structures of some common alkaloids (26-28)

Apart from plants fungi are also important producers of vast variety of alkaloids. After the discovery of ergot alkaloids from *Cleviceps purpurea*, a number of alkaloids of fungal origin have been purified and are shown to be biologically active through biological screening experiments like anticancer, insecticidal, antibacterial, antifungal and cytotoxic activities. Among fungi alkaloids of endophytic fungi e.g. indoles, amines, amides, quinolones etc. have been extensively studied along with their mechanism of action and researchers are trying to find out relationship of endophytic fungi with their host plants as far as the production of alkaloids is concerned [137]. Alkaloids produced by different fungal species like *Boletus*, *Fusarium*,

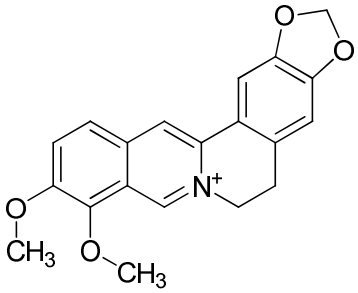
Aspergillus and *Penicillium* species acts as industrially and pharmaceutically important metabolites [138-139]. Some important alkaloids include ergoline **(29)** (Vasoconstriction), Morphine **(30)** (analgesic), Berberine **(31)** (dye wool and leather), Vincristine **(32)** (anticancer drug), and Ephedrine **(33)** (stimulant and antidepressant).



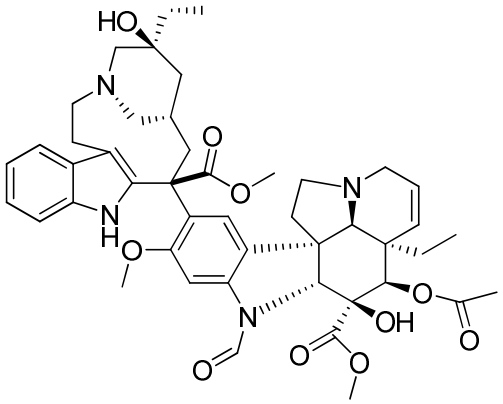
Ergoline (29)



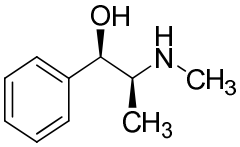
Morphine (30)



Berberine (31)



Vincristine (32)



Ephedrine (33)

Figure 2.16 Structures of some fungal alkaloids which are industrially important (29-33)

Great structural diversity exists among alkaloids and it becomes difficult to classify them on the basis of their structure. Rather alkaloids are classified into different groups on the basis of their biogenic origin [140]. Nitrogen atoms present in the structure of alkaloids are taken from amino acids and usually non proteinogenic amino acids are used in alkaloid biosynthesis.

Different alkaloids are subdivided into various groups on the basis of amino acid precursors. Amino acids used in alkaloid biosynthesis are lysine, ornithene, nicotinic acid, anthranilic acid and histidine. Nitrogen atoms are transferred from amino acids by transamination reactions and rest of the skeleton for the synthesis of alkaloids is derived from either acetate or shikimate. Sometimes alkaloids may originate from terpenoids or steroids but they are termed as pseudoalkaloids [135].

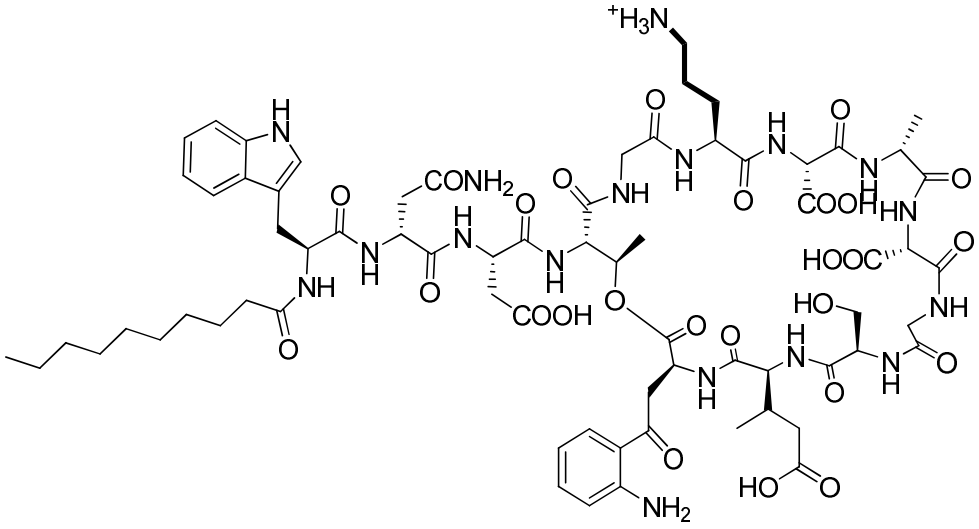
2.2.4 Non ribosomal peptides

Amino acids are organic molecules and the basic building blocks used in synthesis of proteins. Different amino acids are linked to one another by peptide bonds to form a polypeptide and usually ribosome is responsible for catalysis of peptide bond formation. It is reported that apart from ribosome, there is class of multimodular enzymes called non ribosomal peptide synthases (NRPSs) which are responsible for formation and catalysis of peptide bond [141]. Non ribosomal peptides (NRPs) constitute important class of secondary metabolites produced by bacteria and fungi from both proteinogenic and non proteinogenic amino acids by action of NRPSs [142]. The synthesis of these unusual peptides was first reported in *Bacillus* species in 1950s and 1960s [143]. Later genetic studies on *Bacillus subtilus* have unveiled that non ribosomal peptides and their derivatives perform a variety of functions in bacterial cells like regulation of cellular activities, cell differentiation, sporulation and specialization of cellular components [144].

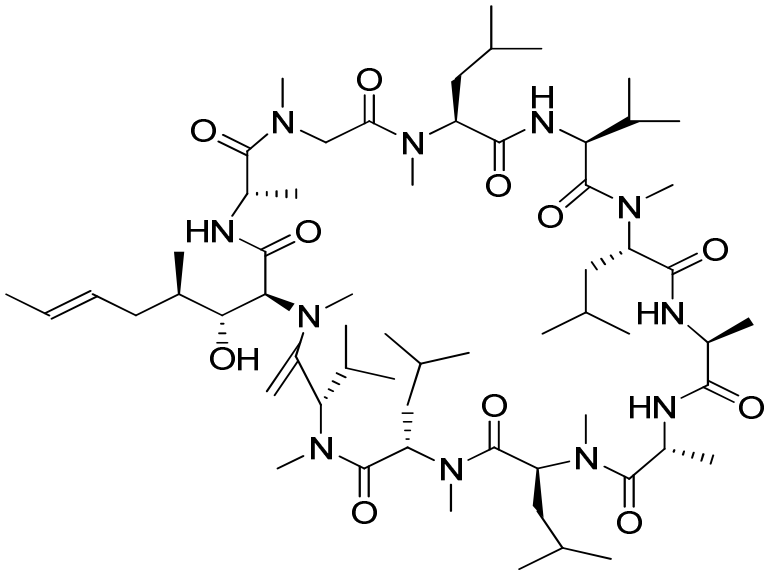
Lipmann (1970s) reported the synthesis of peptide antibiotics like gramicidin S and tyrocidine A through nucleic acid independent mechanisms in *Bacillus* species by complex enzymes (NRPSs). In later years many other peptide antibiotics synthesized through this

mechanism were reported which helped to understand the complex nature of these peptides and still they are the subject of extensive research [145].

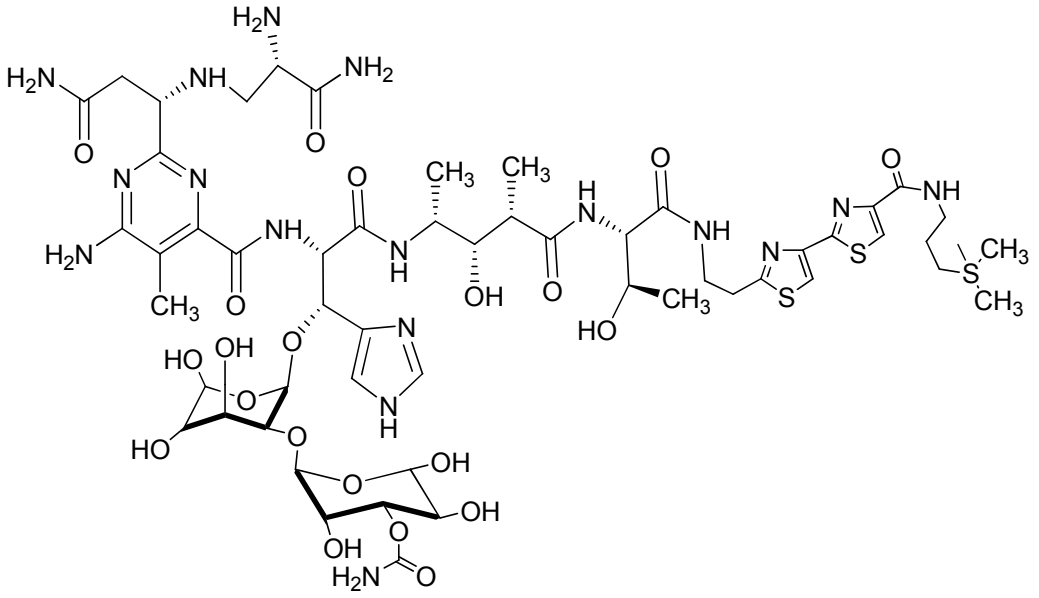
NRPs are one of the diverse groups of natural products which show a broad spectrum of applications in the field of medicine e.g. in infectious disease treatment (Daptomycin **(34)**), immunosuppression (Cyclosporin **(35)**) and cancer (Bleomycin **(36)**) [146].



Daptomycin (34)



Cyclosporin (35)



Bleomycin (36)

Figure 2.17 Structures of some common non ribosomal peptides (34-36)

2.2.4.1 NRPS genes in fungi

Till date genes responsible for the synthesis of NRPSs have only been reported in fungi and bacteria and currently there are no evidences for the presence of *nrps* genes in plants and animals. Among fungi members of *Ascomycetes* are mostly reported to contain many *nrps* genes. Few *nrps* genes have also been reported in the members of *Basidiomyces* and *Chytrids* while one *nrps* gene each has been reported in *Zygomycetes* and yeast *Saccharomyces cerevisae*. Recently genome wide surveys and phylogenetic studies of fungi and bacteria have enabled the scientific community to classify fungal NRPSs into two broader groups. NRPSs with one or two modules which have conserved domains are placed in the first group while in second group complex multidomain NRPSs are placed. In the first group those fungal NRPSs are placed that can cluster with bacterial NRPSs but in second group only fungal NRPSs are placed [147].

Although *nrps* genes are naturally present in fungi and bacteria but just like polyketide synthase (*pks*) genes evidences exists for horizontal gene transfer of *nrps* genes and in some cases even the transfer of these genes have been reported from bacteria to fungi. For example the best studied example of *nrps* gene that can be horizontally transferred is aminoadipate-L-Cys-D-Val (ACV) synthase gene that is responsible for the synthesis of β-lactum antibiotics in *Penicillium chrysogenum*, *Aspergillus nidulans* and *Acremonium chrysogenum*. Another similarity shared by both fungal *pks* and *nrps* genes is that both these genes are often found in clusters with other genes involved in a pathway. Studies conducted on the genomes of several members of *Eurotiomycetes* revealed that just like the genes responsible for production of other secondary metabolites, *nrps* gene clusters are also present in close proximity to telomeric region of the chromosomes. The expression of *nrps* gene clusters is in control of Lae A regulator. The *nrps* gene clusters are one of most diverse gene clusters and this diversity is due to the fact that they are present near subtelomeric regions where genetic recombination occurs at higher rates [148].

2.2.4.2 Important catalytic NRPSs domains

NRPSs usually consists of one or more modules which consists of a minimal of 3 domains i.e. A domain also called adenylation domain, thiolation or peptidyl carrier protein (T) domain and condensation (C) domain. At the C-terminus of NRPSs there is another domain that is responsible for release of synthesized peptides from non ribosomal peptide synthases called thioesterase domain [143]. A brief description of these three domains is given as under.

2.2.4.2.1 Adenylation domain or (A) Domain

Adenylation domain or A domain of NRPSs perform three important functions during NRP synthesis. First the amino acid to be incorporated into to the peptide is selected. Secondly the selected amino acid is charged by activation of its carboxylate group and at last it is transferred to thiolation domain. All these steps are ATP-dependent reactions for which energy is needed [149]. For this reason the A domains are sometimes referred to as the "gate keepers" because they control the first level of subrate activity in NRPSs [150]. A domain is responsible for the structural diversity of the NRPSs because A domain not only incorporate the twenty proteinogenic amino acids but also they have the ability to incorporate more than three hundred (300) amino acid precursors, the presence of which have been confirmed in NRPs [151].

2.2.4.2.2 Thiolation domain or (T) Domain or peptidyl carrier protein (PCP) domain

The proteins that form thiolation (T) domain in NRPSs belong to the same family of proteins in which acyl carrier proteins (ACP) involved in the synthesis of polyketides and fatty acids are placed. Both these proteins are structurally similar as in both these proteins a bundle of four helices are present [152-153]. In NRPSs T domains play many important functions as it is the main site for aminoacylthioester formation. During the formation of non ribosomal peptide the role of T domain is not restricted to interact only with A domains but it also have roles in the formation and modification of peptide bonds. So the role of T domain is very important in NRPSs as it has to interact with multiple partners in an extremely sequential manner [143].

2.2.4.2.3 Condensation domain or C domain

Each NRPSs module begins with a Condensation domain or the C domain. The most important function of C domain is the catalysis of peptide bond formation between the amino acids tethered to the nearby modules in NRPSs i.e. it transfers the acyl substrate to the adjacent module by formation of a peptide bond. C domain on the basis of function is divided into different sites e.g. the site through which it accepts the growing peptide from the previous module is called acceptor site and the site through which this peptide is transferred to the next module is called donor site. Among these two sites the acceptor site is more precise as it has to incorporate the correct substrate [154-155].

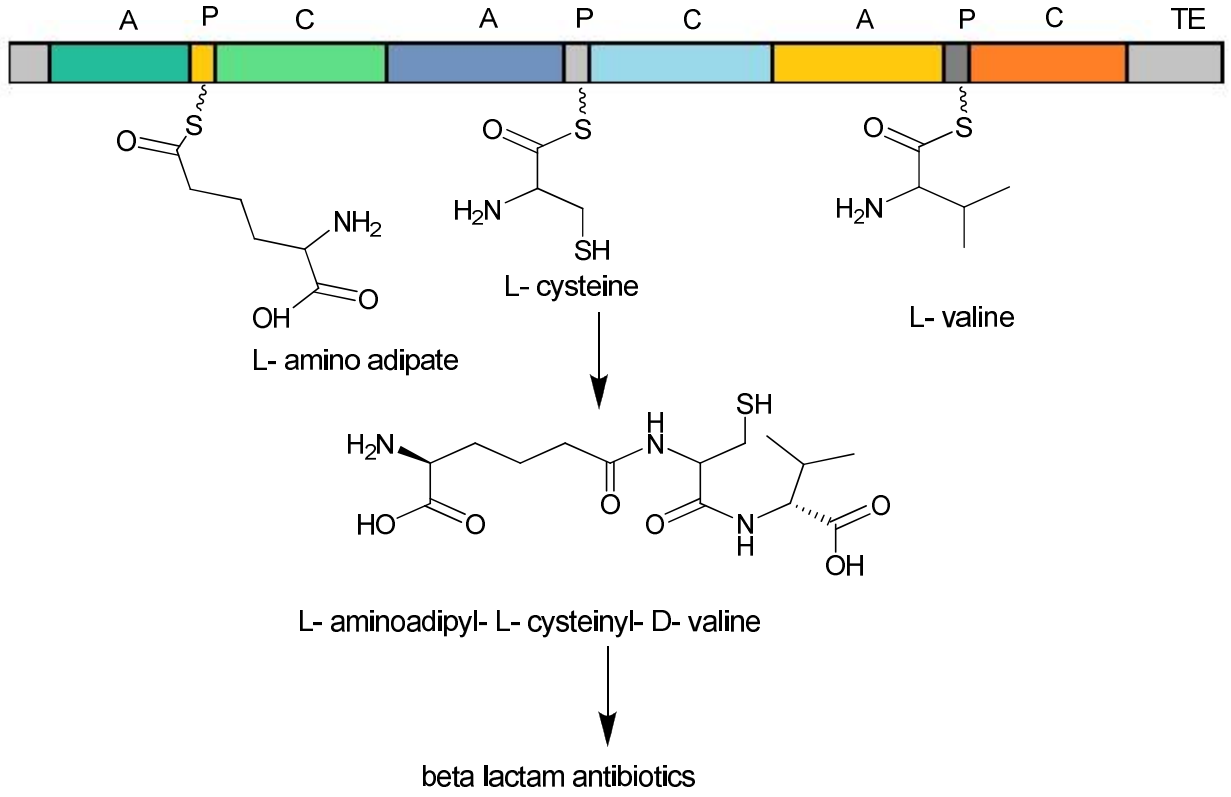


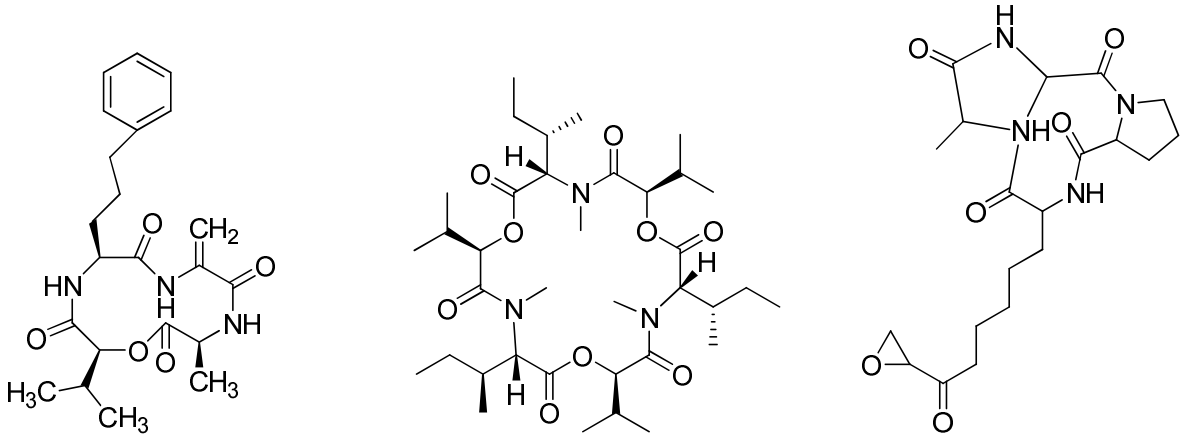
Figure 2.18 Structure and function of AVS synthase that play an important role in Penicillin and Cephalosporin biosynthesis. In figure adenylation domain is represented by A, peptidyl carrier domain is represented by P, C is condensation domain and TE is thioesterase domain

2.2.4.3 Biological functions of NRPSs in fungi

As mentioned earlier the importance of non ribosomal peptides is due to the fact that most of non ribosomal peptides acts as important pharmaceuticals like antibacterial, antifungal, immunosuppressant and anticancer drugs. But enormous amount of work has also been done to find out the biological roles of these important and diverse classes of natural products. These functions includes simple cellular metabolism, pathogenecity, responses to various types of stresses and roles in development of organism. Studies on NRPSs have revealed that mostly mono or bimodular NRPSs of the first group that have conserved domains are mostly involved in cellular processes while the NRPS enzymes belong to second group are species or strain specific and are often involved in synthesis of secondary metabolites [147].

Aminoadipate reductases are NRPSs that are involved in synthesis of metabolites required for normal cellular processes and they also play important role in fungal and bacterial lysine biosynthesis. Aminoadipate reductases are reported in all fungal strains whose genomes have been analyzed so far [156]. NRPSs have been reported in *Ascomycetes*, *Basidiomycetes* and *Schizosaccharomyces pombe* which play an important role in synthesis of siderophores. Siderophores are iron chelating compounds and play important role in acquisition of iron. Siderophores play important roles in cellular processes of many fungi e.g. in *Neurospora crassa* siderophores are shown to be involved in development of conidia [157], in *Fusarium graminearum* they play important role in sexual development and the pathogenecity of *Aspergillus 38umigates* and other plant pathogenic fungi is due to siderophores because they provide abundant quantities of iron to fungi in environments where iron is limiting factor for plants [158-159].

Many NRPs have been identified to directly act as important factors of pathogenecity. For example AM toxin **(37)** (*Alternaria alternaria*) [160] enniatin **(38)** (*Fusarium avenaceum*) [161] and HC toxin **(39)** (*Cochliobolus carbonum*) [162]. Some multimodular NRPSs are reported to have roles in development of fungi e.g. *Alternaria brassicicola* gene *AbNPS2* play important role in the development of conidial wall [154].



AM toxin (37) **Enniatin (38)** **HC toxin (39)**

Figure 2.19 Non ribosomal peptides that act as important factors of fungal pathogenecity (37-39)

2.3 History of antibiotics

The word antibiotic was first used by Selman Waksman in 1941 to those chemical molecules secreted by microorganism that kill or inhibit the growth of other organisms [163]. Penicillin was the first and most famous fungal secondary metabolite used as broad spectrum antibiotic. Penicillin was discovered by Alexander Fleming in 1929 from extract of a mould identified as *Penicillium notatum*. This discovery brought a revolution in pharmaceutical research and it saved millions of lives during the World War II. During the World War II penicillin was purified and its clinical efficacy was determined by Howard Florey, Ernst Chain,

Norman Heatley and coworkers at Oxford University [19]. Initially penicillin yield was very low but the quest for high penicillin yielding strain came to an end when Mary Hunt isolated a strain of *Penicillium crysogenum* from rotting cantaloupe. This strain was later on used for large scale production of penicillin [164]. During the world war, scientists worked hard to optimize the scale up processes for the production of penicillin and to characterize the structure of this antibiotic. It became evident that there were different types of penicillins i.e. the one isolated by Fleming from *Penicillium notatum* and the other isolated from *Penicillium crysogenum* were structurally different. Now a day's these two penicillins are known as 2-pentenylpenicillin or penicillin I and benzyl penicillin or penicillin II respectively [165].

Since 1930's quest for discovery of new bioactive products paced up and during the last fifty years many natural compounds with antimicrobial properties have been successfully reported which greatly helped to decrease the mortality and morbidity rates caused due to bacterial infections [166]. In 1940's penicillin successfully passed clinical trials and pharmaceutical company started to dependent largely on microbial products for the synthesis of new antibiotics particularly on soil actinomycetes and fungi [166-167]. Initially antibiotics and natural products were considered to be the same thing and the term antibiotic was used for those natural products that showed antimicrobial characteristics [168]. More than 50 years ago antibiotics were called wonder drugs and that era is considered the golden age of antibiotic research as a number of drugs were developed during this period e.g. penicillin, sulfonamides, streptomycin, macrolides, tetracyclins, cephalosporin and nalidixic acid [169-170].

In 1967 and 1969 some of the researchers started to believe that due to discovery of antibiotics the infectious diseases have been defeated and according to US surgeon William Stewart the book of infectious diseases be closed for ever. The infectious diseases were

considered to be of lower priority as the existing antibiotics were assumed to be sufficient to cure them. As a result antibiotics discovery and development research experienced a decline and pharmaceutical companies extended support only for synthesis of chronic disease medicines like arthritis, cardiovascular, pain and central nervous system drugs [171-172]. Several factors were involved in abandoning of antibiotics research i.e. the perception that the problem of infectious diseases was solved, lack of investment on antibiotic research and also there was no significant success stories in this field of research in 1980s and 1990s [167]. It was also becoming more and more difficult and tedious to obtain novel metabolites and screening often resulted in the extraction of the similar compounds [173].

Research regarding discovery of antimicrobial agents was at its peak in 20th century but the over optimistic belief that the war against infectious diseases has been won resulted in a major setback. Due to extensive use of antibiotics a variety of pathogenic bacteria started to acquire drug resistance through various mechanisms [174]. In 1990s methicillin resistant *Staphylococcus aureus* (MRSA) became a serious problem. Antibiotic agents like second and third generation cephems were started to be used against Gram negative bacteria instead of Gram positive bacteria. Antibiotics with weak but wide spectrum of activities were preferred to be used against Gram positive bacteria. Through research it was known that most of MRSA strains showed resistance to a number of β-lactum antibiotics because of penicillin binding proteins [175]. In 1997, MRSA strain, different from nosocomial MRSA, was discovered in United States and was termed as community acquired methicillin resistant *Staphylococcus aureus* (CAMRSA) [174].

Multidrug resistant pathogens have become a serious threat to mankind and their incidence is increasing with time. Although scientists are making efforts to synthesize and

discover new antibiotics but there are no enough new drugs in pharmaceutical pipeline to beat the pace at which multidrug resistant bacteria are emerging. In past 30 years only two truly novel antimicrobial agents has been launched in the market i.e. daptomycin and linezolid. Since 1998 only nine antibiotics entered the pharmaceutical pipeline in which only two were new antibiotics. Similarly in 2002, there were no new drugs in FDA approved list of drugs. There are about 550 drugs in developmental process and among them only six are new drugs with antimicrobial properties [166, 176].

This scenario shows that there is an urgent and serious medical need for new bioactive compounds to be discovered to find a cure for infections caused by multidrug resistant pathogens. Only the discovery of new and potent antibacterial compounds will lead to the solution of this problem [177]. If the research area regarding antibiotic production and development is not strengthened, we will be short of antibiotics and there is a serious threat that because of growing population of antibiotic resistant pathogens many infections will be left untreated.

2.4 New habitats for isolation of fungal strains with novel bioactive compounds

In terms of number of species, fungi are the second largest group of eukaryotes after insects and they act as a large reservoir of bioactive natural products. Natural products of microbial origin are mostly unexplored because microorganisms live in a wide range of environments and surely the number of existing microorganisms well exceed beyond the expected number, if millions of microbes living in unreachable environments are explored. It has been reported that microbes living in unique and harsh ecosystems has better potency to produce novel bioactive metabolites because unique habitats generally results in unique organisms with

complex and novel metabolite producing pathways. In general plant associated fungi (endophytic and rhizospheric) marine derived fungi, fungi living in soil of rainforests and mangroves, fungi associated with marine organisms and fungal species living in extreme environments show great chemical diversity and are reported to be important sources of novel natural products [178]. Some important habitats for isolation of bioactive fungal species are given as under.

2.4.1 Endophytic fungi

Endophytic fungi are microorganism that lives inside the internal tissues of plants without causing obvious harm [4]. They colonize a variety of plants including mosses, ferns, grasses and marine algae [179]. According to an estimate about 0.3 million species of plants host more than one million endophytic fungi [180]. They may be in different types of associations with plants like commensalism or mutualism depending upon the type of plants, environmental conditions, nutrient requirements and stages of development [181]. More than twenty thousand compounds have been currently reported from endophytes that include terpenoids, non ribosomal peptides, alkaloids, xanthones, phenols and quinines [178, 182-183]. Endophytic fungi provide a number of advantages to plants. For example, they provide protection to plants against herbivory [184], help the plants to survive under extreme condition of drought [184], help in regulation of growth and other metabolic activities by increasing nutrient absorption capabilities of plants [185] and also provide disease resistant to plants against wide range of plant pathogens [186].

Most of the compounds isolated from endophytic fungi are pharmacologically very important as they show antibacterial, antifungal, anticancer and antidiabetic properties [179, 187-189]. Some secondary fungal metabolites act as a source of potential treatment against

cardiovascular diseases, hypertension, atherosclerosis, ageing and degenerative brain diseases like Alzheimer disease [190-192].

2.4.2 Rhizospheric fungi

The term rhizosphere was first used by Lorenz Hiltner in 1904 and since that time this word is frequently used by scientists to refer to the area of soil around the plant roots. Rhizosphere is the zone of soil present immediately around roots of plants which contain variety of microorganisms particularly bacteria and fungi. It is a highly dynamic and biologically active zone because both plant roots and microorganisms secretes variety of chemicals by a process called rhizodeposition. Rhizosphere is very important zone as it plays an important role in crop productivity and supports large number of microbial communities that provides additional advantages to plants like assisting plants in acquisition of nutrients and conferring them disease resistance. That's why it the main focus of research in different fields of science [193-194].

Another important aspect of rhizosphere is that it is a zone where complex ecological interactions that occur continuously between a rhizosphere inhabiting microorganisms which includes competition, mutualism and parasitism. All these microbes interact with one another and with the plant roots by releasing a variety of chemicals. So in other words it is a zone of continuous chemical warfare [194]. Microorganisms residing in rhizosphere are highly dynamic as at one side they are interacting with the soil and other microbes present in the soil while on the other hand they are in communication with the plant roots. Although rhizosphere represents a picture of complex ecosystem where variety of organisms are present depending upon the type and age of the plant but this zone is predominantly occupied by bacteria and fungi. Among fungal groups saprotrophs (organotrophs) are mostly present in rhizosphere which is confirmed by biochemical analysis and PCR technique. These organotrophic fungal strains are mostly

yeasts and filamentous fungi which in most of the cases are members of *Ascomycetes* and *Basidiomycetes* [195].

DNA hybridization studies, 16s rRNA sequence analysis and fatty acid metabolic profiling has confirmed that rhizospheric microorganisms are different from other soil inhabiting microorganisms. It has been successfully shown that the diversity of rhizospheric microorganisms is strongly influenced by the type of plant species with which they are associated and nature of environment. Even it has been reported that the same fungal strain if associated with the roots of two different plant species growing in different environments will show chemical diversity. These factors suggest that rhizospheric microorganisms can be strong candidates for harvesting of biologically active secondary metabolites. But unfortunately, as compared to endophytic microorganisms much less research has been conducted on rhizospheric fungi and other rhizosphere microorganisms. Although recently actinomycetes isolated from rhizosphere of *Artemisia tridentate* (big sagebrush) have been reported for production of antifungal compounds and forty-one compounds including eighteen new compounds have been reported from fungi associated with rhizosphere of Sonoran desert plant [194].

2.4.3 Marine fungi

Due to the emergence of new infectious diseases and multidrug resistant pathogens, scientists are trying unconventional habitats of microorganisms for isolation of novel bioactive compounds to overcome this problem. For this marine habitat turned to be an attractive source for the isolation of bioactive metabolites [196]. Marine ecosystem covers more than one third of the world ecosystem where immense biodiversity is observed [197]. However, marine fungi and bacteria greatly attracted the interest of scientists as reservoirs of novel bioactive compounds.

Marine fungi have been isolated from diverse sources like sea sediments, attached to rocks, marine algae and marine organisms like sponges. Till year 2000 more than forty metabolites from fifteen different fungal strains have been reported alone from fungi associated with marine sponges [198].

Marine fungal strains live within or upon the bodies of these soft bodied organisms in marine habitat. Fungal inhabitants of these marine organisms provide them immense advantage as these marine organisms lack a definite defense mechanism. Such marine organisms completely rely on chemical defense of their fungal partner by production of bioactive natural products to survive in marine habitat. A steady increase has been observed since last decades in number of biologically active natural products isolated form marine fungi and bacteria, as alone in the year 2007 more than 960 novel compounds were isolated form microorganisms of marine habitats. As compared to the number of compounds reported in year 2006 this reflects more than 24% increase in one year. This reflects that marine microorganisms are promising sources for isolation of a large number of bioactive natural products. These marine microorganisms mostly reside in harsh environmental conditions such as high pressure, low temperature and low light and produce a diverse array of compounds for their survival [199].

Natural products isolated from marine microorganisms till date play very important role in development of new drugs. Marine natural products are either directly used as sources of new drugs or new drugs are derived from their carbon skeleton by chemical synthesis [200]. Natural products isolated from marine sources show diverse range of activities like antibacterial antifungal and antidiabetic activities but an important aspect of marine natural products is that they are potential candidates for anticancer drugs [199]. Advances in bioassay screening methods and technological advances in structure elucidation of natural products have greatly helped in

isolation of large number of anticancer compounds from marine organisms. These compounds show great structural diversity as some of these compounds are simple and occur in the form of linear peptides for example dolastatin 10 while some occur in the form of complex Polyethers with large cyclic structures like halichondrin B. They not only show structural diversity but also have complex mechanisms of action [201].

2.4.4 Soil borne fungi

The exploitation of fungi for the production of therapeutically important natural products is an interesting and challenging research field and for this reason fungi residing in many different habitats have been screened for extraction of bioactive secondary metabolites. Like marine habitat soil also supports the growth of diverse array of microorganisms and among these microorganisms soil inhabiting fungi have been reported to be an important source of bioactive natural products [202]. It has been describe that more than eighty thousand fungal species occur in soil or are associated with soil at some stage of their development. Fungi perform a variety of functions in the soil but the main function of soil fungi is that they act as degraders. Other important functions include their role as mycorrhizae; some are plant pathogens while some confer the plants disease resistance. Fungi may be present in the soil as free living organisms or they may be found in associations with other organisms such as plant, nematodes and arthropods [203].

The role of soil microorganisms particularly that of fungi and soil actinomycetes in production of bioactive secondary metabolites cannot be ignored as the pioneering drugs that successfully passed clinical trials were obtained from fungi and actinomycetes. The discovery of penicillin in 1928 by Alexander Fleming from a soil derived fungus *Penicillium notatum* was the

beginning of this field of science which attracted the attention of scientists all over the world towards soil fungi for the production of bioactive metabolites. Few years later, streptomycin was isolated from soil actinomycetes *Streptomyces griseus,* which proved very effective particularly against *Mycobacterium tuberculosis*. Vigorous searching for soil borne antibiotic producing strains of microorganisms resulted in the isolation of a number of antibiotic compounds which showed powerful activities against a wide range of pathogens particularly against Gram positive and Gram negative bacteria [204].

According to studies conducted till 2003, more than two thousand antibiotic compounds have been isolated so far from soil fungi among which more than fifty are commercially used as therapeutic agents [205]. Some examples of antibiotics produced by soil fungi which are commercially available in the market include penicillins, fusidic acid and cephalosporin. All these are broad spectrum antibiotics used to treat both fungal and bacterial infections [206]. Of the total antibiotics discovered from soil derived organisms, more than 20% have been isolated from fungi, 70% from soil actinomycetes and 10% from eubacteria [204].

In the quest for novel bioactive compounds scientists have isolated biologically active fungal strains from a variety of soil types. It has been reported that soil health and type greatly affects the soil micro flora and also promote chemical diversity. For example, bioactive fungi have been isolated from acid heath lands. Heathlands are nutritionally poor lands with acidic pH and thus have less economic value as they cannot support growth of vegetation. But such lands support the growth of fungi in which fungi release certain chemicals that kills other fungi and bacteria. It has been reported that these chemicals are antibiotics which are released by fungal strains particularly present in these soils. Such soils have different chemical and physical

properties and due to such differences in physiochemical properties one can expect differences in micro flora of such soils [207].

Cerrado is a type of land mostly encountered in countries like Brazil which is characterized by poor vegetation and as nutrient poor acidic soils with high aluminium content. It is a type of land with harsh environmental conditions and only those microorganisms can survive in such lands which have adapted their metabolism accordingly. It has been reported that microorganisms thriving in these lands have better potential for production of novel bioactive metabolites [208].

Another type of land that have been the focus of attention for isolation of bioactive fungal species include mangrove soils which are wetlands situated between sea and land. Mangroves cover about 18.1 M hectares of land and most frequently found in countries like Australia, Brazil and Indonesia. Mangroves are considered as highly productive ecosystems that support the growth of wide range of organisms including fungi, bacteria and plants [209]. Other examples of soil types which have resulted in isolation of wide variety of antibiotic producing microorganisms include agricultural lands [210] and rainforest soil [211].

2.5 Isolation of fungi

According to a widely accepted estimate more than 1.5 million species of fungi are present on Earth of which only 70,000 species have been described so far. This means that till now only 0.07 million species of fungi have been isolated and identified and more than 1.43 million species still await isolation and identification. This is because fungi live in wide variety of habitats such as different types of soils, rhizosphere of plants, on and within the bodies of plants and animals, rainforests, associated with lichens and also some fungi lives in symbiotic

associations with insects. One major problem is the isolation and culturing of fungi from these habitats because sometimes we do not have efficient isolation protocols or the fungal strain is very difficult to culture on laboratory media. The isolation and identification of cryptic fungal species is a challenge for biologists and development of new isolation techniques from unexplored habitats will lead to isolation of novel strains which will lead to isolation of new bioactive compounds. Some people are of the opinion that molecular sequencing is the sole answer to describe fungal biodiversity but each year approximately eighteen hundred new fungal species are reported and less than hundred species are sequenced each year. So if we rely solely on molecular approaches the gap will widen than narrowing. Scientists suggest that both molecular and descriptive approaches supplemented with electronic and internet data should be used to identify fungal strains [212].

In order to understand fungal diversity, isolation techniques have been established for isolation of fungal strains from diverse habitats. Some isolation techniques will be discussed in the following section.

2.5.1 Isolation of fungi from soil

Soil is a complex ecosystem that supports the growth of many diverse organisms and it becomes extremely difficult to isolate a fungus by direct picking from soil by microscopic observation. And also soil contain many different types of fungi one method is not sufficient for isolation of different types of fungi from soil, therefore different techniques have been described for isolation of fungi from soil. Some important techniques for isolation of soil fungi include serial dilution agar plating method, soil washing technique, Warcup soil plating method also known as direct soil plate method, soil sieving and soil floatation method. Among all these

methods serial dilution agar plating method and Warcup soil plating method is widely used for isolation of fungi from soil [213]. All these methods are briefly describe as under

2.5.1.1 Serial dilution agar plating method

Although many different methods are available for isolation of microorganisms from soil but serial dilution agar plate method also known as viable plate count method is one of the widely used methods for isolation and counting of bacteria, fungi and actinomycetes from soil. This method work on a principle that when material containing bacteria and fungi is plated on a suitable medium they will start growing and will form colonies. According to this method soil samples are collected from different depths depending upon the type of soil and then known amount of soil is suspended in known amount of autoclaved distilled water or normal saline. The sample is mixed and serial dilutions of 10^{-2}, 10^{-3}, and 10^{-4} up to 10^{-7} are prepared by pipetting. At last one mL of samples of different dilutions are added to already prepare sterile agar medium. Usually Glycerol yeast agar (GYA) is used for isolation of actinomycetes, Nutrient agar (NA) is used for isolation of bacteria and for fungal isolation Potato dextrose agar (PDA), Saboraud dextrose agar (SDA), or Czapec Dox agar (CDA) is used. To prevent bacterial contamination fungal culture medium may also be supplemented with antibiotics like streptomycin or tetracycline. Plates are incubated at 25 to 30 ^{o}C for 3 to 7 days. The dilutions 10^{-2} to 10^{-5} are selected for enumeration of fungi in sample while dilutions 10^{-3} to 10^{-6} and 10^{-4} to 10^{-7} are selected for enumeration of actinomycetes and bacteria respectively [214].

2.5.1.2 Warcup soil plating method or direct soil plate method

Warcup soil plating method was developed by Warcup in 1950s and as compared to soil dilution plating method, Warcup method or direct soil plating method is easy to use, samples can

be processed in less time and provide efficient results. According to this method soil samples are randomly collected in sterile containers and then mixed forming a composite sample. A small amount of the sample soil i.e. 0.005 to 0.15 g is dispensed in a sterile petriplate and is agitated slowly so that the soil sample spread throughout the petriplate evenly. Autoclaved agar medium i.e. PDA, CDA or SDA cooled to 45 ^{o}C and supplemented with antibiotics and rose bengal is added to the petriplate containing the soil sample. After solidification the plates are incubated at 25 to 30 ^{o}C for five to fifteen days [215].

2.5.1.3 Soil washing technique

In this method after collection of soil samples, definite amount of soil is measured and added to glass funnel lined with muslin clothe of 0.5 millimeter pore size. The soil samples are washed with sufficient amount of sterile autoclaved water and the out flowing water is collected in the funnel lined with muslin. The procedure is repeated several times. Muslin along with its contents is transferred into a sterile petriplate. Washed soil particles are then plated on PDA or CDA containing rose bengal and antibiotics. Like other methods plates are incubated at 25 to 30 ^{o}C for 7 to 15 days and the growth is observed regularly. Soil washing technique is considered to be an efficient technique for isolation of fungal hyphae in the soil [216].

2.5.1.4 Soil sieving method

Sieving method is an efficient method for isolation of Vesicular Arbuscular (VA) mycorrhizal spores from soil samples. For this after collection of soil samples about 50g of soil is mixed well with preheated 200mL distilled water so that all the soil aggregates separates and

leave behind a uniform suspension. In order to remove the larger particles such as plant roots and organic matter the suspension is passed through a 710 µm sieve. 200 mL water is added and the suspension is again mixed and passed through 710 µm sieve. Then this suspension is serially passed through 250, 75 and 45 µm sieves. On 45 µm sieve the residues are collected, washed and spores are isolated [213].

2.5.1.5 Soil floatation method

It is another method used for isolation of VA mycorrhizal spores from soil samples. In this method the soil sample is blended with the help of a blender for 2 to 3 minutes to make the spores free which may be attached to roots, enclosed within the sporocarps or attached to soil particles. These particles are passed through a fine sieve and then thoroughly wash with water so that it becomes a colloidal like solution. In a sterile centrifugation tube first 10 mL of 20% then 10 mL of 40% and then 10 mL of 60% sucrose solution is added to form different layers. Now about 15 mL of sample solution is added to this tube containing different percentages of sucrose solution and centrifuged at 300 rpm for 3 to 5 minutes. The debris is removed and the spores are rinsed on the surface of a fine sieve using a comparatively strong stream of water which removes the sucrose leaving behind the spores which can be collected from the sieve [213].

2.5.2 Isolation of endophytic fungi

Since 1970s the term endophytes have been frequently used in mycological literature to describe those fungi which are present internally in plant tissues. These fungi do not apparently cause any harm to plant tissues and it is rather a symptomless association between plants and

fungi. Endophytes have been reported to colonize both herbaceous and woody plants of nearly all regions of the world i.e. temperate, tropical and boreal forests and plants of xeric, arctic and alpine habitats [217]. Exploration of endophytic fungi was one of the attractive topics of 1970s and 1980s and even today because of their diverse nature isolation of bioactive compounds from endophytic fungi is the focus of attention of many scientists around the world. Endophytes are distinguished from mycorrhizal fungi as it is believed that mycorrhizal fungi generally colonize the roots but endophytic fungi usually colonized the aerial parts of the plants such as stem, leaves, petioles, bark and floral parts. However this distinction is not very clear because endophytes have also been isolated from the roots of a number of plants [218].

Isolation of endophytic fungi is a comparatively simple and routine method used for isolation of endophytic fungi from plants employs the use of surface sterilized tissues. The plant part from which endophytic fungi is to be isolated is first rinsed with autoclaved water and then surface sterilized with the help of chemicals. The most common surface sterilizer used is 2 to 10% solution of house hold bleach (NaOCl). Other effective disinfectants commonly used in laboratories include 2% $KMnO_4$ solution and 35% H_2O_2. Sometimes along with surface sterilizer a wetting agent is also necessary particularly in case of hydrophobic plant materials. Wetting agent greatly improves the performance of the surface sterilizer. Ethanol and Tween 80 are generally used as wetting agents in most of the cases. The concentration and exposure time of different surface sterilizers is different for different types of plants but usually they are used for one to three minutes [218].

There are some other sterilants such as mercuric chloride, ethylene oxide, silver nitrate and formalin which are not commonly used in isolation of endophytic fungi. However, literature provides evidence of the use of these surface sterilizers for surface sterilization of plant tissues

[219]. For example, silver nitrate in concentration of 1% is used for surface sterilization of stem and root of several grasses and silver nitrate can be precipitated after sterilization by washing the roots with 5% sodium chloride solution. A 0.01% concentration of mercuric chloride for one minute can be use for sterilization of leaves and roots of plants [220] and a 30-50% solution of formalin is also reported to be an effective surface sterilizer [219, 221] Propylene oxide and ethylene oxide are very toxic and also because of their slow rate of permeability they are not favored as surface sterilizers but can be used for sterilization of field equipments and surface disinfection of woody plants.

For isolation of endophytes the part from which isolation is performed is cut into pieces of 2-3 millimeter and then it is surface sterilized. These pieces after surface sterilization are rinsed with autoclaved distilled water to wash the surface sterilizer. The pieces are micro dissected with the help of a sterilized blade and pressed into one of the routinely used mycological media such as PDA or CDA and incubated at 28 ^{o}C for three to four weeks. Endophytes are generally slow growing and if rapidly growing fungi appear in the medium within first two weeks they must be discarded. After two to three weeks endophytes usually starts to grow on the medium which can be recognized from their white or half white colonies. Seeds can also be used for isolation of endophytic fungi but first the glume of seeds must be removed. Seeds also require a strong surface sterilizing solution and for more time [217].

2.5.3 Isolation of marine fungi

Marine fungi are defined as those fungal species whose growth and development completely occurs in sea water or in saline conditions. According to this definition salt tolerant fungi are considered to be marine fungi. Marine fungi show great diversity in form, function and

biochemistry. The type of isolation technique for the isolation of marine fungi depends largely on the substrate from which the fungus is being isolated e.g. marine algae and plants, marine animals and sponges, sea water, sea foam, marine rocks and sediments. Some commonly used methods for isolation of marine fungi includes floatation method, single spore isolation method and growth on sea medium [222].

One of the most important and tedious step in the isolation of marine fungi is the collection of samples from the site of fungal isolation. For the isolation of fungi from marine water, water samples are collected from different depths of the sea with help of sterilized screw capped bottles. The bottle is dipped in water with the cap on and the cap is removed at the site of sampling underwater. When the bottle is filled with water it is again recapped while still in water. For the isolation of fungi from marine water samples two methods are commonly used i.e. membrane filtration method and agar plating method. In membrane filtration method water sample is filtered through a membrane filter of 0.45 μm pore size so that all bacteria pass through the filter. The filter is then placed in the centre of routinely used mycological agar media and sometimes supplemented with additional supplements. The media is incubated at 20- 22 ^{o}C for 3 to 10 days. In agar plating method about 500 μl of marine water is spread on either Malt extract agar or Saboraud glucose agar media containing antibiotics for prevention of bacterial growth with help of sterilized spreader. The media is incubated at 22 ^{o}C for 3 to 10 days for isolation of fungi [223].

Deep sea sediments are collected with the help of corers which may be multiple corers, box corers or long gravity corers. The corer is lowered into the sea and penetrated into the bottom. When pulled out the top and bottom of the corer automatically closes. Box corer has the ability to take about 20 cm long sample. Research submarines are designed for collection of deep

sea sediment samples [224-225]. For isolation of fungi from marine sediments isolation procedure described by Bills and Polishook (1993) is efficiently used. For this one gram of sea sediment is mixed in sterile sea water and filtered through two meshes of 200 and 100 μm. The particles that remain on 100 μm mesh are plated on mycological culture media containing antibiotics. The media plates are incubated under pressure at 5 ^{o}C for 30 days for isolation of fungi [226].

Fungi isolated from marine sponges have been reported to be rich sources of bioactive secondary metabolites. For isolation of fungal strains from marine derived sponges, the sponges are collected from different depths of the sea water. Depending upon the sponge genera some samples needs to be processed immediately while some can be stored for longer time. The sponge material can be stored in sterilized sea water supplemented with antibiotics at -20 ^{o}C. After surface sterilization the sponges are gently cut to expose internal tissues which are again sterilized with autoclaved artificial sea water. Internal tissues are cut into small cubes of about 0.5cm^3 and placed on mycological agar media. Different culture media are used for fungal strains isolation from marine sponges but some commonly used media include Glucose peptone yeast extract, Biomalt agar Malt extract soya meal agar, Cornmeal agar, Potato carrot agar, Cellulose agar and Glucose peptone yeast extract agar. These media are supplemented with antibiotics to reduce bacterial contamination and also sometimes cyclosporine is added to media to retard the growth of rapidly growing fungal strains. After inoculation the plates are incubated at 20 ^{o}C for 15 days for isolation of fungi [198].

Different isolation techniques have been suggested for the isolation of endophytic fungi of marine weeds. Endophytic fungi of algae can be isolated by surface sterilization of algal filaments with 80% ethanol and then by sterile distilled water. The algal fragments are then cut

into smaller pieces and are placed in a suitable fungal culture medium such as malt extract agar and placed in incubator at 25 ^{o}C. Growth of fungal hyphae from the cut ends of the algal fragments is observed for two weeks.

2.6 Bioassay screening

Large numbers of bioactive compounds are reported each year from variety of organisms such as plant, fungi and actinomycetes. A good amount of these compounds are currently benefiting human beings as they act as important therapeutics and other drugs for example they are used as antimicrobials, immunosuppressive, anticancer and cholesterol lowering agents or these compounds may be used as pesticides or herbicides. But question arises how do we know which compounds are beneficial and have the desired properties? The property of bioactive natural products is that they will exert their effect on other living organisms. Techniques are available in science through which we can measure and compare the effect of different compounds or even the same compound under different conditions. These techniques are collectively termed as bioassay screenings which are defined as techniques used for estimation of potency of a compound by measurement of biological response that it produces against other organisms [227-229].

Different bioassay screening methods are in use now a day's which may be of biological, biochemical or pharmacological nature. However, bioassay screening methods used for isolation of biologically active natural products should fulfill some conditions i.e.

a) **Simplicity:** which means that particular bioassay can be operated on small scale, the procedure should be simple and it should require small amount of test sample.

b) **Rapidity:** This means that the described procedure will provide accurate results in less time.

c) **Reproducibility:** The same experimental procedure if repeated should provide similar results at different occasions.

d) **Comparability:** The results obtained must be comprehensive and experimental data should be proved numerically. The data obtained should be comparable to other research data conducted using similar procedures [230].

Different types of bioassay screening methods are available which are suitable for screening of crude extracts of plants, fungi and actinomycetes but selection of particular bioassay protocol and its execution depends upon the expertise and resources available. Some frontline invitro bioassays which we have used in our research are antibacterial activities, antifungal activities, brine shrimp lethality assays or cytotoxic activities, phytotoxic activities and herbicidal activities. All these activities are briefly discussed in the following section.

2.6.1 Antibacterial activities

To test the extracts for antibiotic activity different methods can be used depending upon the expertise, availability of resources and purpose. One of early used important methods for antibacterial testing includes bioautographic method in which bioassay screening of known microorganisms is performed by the utilization of either thin layer chromatography (TLC) or paper chromatography. The advantage of this method is that it is not only rapid but also provides qualitative results [231]. However quantitative methods for antibiotics assays are also available which include turbidimetric method [232] and agar plate diffusion assays [233].

As antimicrobial activity testing is the first step toward searching for bioactive natural products which should be more precise and for this qualitative and sensitive bioassay screening methods should be employed. The selection of test organisms can also exert significant effects on the results obtained. For example in preliminary testing of antibacterial properties of a compound pathogenic stains of common test organisms i.e. *Staphylococcus aureus, Bacillus subtilus*, *Pseudomonas aeruginosa* and *Escherichia coli* should be used. But specific antibiotic activities of bioactive compounds can also be performed using specific strains of organisms. it has been suggested that the main frontline bioassays should be simple, inexpensive, sensitive should provide results in less time, and should have a broad range of activities [234].

Finding antibacterial activities of compounds by determining minimum inhibitory concentration (MIC) against the test organisms is one of the simple bioassay methods available. According to this method, serial dilutions of the test samples are prepared and incorporated into known concentration of suitable agar growth medium. Fixed volume of agar medium and desired concentration is poured into petriplate and allowed to solidify. 20 µL of bacterial broth culture diluted to 10^5 colony forming unites (CFU) per mL are inoculated onto the agar medium and incubated along with positive and negative controls for 24 or 36 hours. The lowest concentration at which the test organisms are inhibited is considered as MIC. MIC values are measured in µg/ mL and lowest values are of particular interest. In determination of MICs different factors are brought into considerations for example whether Gram positive or Gram negative bacteria, strain of pathogenic bacterium being used and density of the inoculum [235].

Agar diffusion method is perhaps the most widely used methods for evaluation of antibacterial ability of natural compounds because it is a simple, sensitive and cost effective technique in which the results are obtained simply by measuring the zone of inhibition produced

by organisms sensitive to that compound. Agar diffusion assays are further of two types. The first one is called paper disc diffusion method in which the test compound is loaded on paper discs, allowed to dry and placed on agar plates preinoculated uniformly with pathogenic bacteria. Both positive and negative controls are used in this method. After a definite time period when the zones of inhibition become clearly visible, are measured in millimeters to obtain the results. Other method is termed as agar well diffusion technique which is similar to paper disc diffusion technique but in this small wells of known size are made in agar medium with the help of a sterilized cork borer. Dilutions of the test compound are made and a known amount is carefully poured into the wells with the help of a micropipette which slowly and uniformly diffuses into the agar medium. In this method also both positive and negative controls are used and after incubation for specific time the zones of inhibition are measured to record the results. However, the size of zone of inhibition is effected by various factors like density of the inoculum, volume of the compound in the well and time duration for which the assay is allowed to get the results [236-237].

2.6.2 Antifungal activities

Antifungal activities of natural bioactive compounds can be determined by a number of bioassay techniques. Some important invitro antifungal bioassay techniques provide the results by testing the effect of compound or extract on germination of fungal spores, on linear growth of fungi or on fungal biomass. Fungal cultures for bioassay screening can be obtained from a number of sources but it is recommended that only vauchered fungal isolates should be used. It is also important to use the same fungal isolate throughout the bioassay as the same fungus isolated from different sources may show different susceptibility pattern [236]. Furthermore, it is also

suggested that the same culture media or even the same batch of culture medium should be used throughout the antifungal bioassay screening as batch to batch variation in the medium can lead to changes in status and percentage of nutrients available to fungi. This can affect the susceptibility results of antifungal bioassays to a particular test compound [238].

Fungal spore germination assays are conducted in order to evaluate the effects of natural products on the germination of fungal spores and also on the growth of germ tube. This assay also provides an advantage to know that whether the compound under consideration is a fungicidal or fungistatic. Both liquid and solid culture media can be utilized to conducting this type of antifungal bioassay. In order to test whether the test compound or extract is fungistatic or fungicidal the non geminated spores are collected and transferred to fresh medium. These spores are incubated at optimum temperature for definite period of time. If spores show germination the test compound or extract is considered as fungistatic otherwise the effect of compound or extract is recorded as fungicidal [239-240].

Like for antibacterial activities a compound or extract can be evaluated for antifungal activities using agar diffusion assays as described above. Both agar well diffusion method and paper disc diffusion methods are equally applicable. In both methods plates after inoculation of fungi are incubated at optimum conditions and are examined regularly during the incubation period to check the growth pattern of fungi under observation. Any deviation or deformation from the normal fungal growth pattern like retardation in growth, change in colony color and sporulation time etc. are recorded which indicate antifungal activity [236]. Agar diffusion assays are the most widely used assays for determination of antibacterial and antifungal activities and numerous antifungal compounds have been identified through these techniques. Some of the

important antifungal compounds identified through these techniques include Flavonoids, alkaloids, monoterpenes and naphthoquinones [241].

Antifungal activities can also be determined by determining the effect of a compound or crude extract on growth of fungi. For this both radial and linear growth of fungus can be used. Agar media along with known concentration of the test compound is added into petriplates and a plug of test fungus is placed at the center of the plate. Colony diameter is measured regularly and compared with control. From the recorded data fungal growth rate can be determined by plotting colony diameter against the time. Similarly antifungal activities can also be determined from the linear growth of fungi but for this agar media is prepared in test tubes to make agar slants. In this method linear growth of fungi is measured and compared with control to find out percentage of inhibition [236, 242].

2.6.3 Cytotoxic activities

Brine shrimps belong to a group of aquatic crustaceans and are placed in the genus *Artemia* which have eight species which mostly live in waters of high salt concentrations. The eggs and larvae of brine shrimps particularly those of *Artemia nauplii* are commercially used as fish feed. One of the important properties of *Artemia* species is that they produce dormant eggs called cysts which can be stored for longer periods of time and can be hatched whenever needed [243-244].

In modern times brine shrimps are considered as standard organisms for testing the toxicity of chemicals, plant extracts and fungal toxins. Brine shrimp toxicity assay was first proposed in 1956 by Michael and coworkers [245] and this technique is based on the ability of a chemical or toxin to kill laboratory cultured brine shrimps. It is a very effective technique for

preliminary cytotoxicity screening of plant, fungal and algal extracts. Brine shrimp lethality assays have also been used for testing the cytotoxicity of heavy metals, dental material and pesticides. The brine shrimps eggs are hatched by placing them in sterilized artificial sea water and a known concentration of the test compound within 48 hours. Cytotoxic activities of the test compound are determined by counting the number of shrimps that survive within 24 hours [246].

Apart from brine shrimps lethality assays a variety of other bioassay screening methods have been proposed for testing of crude extracts for antitumor or cytotoxic activities. For example crown gal tumor bioassay (CGTB), star fish bioassay (SFB), sea urchin bioassay (SUB) and bioassay techniques that involve the utilization of human lung carcinoma (HLC) and colon carcinoma (HCC) cells. CGTB is a simple, fast and inexpensive bioassay screening method which can be used for detection of antitumor activities of crude extracts of plants and fungi. This method utilizes *Agrobacterium tumefaciens* a Gram negative bacterium that cause crown galls or tumors in the plants by insertion of its Ti plasmids. According to this method surface sterilized potato tuber discs are placed on autoclaved water agar medium in petriplates and different concentrations of the extracts dissolved in some appropriate solvent are spread on potato discs. After evaporation of the solvent potato slices or discs are inoculated with 0.1 % of *A. tumefaciens* broth culture and incubated at suitable temperature. By this assay the inhibitory effect of test extracts on tumor cells can be easily measured on the potato discs. There is one limitation of this technique that it cannot be used for extracts that show antibacterial activity as it will inhibit *A. tumefaciens* and tumors will not be induced on potato discs [234, 247].

In SFB fertilized star fish (*Asterina pectinifera*) eggs are used to test the cytotoxic activities of different antineoplastic chemicals and crude extracts because the plasma membrane of *A. pectinifera* eggs are permeable to variety of chemicals. When these fertilized eggs are

exposed to different compounds, depending upon the nature of compound different results are obtained e.g. aphidicolin caused inhibition of DNA polymerase, vinblastine inhibited cell division by inhibition of microtubule assembly and cycloheximide proved as protein inhibitor against *A. pectinifera.* SUB is a relatively similar technique in which fertilized sea urchin eggs are used in place of star fish eggs but as this bioassay is not applicable for some antitumor agents so SFB is mostly preferred [234]. Antitumor or anticancer activities of the compounds can also be evaluated by utilization of human cancer cell lines invitro [246, 248].

2.6.4 Phytotoxic activities

Natural products with phytotoxic properties have been reported which have played a lead role in development of new herbicides. Plants are considered important sources of phytotoxic substances which are secreted into the surrounding environment by living tissues and are termed as allelochemicals [249]. However, alternative to plants fungi have recently been utilized for production of phytotoxic substances. Comparative to plants fungi are easy to isolate and grow in laboratory conditions many researchers have focused their attention on phytotoxic substances of fungal origin [250].

For testing the phytotoxic nature of secondary metabolites different phytotoxic tests are performed which mostly uses fresh water organisms like algae and duck weeds. Other organisms like diatoms, whole plants and seeds can also be used for phytotoxicity testing of natural products [251]. The term duck weeds are generally referred to small fresh water floating plants that consists of two parts i.e. fronds and roots and belong to family *Lemnaceae.* This family has four genera i.e. *Lemna, Wolffia, Spirodela,* and *Wolffiella*. Among these, species of *Lemna* such as *Lemna minor* and *Lemna gibba* have been widely used for phytotoxicity testing. These species

are considered as standard organisms for phytotoxicity testing in aquatic environments such as lakes, rivers and streams. They show an active growth rate and wide range of distribution. They are easy to grow and test in laboratory [252].

Toxicity tests with *Lemna* species can be conducted both in static and flow through experiments. Generally static experiments are used which are easy to conduct, economical and particularly helpful for screening of samples with uncharacterized metabolites [253]. The static test is conducted under controlled environment at a temperature of 28 ^{o}C, light of cool white fluorescent quality, light intensity of 9000 lux and continuous photoperiod. The test can be conducted in glass vessels such as glass beakers, jars, petriplates or flat bottom test tubes but plastic vessels are generally avoided as *Lemna* has the tendency to adhere to plastic vessels. It is recommended that only one type of test vessel should be used throughout the experiment and it should be covered to avoid excess of evaporation. The test sample should be in liquid form with a liquid height of more than 5 centimeter in the test vessel so that *Lemna* plants are fully accommodated in it. A variety of nutrient media are used for *Lemna* bioassay in which sufficient amount of nutrients are added for optimum growth and multiplication of *Lemna* [252] but the most widely used medium for phytotoxicity testing that involves *Lemna* is Hoagland's E-medium [254].

For duck weeds phytotoxicity testing two types of controls are used i.e. negative control and positive control. In most of the cases negative control is used in which no standard phytotoxic substance is added and negative control acts as quality control to which the test sample is compared. Positive controls are very much important like negative controls and in this some phytotoxic substance is used as reference. Different organic and inorganic substances have been used as reference toxicants in phytotoxic bioassay screenings such as phenol, chromate,

pentachlorophenol, sodium dodecyl sulfate and sodium chloride. When *Lemna* plants are under stress or when they are exposed to toxic substances they show different symptoms like colony break ups, chlorosis, necrosis, loss of buoyancy and deformation. Other effects such as effect on biomass and physiology are also used as end points in *Lemna* phytotoxicity tests [252].

2.6.5 Herbicidal activities

In recent times determination of herbicidal activities of plant and microbial extracts is attracting the interest of scientists and a variety of target based bioassays have been proposed for screening of natural extracts for herbicidal activities. For determination of primary herbicidal activities of microbial products surface sterilized seeds of grasses or weeds are used. The seeds are placed on filter paper inside the petridish and the filter paper is then moistened with different concentration of test extracts. Surface sterilized seeds of the test plants are then carefully placed on the filter paper in the petriplates. Depending upon size of seeds the number of seeds placed on the filter paper could be varied. The seeds are then incubated at room temperature for a definite time period and after germination the data such as percent germination, root growth, shoot growth and biomass is collected by comparing the samples with control plates. This method has been used for screening of more than 1500 extracts of soil microorganisms particularly actinomycetes for herbicidal activities [255].

Another important and simple method for screening of crude extracts for herbicidal activities is the detached leaf technique. In this method a leaf is detached from the target plant, washed and a small puncture is made in the leaf. A few micro litters of the test extract are placed on the leaf so that it could penetrate into the leaf tissues. The leaf is then carefully placed on filter paper soaked with water in petriplates. The petriplates are then sealed with parafilm and are

incubated in growth chamber at optimum conditions of temperature, light intensity and humidity. Herbicidal activities are recorded by visual observation of the leaf for necrotic or chlorotic spots. However a number of problems could interfere with the results in this method such as solubility of the extract and test species of plants. Sometimes the extracts are not soluble in commonly used solvents and also different extracts show different results in different plant species. Tissue culture techniques have also been found useful in determination of herbicidal potential of crude extracts of plants and microorganisms [256-258].

2.7 Bioassay guided isolation of compounds

One of the methods for natural product isolation is the use of proper bioassays and in most cases more than one bioassay screening test is used for proper detection of compounds. Bioassay guided isolation of compounds is a simple but repetitive process in which the compounds isolated from an organism in crude form are divided into different fractions and each fraction is bioassayed for biological activities of choice. Bioactive compounds are further fractioned till a pure compound is isolated. The discovery of new bioactive compounds using only bioassays purely depend upon luck because the activity of crude extracts could be because of higher concentration of a particular compound or in most of the cases the active compound is a known compound. However, bioassay guided isolation is a standard method which have became easier in modern times because of the availability of highly sophisticated analytical instruments and simple bioassay techniques [259].

For discovery of bioactive compounds the extraction is performed with a series of solvents with increasing or decreasing polarity. Biologically active fractions of the crude are further fractioned by the utilization of chromatographic techniques. The use of modern

equipments such as HPLC, LCMS and NMR have simplified bioassay guided isolation and identification of bioactive compounds [260].

2.8 Techniques for isolation of bioactive compounds from fungi

Fungi produce a wide range of secondary metabolites that belong to different classes of compounds such as alkaloids, polyketides and terpenoids etc. These secondary metabolites are often produced in response to self protection of fungi against both biotic and abiotic stresses. These secondary metabolites are very important for the human beings because of their interesting chemical and pharmacological properties. Some of these compounds are used for treatment and prevention of diseases, while some are used as dietary supplements and others are used as anticancer drugs. As secondary metabolites belong to different class of compounds and are of biological importance therefore, all the natural sources including fungi can be exploited for isolation of secondary metabolites using different state of the art techniques and equipments, using Column Chromatography Technique (CCT), liquid chromatography, mass spectrometry (LCMS), and preparative high performance liquid chromatography (HPLC).

Secondary metabolites can be isolated using different solvents based on their different chemical properties and solubility in different solvents such as methanol, ethanol and ethyl acetate etc. and further characterization and structure elucidation is performed using various spectroscopic techniques especially NMR [261]. Some of common and important techniques usually used for the isolation of fungal secondary metabolites are briefly discussed as under.

2.8.1 Thin layer chromatography (TLC)

TLC is a chromatographic method available for the estimation of the number of compounds in a mixture of compounds. Izamailov and Schreiber discovered TLC in 1938 which was later on standardized and also was given mane as TLC by Stahl in 1950s. Even by the discovery of so many sophisticated chromatographic techniques TLC is as important as it was before. Now a days' preparative TLC is also available which is considered to be an effective technique for separation of compounds from a mixture of a few compounds. TLC is a simple technique in which different steps can be carried out independently and it is less time consuming with other advantages like cost effectiveness, choice of a wide range of mobile phases, low consumption of solvents, high capacity of sample loading and little need for laboratory facilities. However there are few disadvantages of this technique for example separation efficiency is very poor and the results are affected by environmental conditions [262].

Drop chromatography technique was first introduced by Izamailov and Schreiber with the help of which they separated different medicinal compounds using alumina on glass plates. Ten years later Meinhard and Hall separated Fe^{2+} from Zn^{2+} by using a mixture of alumina and Celite binder. This provides the first evidence of using TLC for separation of inorganic substances. Some important fields of inorganic TLC applications include rock analysis and analysis of biological, pharmaceutical, water, industrial wastewater and food samples. The work published on inorganic TLC till 1972 was reviewed by Brinkman *et al* and that published between years 1973-1994 was reviewed by Muhammad *et al*. A comprehensive review that describes the historical background, principle, mechanism and applications of TLC was published in 1967. Commercialization of TLC began in 1965 when TLC plates were offered for sale which quickly became very popular and regarded as a quick and efficient means of compounds separation. It

was soon realized that silica gel of 60 Å pore size was the most appropriate sorbent for TLC on which most companies focused their attention in preparation of TLC plates [262, 263].

One of the major advances in TLC was the discovery of high performance thin layer chromatography (HPTLC) by Halpaap in 1973 who realized the advantages of using smaller size silica gel in preparation of TLC plates. In the mid 1970s the importance of HPTLC was recognized as it was more precise technique that could be operated in less time and less mobile phases were required. In 1980 Halpaap reported reverse phase HPTLC which soon became commercially available in the form of pre coated plates. Later in 1982 amino modified HPTLC plates were introduced by Jost and Hauck which was soon followed by cyano bonded and diol bonded plates in 1985 and 1987 respectively [263, 264].

TLC is a type of liquid chromatography in which the sample is applied on a glass, metal, aluminium or plastic plate in the form of a small spot or as thin layer of about 0.10 to 0.25 millimeter thickness. Glass plates are more commonly used in this process but aluminium and plastic plates are also utilized as they are flexible and can be cut into any size easily without disturbing the sorbent layer. Large number of substances has been employed as sorbents but silica gel, aluminum oxide, cellulose and polyamides are more frequently used sorbents. A small amount of sample is dissolved in some suitable solvent and applied as thin spot on one side of the sorbent layer. The sorbent is placed in a solvent system termed as eluent at a depth of five millimeter in a glass tank and is covered with a lid. As the eluent migrates through the sorbent the sample also moves by capillary action but at different rates which results in separation of the components of sample. The plate is removed from the tank when the solvent front reaches near the top of the sorbent plate and is allowed to dry. The bands or spots appeared on TLC plates are then visualized in UV light [263].

Although TLC is an important technique alone but recently in has been integrated to modern chromatographic and spectroscopic techniques for efficient separation and analysis of bioactive compounds. For example TLC have been combines with HPLC, Fourier transform infrared (FTIR), Mass spectroscopy (MS) and Raman spectroscopy to obtain detail data on purified compounds. Recently peak purity of compounds has been determined by utilization of UV/ visible diode array technique in TLC [262].

2.8.2 Column chromatography

Column chromatography is classical technique used in the field of chemistry and/or biological sciences to isolate a single compound from the mixtures of compounds. It is often used for isolation of compounds from micrograms up to kilograms depending on the size of column and type of sample. Normally column is a glass tube with a diameter from 5 mm to 50 mm and a height of 5 cm to 1m. Column chromatography is still used in laboratories throughout the world for preparative purposes because it is simple to use and low cost than the use of instruments for isolations. Column chromatography is divided into two types based on the flow of solvent through the column. When the solvents flows down through the column by gravitational force it is termed as gravity (Gravity or Gravitational, please Confirm this term) column chromatography and when the solvent is forced to pass down through the column by using air pressure along with gravitational force then it is called as flash column chromatography [265].

In conventional column chromatography a solid support often of silica gel is prepared and the sample which needs to be purified is placed on top of silica gel. The column is then filled with desired solvent or mixture of solvents to achieve mobile phase of desired polarity which

runs down through the silica gel in the column. As a result different components of the mixture travel through the column at different flow rates which can be collected separately at the lower part of the column. However due to small pore size the rate at which the solvent passes through silica gel is very slow for which flash chromatography can be used to speed up the percolation of solvents through the column. Flash chromatography technique is different from conventional chromatography technique is two ways i.e. in flash chromatography silica gel of smaller size is used and air pressure is used to forcefully pass the solvent through the column [266].

One of the important factors for successful separation is the use of appropriate absorbent. Silica gel (SiO_2) and alumina (Al_2O_3) are two commonly used absorbents which are available in different mesh sizes indicated by numbers written on the bottle label. Solvent system used in column chromatography is usually a mixture of two solvents i.e. a polar and a non polar solvent. Occasionally one solvent may also be used. Some of the important solvents used in column chromatography are hexanes, ethyl acetate, ethanol, methanol, ether, petroleum ether and dichloromethane [267].

2.8.3 High performance liquid chromatography (HPLC)

HPLC is highly sophisticated form of chromatography which is used for separation, identification and quantification of chemical compounds either synthetic or from the natural source. HPLC mainly consists of a column called stationary phase, pump the driver of mobile phase through the column and a detector that detects the chemical compounds, which are eluted from the column by their retention time. The different retention times of the chemical compounds are due their interaction with stationary phase and solvent system. The sample is mixed with a small amount of solvent and injected into the column [268, 269].

Depending upon the type of phase system used in HPLC, it can be differentiated in different types, i.e., normal phase chromatography (NPC), reverse phase chromatography (RPC), ion exchange chromatography (IEC) and size exclusion chromatography (SEC). Normal phase chromatography also termed as normal phase high-performance liquid chromatography (NP-HPLC) which separates different compounds in a mixture on the basis of their polarity. In this type of chromatography the stationary phase is polar and the mobile phase is non polar. The polar samples interact with the polar stationary phase and are retained for longer time in the stationary phase. In RPC or RP- HPLC the stationary phase is non polar while the mobile phase is somewhat polar. This type of HPLC functions on the principle of hydrophobic interactions that occur between non polar stationary phase, relatively non polar sample and a comparatively polar mobile phase. SEC also known as gel filtration or gel permeation chromatography separates compounds from a mixture on the basis of their size. This type of chromatography is particularly helpful in determination of molecular mass of polysaccharides and also in determination of tertiary and quaternary structures of proteins and amino acids. In IEC the compounds are retained as a result of attraction between molecules of the sample and charge sites on the stationary phase. Ions of similar charges are eluded faster while those of opposite charges are retained for longer time. IEC is widely used in purification of proteins, carbohydrates and water [270].

While analyzing compounds with the help of HPLC some standard parameters are used which can greatly affect the results if some changes occur in these parameters. Some commonly used parameters include internal diameter of HPLC column, pore size, particle size and pressure of pump. Depending upon the nature and properties of compounds these parameters can be changed accordingly. Diameter of HPLC column is a sensitive issue as larger columns are used

in industrial purification of pharmaceutical products while columns with smaller diameter are more sensitive and less solvent consumption takes place. In most cases HPLC columns are porous which increases the surface area. Columns with small pore sizes offer larger surface area while columns of bigger pore sizes provide better kinetics through the column especially for larger molecules. The column is attached to the outside through small beads of silica particles. Smaller particles are more frequently used as they provide more surface area and thus provide better separation. HPLC pumps work at higher pressures which enabled the scientists to use columns of very small pore sizes. Generally HPLC pumps with higher pressure capability and constant flow rates are preferred [265, 270].

The compound which needs to be analyzed is injected into the HPLC machine. Two types of injectors are commonly utilized i.e. septum injector and loop injector which provide the advantage of reproducible results. After injection the sample flows through the column and detector detects the compounds. The detectors can detect the compounds in many different ways for example to some HPLC machines UV spectroscopy is attached which can detect organic compounds by their UV absorption spectra. The amount of light absorbed is directly proportional to the amount of compound present in the sample. A number of information can be obtained using HPLC for example purification, identification, quantification and separation of compounds. Preparative HPLC is used for isolation and purification of compounds. It is different from analytical HPLC which is only used for analysis of a particular compound present in the sample [270].

2.9 Nuclear magnetic resonance (NMR)

Nuclear magnetic resonance (NMR) spectroscopy is a powerful technique which has a number of applications in different field of science such as biology, chemistry, medicine and

physics. However, NMR spectroscopy is considered as an essential technique for chemists interested in structural elucidation and estimation of the biosynthetic pathways of molecules. NMR spectroscopy was discovered by two groups of scientists i.e. Felix Bloch *et al* and Edward Purcel *et al* who worked independently at the end of World War II and won the Nobel Prize for this discovery in 1952. A few years later after the discovery of NMR, Proctor and Yu first used NMR spectroscopy in chemistry and discovered that two nitrogen atoms in NH_4NO_3 give rise to two different signals. The first commercial Varian NMR spectrometer for ^{1}H operating at 300 MHz was developed in 1952. At this time NMR spectrometry was rather insensitive technique for which pure solvents were required to make it operational for ^{13}C spectra. This technique was further developed to reach 200 MHz for ^{1}H in 1960s. In 1966 Ernest and Anderson made NMR spectroscopy a more sensitive technique when they introduced Fourier Transformed (FT) NMR spectroscopy. NMR spectroscopy was first used for analysis of biological molecules in 1957 when bovine pancreatic ribonuclease and some common amino acids were analyzed. After failing to observe a spectrum, H_2O was replaced with D_2O which showed four spectral lines corresponding to different types of protons present in aliphatic and aromatic amino acids. A major advancement in NMR spectroscopy was the discovery of two dimensional (2D) NMR by Ernest and his coworkers in 1976 for which he won the Nobel Prize in chemistry in 1991. 2D NMR was later on used for the structural elucidation of biomolecules and soon it was started to be used as an important structural tool for analysis of proteins. Soon 3D NMR was introduced using unlabelled proteins, which was later on developed using ^{15}N and ^{13}C labeled samples. Kurt Wuthrich received Nobel Prize for discovery of 3D NMR spectroscopy [271].

After the completion of human genome project another challenge for the biologists was the structural elucidation of biological molecules like proteins and it was shown that two

techniques i.e. X-ray crystallography and biological NMR spectroscopy can provide the structures of biological molecules at atomic resolution. Currently both techniques are well established and widely used in biological and pharmaceutical research. X ray crystallography requires crystals while for NMR the sample is prepared in solution form. Modern NMR instruments not only provide structural information about the compounds but also provide detailed data of conformation equilibrium, folding and intermolecular and intermolecular interactions. When a sample is injected into NMR it does not directly provide the structure of compound but rather it provides spectra and other information from which the structure of a compound is determined by extensive data analysis and computer based calculations. Now a day's NMR is an established technique and frequently used for structural determination of biological macromolecules with molecular mass up to 30 kDa [272].

The basic principle of NMR spectroscopy is that every nucleus spins around their axis when magnetic field is applied. This spin is due to the subatomic particles present in the nucleus. If the number of protons and neutrons are even the nucleus shows no spin but if the number of protons and neutrons is odd then the nucleus shows half integer spin (1/2). In NMR the nuclei of biological molecules have only two types of spins i.e. spin up and spin down. With this spin of nuclei a magnetic moment is associated which is termed as magnetic dipole which can either orient is parallel or antiparallel direction in an external magnetic field. The two orientations corresponds to different energy states and when spins jump from one energy state to the other energy is either absorbed or emitted in the form of electromagnetic radiations. This difference of energy between the two states is very small even if strong magnetic field is applied. With the help of NMR spectroscopy a very small difference in energy between parallel and antiparallel spins can be detected which results in the production of NMR signal [273-274].

NMR spectrometer has two important components i.e. a superconducting magnet which produces a strong and homogeneous magnetic field and a console which can produce electromagnetic radiations of different wavelengths. For NMR analysis the test sample is mixed in NMR solution in a glass tube and is placed in room temperature bore of superconductive magnet. Due small imbalance of nuclear magnetic moments polarization of nuclear spin occurs in the sample which results in net macroscopic magnetization. This magnetization is then irradiated with electromagnetic radiation. The frequency of radiations is in range of 50 to 800 MHz and is termed as radio frequency (*rf*) which is applied for very short time [272].

Important feature explored in NMR spectroscopy is that magnetic moments of individual nuclei interact with magnetic fields created by spins of nearby nuclei which can be used for correlation of different nuclei within a molecule. These nuclei either directly interact with one another through space or they interact indirectly through bond formation. The former is known as spin-spin coupling or *J* coupling while the latter form the basis for nuclear overhauser effect (NOE) which helps in the measurement of the distance between hydrogen nuclei. The detailed NMR spectra is based on both space and bond correlation between the nuclei. The *J* coupling is often used to correlate different nuclei that's why *J* coupling is termed as correlation spectroscopy (COSY). COSY is used to transfer magnetization between different types of nuclei which provide an advantage that we start experiment with one type of nucleus and during the course of experiment the magnetization can be transferred to several other nuclei. This results in achievement of wide range of sensitivities with different type of nuclei. However, in many NMR experiments the magnetization is often started with proton nuclei because they are the most sensitive type of nuclei. From proton nuclei the signal is transferred through some heteronuclei like carbon and nitrogen and returned back to protons in order to record NMR signal called free

induction decay (FID) with maximum sensitivity. The space correlation provides the basis for geometric information which is then used for structural elucidation of macromolecules with the help of NOE [272, 275].

2.10 Mass spectrometry

Mass spectrometry is an important technique which helps to determine the mass of the compound by production of ions which are then separated from one another on the basis of their charge to mass ratio *(m/z)*. These ions are then detected by the detector to plot a mass spectrum. Mass spectrometer (MS) is a highly advanced and computer based equipment. The Mass spectrometer is consist of five parts i.e. samples injector, ionization chamber, analysis of mass of ions, detection of ions and data acquisition part. MS is often used in combination with liquid chromatography (LC) and is termed as LCMS which provides an additional value in detection of compounds. LCMS is a common technique in laboratories where qualitative and quantitative analysis of drugs or biological samples such as plant or fungal crude extracts, blood, urine and plasma etc. needs to be performed. Many different ionization techniques are used for MS which are divided into two types i.e. hard and soft depending upon the extent of separation that occurs during the process of ionization. For example electron ionization (EI) is a type of hard ionization method while the most widely used ionization techniques such as electro spray ionization and matrix assisted laser desorption ionization (MALDI) are examples of soft ionization methods [276].

MALDI is the most widely used ionization technique in the analysis of large and complex biomolecules in MS which was discovered by Tanaka *et al* and Hillenkamp and Karas in 1988 [277-278]. In modern times MALDI is widely used in characterization of proteins, synthetic

polymers, oligonucleotides and sugars. In this technique a sample is applied on the target and crystallized with a solid matrix. The target is placed in a vacuum and bombarded with laser beam of photons for which nitrogen laser of 337 nm is used now a days. Although, process of ionization in this technique is not completely understood but MALDI is efficiently use to analyze compounds of high molecular mass i.e. more than 200 kDa [279].

LCMS is becoming the most important and powerful tool of liquid chromatography as in this analytical technique the separation power of liquid chromatography is combined with the detection specificity of mass spectrometry. In LCMS the sample is first separated into individual compounds by liquid chromatography and then transferred to mass spectrometer which creates charged ions and then detects them on the basis of their mass to charge ratio. The data provided by LCMS can be helpful in determination of molecular mass, structure elucidation, identification of compounds and for determination of quantity of specific compound in a given sample. With this technique the molecular mass of compounds can be determined with accuracy of 0.01% of the total molecular mass of the sample. Structural information of compounds can also be derived with the help of spectrometers called tandem mass spectrometers which have multiple analyzers [280].

CHAPTER 3

MATERIAL AND METHODS

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3.1 Culture media used

As Fungi are the main experimental organisms in this project, therefore, different mycological and other nutrient media were used for cultivation of fungi and to carry out experiments 1 to 13. A list of the culture media used in this study along with their composition is provided in the table 3.1.

Table 3.1 List of culture media used in this study

S. No	Name of the Culture Medium	Composition of the Culture Medium Used
1	**Potato dextrose agar (PDA)**	Potato starch (4 gm), Dextrose (20 gm) Agar (15 gm) dissolved in 1000 mL de–ionized water and autoclaved at 121 ^{o}C temperature for 15 to 20 minutes at 15 psi pressure.
2	**Potato dextrose broth (PDB)**	Composition of PDB is the same as PDA but in PDB agar is not added.
3	**Sabouraud dextrose agar (SDA)**	Peptones (10 gm), D–Glucose (Dextrose) (40 gm), agar (15 gm) dissolved in 1000 mL de–ionized water and autoclaved at 121 ^{o}C temperature for 15 to 20 minutes at 15 psi pressure.
4	**Czapec yeast extract agar (CYA)**	For preparation of CYA medium first we need to prepare three different solutions i.e. solution A, B, and C. Solution A is prepared by dissolving 5 gm of $MgSO_4.7H_2O$, 20 gm of $NaNO_3$, 0.1 gm $FeSO_4.7\ H_2O$ and 5 gm of KCl in 500 mL de–ionized water. For preparation of Solution B, 10 gm

		K_2HPO_4 was dissolved in 500 mL de–ionized water while solution C was prepared by dissolving 1 gm of $CuSO_4.5H_2O$ and 2 gm of $ZnSO_4.7H_2O$ in 200 mL de–ionized water. CYA is prepared by adding 5 gm Yeast extract, 30 gm saccharose, 15 gm agar, 50 mL each of solution A and solution B and 1 mL of solution C in 1000 mL de–ionized water and autoclaved at 121 ^oC temperature for 15 to 20 minutes at 15 psi pressure [281].
5	**Czapec yeast extract broth (CYB)**	CYB has the same nutrient composition as that of CYA but agar is not added in CYB [282].
6	**Czapec Dox agar (CDA)**	CDA medium was prepared by mixing 30 gm sucrose, 10 gm peptone, 0.5 gm of KCl, 3gm $NaNO_3$, 0.5 gm of $MgSO_4.7H_2O$, 0.01 gm, $FeSO_4.7H_2O$, 1 gm K_2HPO_4 and 15 gm of agar in 1000 mL de–ionized water and autoclaved at 121 ^oC temperature for 15 to 20 minutes at 15 psi pressure [281].
7	**Nutrient agar medium (NAM)**	For preparation of NAM five grams of peptones, 3 gm of yeast extract and 15 gm Agar was in 1000 mL distilled water and autoclaved at 121 ^oC temperature for 15 to 20 minutes at 15 psi pressure.
8	**Nutrient broth medium (NBM)**	The composition of NBM is the same as that of NAM except agar is not used in NBM.
9	**E. medium**	E. Medium was prepared by dissolving 1.515 gm of KNO_3,

		0.492 gm of $MgSO_4.7H_2O$, 0.86 gm of KH_2PO_4, 0.00286 gm of H_3BO_3, 1.18 gm of Ca $(NO_3)_2.4H_2O$, 0.00540 gm of $FeCl_3.6H_2O$, 0.00362 gm of $MnCl_2.4H_2O$, 0.00022 gm $CuSO_4.5H_2O$, 0.00012 gm of $Na_2MO_4.2H_2O$ and 00.00022g of $ZnSO_4.5H_2O$ in 1000 mL distilled water and autoclaved at 121 ^oC temperature for 15 to 20 minutes at 15 psi pressure [252].
10	**Artificial sea medium (ASM)**	Artificial sea medium was prepared by mixing 3.8 gm of sea salt in 1000 mL double distilled water and autoclaved at 121 ^oC temperature for 15 to 20 minutes at 15 psi pressure [242].

3.2 Methodology Phase–I

The present research project was completed in following stages

- ✓ An extensive review of literature was conducted on the proposed topic.
- ✓ Samples were collected from soil and rhizosphere of *Mentha piperita* for isolation of biologically active fungal species.
- ✓ After isolation purification and identification of the bioactive fungal species were performed.
- ✓ Biological activities like antibacterial, antifungal, cytotoxic, phytotoxic and herbicidal activities of the crude extract of two isolated fungal strains were performed.

3.2.1 Isolation of fungi

For production of bioactive secondary metabolites fungi were isolated from two different sources i.e. from soil and rhizosphere. Soil samples were collected randomly from soil of Malakandeer agricultural farm of the University of Agriculture Peshawar in clean polyethene bags. Rhizosphere samples were collected carefully from the rhizosphere of *Mentha piperita* in clean polyethene bags and both samples were transferred to Microbiology research Laboratory at the Centre of Biotechnology and Microbiology University of Peshawar, Pakistan.

Potato dextrose agar (PDA) medium was prepared according to the instructions and autoclaved along with petriplates wrapped in paper for 15 to 20 minutes at 121 ^{o}C at 15 psi pressure in order to sterilize both media and petriplates. After autoclaving PDA medium was transferred to Laminar Flow Hood (LFH) so that it cools down to 60 ^{o}C and petriplates were kept in oven at 190 ^{o}C for dryness. To prevent bacterial contamination fungal culture medium was also supplemented with streptomycin. After dryness the petriplates were also shifted to LFH and

20 -22 mL of PDA medium was carefully poured into each petriplates. The medium was allowed to solidify and after solidification the petriplates were wrapped with parafilm. Petriplates were then kept overnight at 30 ^{o}C to check for sterility.

Both soil and rhizosphere samples were separately processed and dissolved in autoclaved distilled water to prevent microbial contamination. For isolation of fungi serial dilution agar plate method was used which is one of the widely used method for isolation of fungi. For this, 3 gm of soil was suspended in 100 mL autoclaved water and ten dilutions of each sample were prepared. At last one mL of sample from the final dilution was added to already prepared PDA medium and spread it with the help of sterile glass spreader. The plates were allowed to dry for 10 minutes in LFH and then wrapped with parafilm. All these steps were carried out in LFH. Plates were then incubated at 25 to 30 ^{o}C for 3 to 7 days [214]. In order to isolate and purify the fungal strains these experiments were repeated several times. After isolation and purification the fungal strains were kept at 4 ^{o}C in the refrigerator for use in the next experiment.

3.2.1.1 Experiment No. 1

The purpose of conducting experiment 1 was to isolate and purify soil and rhizosphere inhabiting fungal species for production of bioactive secondary metabolites.

3.2.2 Preliminary antibacterial activity of isolated fungi

For preliminary antibacterial testing the pure isolated fungal strains were cultured in petriplates. For culturing of bacterial strains nutrient broth medium (Sigma Aldrich, Germany) was prepared and 10 mL media was transferred to each test tube. The test tubes were plugged with a cotton plug and carefully placed in a beaker. Nutrient agar medium was also prepared in a

flask which was also plugged with a cotton plug. Both nutrient agar and nutrient broth media along with petriplates wrapped in paper, metallic cork borer and cotton swabs were transferred to autoclave for sterilization.

After autoclaving both the nutrient media were transferred to LFH while petriplates were placed in dry air oven at 190 ^{o}C for dryness. After dryness the petriplates were also transferred to LFH. Inside LFH the nutrient agar medium was poured into petriplates and after solidification petriplates were tightly sealed with parafilm. These petriplates were labeled and were kept in incubator overnight for sterility checking. For culturing of pathogenic bacteria into broth medium a small portion of bacterial culture was transferred with the help of sterile wire loop into the test tubes containing sterile nutrient broth medium and were tightly plugged with a cotton plug. The test tubes were labeled accordingly, were placed in a beaker and transferred to incubator for incubation at 37 ^{o}C for 18 hours.

After incubation bacterial culture along with petriplates were transferred to LFH. Now with the help of sterile cotton swabs a uniform lawn of experimental bacteria were made on nutrient agar plates and allowed for 5 minutes. Now with the help of sterile metallic cork borer pustules were taken from all the seven days old fungal cultures and were placed on the lawn of bacteria in the nutrient agar plates. After covering with parafilm the petriplates were transferred to incubator for 24 hrs at 37 ^{o}C for determination of zone of inhibition. This experiment was performed in triplicate to record an accurate zone of inhibition and to identify bioactive fungal strains [14, 283].

Those fungal strains which showed considerable antibacterial activity were further processed for bioassay screening and for production of bioactive secondary metabolites.

3.2.2.1 Experiment No. 2

Through this experiment the effect of secondary metabolites produced by fungi was determined against the pathogenic bacteria and those fungal strains which produced active zone of inhibition were further preceded for production and isolation of biologically active secondary metabolites. The test pathogenic bacteria used in these experiments were *Xanthomonas oryzae, Klebsiella pneumonia, Bacillus subtilus, Proteus vulgaris, Staphylococcus aureus, Escherichia coli, Salmonella typhi* and *Shigella flexeneri*.

3.2.3 Preservation of fungal spores

Those fungal strains which showed considerable antibacterial activities were cultured on CYA medium for 10 days at 28 ^{o}C for production of spores. After completion of incubation period the fungal spores were collected with the help of 0.01% Tween–80 solution. 10 mL of the said solution was spread on the petriplate and with the help of a sterile blade the fungal spores were carefully scratched and poured into 50% glycerol solution by a sterile syringe in 15 mL falcon tubes. Spore suspensions of different fungi were prepared and labeled accordingly for future use. The spore suspensions were preserved in –20 ^{o}C freezer.

3.2.3.1 Experiment No. 3

The purpose of experiment number 3 was to preserve the spore of bioactive fungal species and to keep them viable for longer period. Keeping the spores in glycerol solution at –20 ^{o}C prevented them from germination.

3.2.4 Identification of fungal strains

The colony morphology, pigmentation and spore structure of the pure fungal isolates was studied using light microscope (100-200 magnification). Fungal strains were identified by studying traits e.g., structure of hyphae, colony structure and arrangement of spores. Coloration of the colonies was also observed by growing the fungal strains on PDA and Czapec Dox Agar (CDA) medium. For further confirmation, the isolates were sent for identification to Department of Plant Pathology, University of Agriculture Peshawar.

3.2.4.1 Experiment No. 4

Experiment 4 was conducted for microscopic and morphological identification of isolated fungal strains which were selected for production of biologically active secondary metabolites.

3.2.5 Culturing of bioactive fungal strains in liquid culture medium

Czapec Yeast Broth (CYB) medium was prepared according to the instructions in 500 mL conical flasks and after autoclaving each fungal strain was inoculated to their respective broth medium for production of secondary metabolites. This process was carried out under sterile conditions in LFH. The flasks were then transferred to shaking incubator at 28 ^{o}C and 150 rpm for 14 days. The flasks were frequently observed for first three days to trace bacterial contamination in the medium which can spoil the results.

Figure 3.1 Liquid culture of *Aspergillus* species

Figure 3.2 Liquid culture of *Penicillium* species

3.2.6 Extraction of fungal metabolites from liquid culture medium

After 14 days of incubation, 250 µL of 40% HCl was added to each flask and allowed for 5 minutes. This helped to acidify the culture medium and improves settling down of media components. After this the fungal mycelia were grinded with the help of electrical blender and equal volume of ethyl acetate was added to each flask. Mixing and shaking was performed from time to time for 30 minutes. After through mixing and shaking the mycelia were filtered with the help of cheese cloth.

After filtration the remaining mixture was transferred to separating funnel and allowed to settle down for 10 minutes. In separating funnel two different layers were observed i.e. upper organic layer formed by ethyl acetate and lower layer formed by media and water. Water and media components were removed and upper organic layer was washed with 2M brine solution by mixing and shaking for 5 minutes. The organic portion was further purified and dehydrated by adding anhydrous sodium sulphate (Na_2SO_4). After extraction crude extract was concentrated by rotary evaporator at 45 °C and 120 rpm. The dry crude extract was recovered from the flask of rotary evaporator by rinsing it with methanol.

This work was performed in Microbiology research Laboratory, at the Centre of Biotechnology and Microbiology University of Peshawar, Pakistan and in the Department of Agricultural Chemistry, The University of Agriculture Peshawar, Pakistan.

3.2.6.1 Experiment No. 5

The main aim of performing these experiments was to isolate secondary metabolites of bioactive fungal strains in crude form and to further process it for biological screening using activities like antibacterial, antifungal, cytotoxic, phytotoxic and herbicidal activities.

3.2.7 Antibacterial activities

Ethyl acetate and *n*–Hexane fractions of *Aspergillus* and *Penicillium* species were screened against eight pathogenic bacterial strains i.e. *Xanthomonas oryzae, Klebsiella pneumonia, Bacillus subtilus, Proteus vulgaris, Staphylococcus aureus, Escherichia coli, Salmonella typhi* and *Shigella flexeneri*.

For screening of antibacterial activities agar well diffusion method was employed [236-237]. For preparation of fresh bacterial cultures 50 mL nutrient broth medium was prepared and 5 mL medium was dispensed into each test tube (5 mL x 8). The test tubes were plugged with cotton plug and were autoclaved along with nutrient agar medium, petriplates wrapped in paper, cotton swabs, normal saline and metallic cork borer. After autoclaving nutrient agar medium and test tubes containing nutrient broth medium was transferred to LFH while petriplates were dried in dry air oven. After drying 22 mL nutrient agar medium was added to each petri dish in LFH and the plates were kept for solidification. After solidification nutrient agar plates were sealed with parafilm and transferred to incubator at 37 ^{o}C for 24 hours to check for sterility. Pathogenic bacteria were inoculated into sterile nutrient broth medium in test tubes and after inoculation they were tightly plugged with cotton plug. The test tubes were labeled and transferred to incubator at 37 ^{o}C for 24 hours.

McFarland turbidity standard was prepared by adding 99.5 mL of 1% sulfuric acid and 0.5 mL of 1.175% barium chloride. Turbidity of this solution was checked using spectrophotometer and after pouring this solution into screw capped test tube was stored in refrigerator. 0.5 McFarland's turbidity standard provides turbidity comparable to 1.5 x 10^{8} C.F.U/mL [284].

After completion of the incubation period turbidity of the bacterial strains was adjusted using 0.5 McFarland's turbidity standard to 1.5 x 10^8 C.F.U/mL and 20 µL of each experimental organism was inoculated on nutrient agar plates to prepare a uniform lawn and allowed for 10 minutes. Desired numbers of wells were made in nutrient agar plates keeping appropriate distance using sterile metal borer. Using sterile di-methyl sulfa oxide (DMSO), a stock solution of 12 mg/mL was prepared which was further used to prepare dose concentrations of 10, 100, 200, 250 and 500 µg/ml. 80 µL of each concentration was added in the respective well. Ciprofloxacin was used as reference antibiotic with a dose concentration of 100 µg/mL. To allow the proper diffusion of the samples into the nutrient agar medium, the plates were left at 25 ^oC for 2–3 hrs and then transferred to incubator at 37 ^{o} C for 24 hours for the screening of antibacterial activities. The experiment was repeated two times and zone of inhibition was measured from the centre to the edge of the zone in millimeters.

3.2.7.1 Experiment No. 6

These experiments were performed to evaluate the antibacterial effect of both ethyl acetate and *n*–Hexane fractions of fungal crude extracts against pathogenic bacterial strains.

3.2.8 Antifungal Activities

Both *n*–Hexane and ethyl acetate extracts of *Aspergillus* and *Penicillium* species were screened for antifungal activities against six pathogenic fungal strains i.e. *Microsporium canis Trichophyton longifusus, Candida albicans, Candida glabrata, Aspergillus flavus* and *Fusarium solani.* For determination of Antifungal activities agar tube diffusion technique was employed [242]. PDA medium was prepared according to the manufacturer's instructions in

sterile distilled water and homogenized it by heating. 10 mL media was poured into each screw capped test tube and autoclaved at 121 ^{o}C for 15 minutes. After autoclaving the media was transferred to LFH and was allowed to cool down to 50 ^{o}C. The stock solution i.e. 24 mg/mL of both ethyl acetate and *n*-Hexane fractions of both the fungal strains were prepared using sterile DMSO. Test samples with dose concentration of 10,100, 250, 500 and 1000 µg/mL were prepared from stock solution and were added to the non solidified medium in the test tubes. The test tubes were slightly vertexed to homogenize the media and test samples and after this the test tubes were placed in slanted position at room temperature to solidify the medium. After solidification a small piece of fungal inoculum was removed from a 10 days old fungal culture and implanted in the center of the slant. Meconazole in concentration of 100 µg/mL was used as standard drug in this experiment. These test tubes were then incubated at 28 ^{o}C for seven days to visually observe fungal growth inhibition. These experiments were performed in duplicate and the linear growth of fungi was calculated using the following formula [242].

$$\text{Growth inhibition (\%)} = 100 - \frac{\text{linear growth in test (mm)}}{\text{linear growth in control (mm)}} \times 100$$

3.2.8.1 Experiment No. 7

In these experiments the antifungal effect of ethyl acetate and *n*- Hexane fractions of fungal crude extract was determined against pathogenic fungal strains. Antifungal activities of the crude extract were compared with Meconazole which was used as drug of choice in this experiment.

3.2.9 Brine shrimp lethality testing

The organic extracts of *Aspergillus* and *Penicillium* species were screened against Brine shrimps eggs (*Artemia salina*) for determination of cytotoxic activities. *Artemia salina* eggs were hatched in artificial sea medium which was prepared by dissolving 3.8 g of sea salt in 1 liter of autoclaved double distilled water. 2 mg of brine shrimp eggs were measured using electrical balance and were added to the medium in a large glass bottle. This bottle was covered with aluminium foil and was placed at room temperature for 48 hours in ordinary light. After two days numerous shrimps were obtained in form of small larvae.

Stock solution i.e. (20 mg/mL) was prepared in Ethanol which was further used to prepare doze concentrations of 10, 100 and 1000 µg/mL. Equal volume of the different test samples were added to autoclaved artificial sea water medium and then 30 shrimps were transferred to each beaker. The beakers were covered with aluminium foil and were placed in ordinary light at room temperature for 24 hours. After 24 hours the number of shrimps survived were counted and lethality percentage was calculated [242, 285].

3.2.9.1 Experiment No. 8

These experiments were conducted to evaluate the cytotoxic properties of the crude extracts of *Aspergillus* and *Penicillium* species against *Artemia salina*. Cytotoxic metabolites of many fungi have been previously reported which can act as novel anticancer drugs.

3.2.10 Phytotoxic Activities

Ethyl acetate extract and *n*-Hexane fractions of *Aspergillus* and *Penicillium* species were subjected for phytotoxic activities against *Lemna* plant. E- Medium was prepared by adding

different inorganic salts in deionized water (1L) and final pH of the medium was adjusted between 5.5- 6.5 by adding 2M solution of sodium hydroxide.

E- Medium was prepared by mixing different chemicals given in table 3.1 and autoclaved at 121 ^{o}C temperature for 15 to 20 minutes at 15 psi pressure. Stock solution i.e. 30 mg/1.5 mL was prepared in sterile DMSO which was further used to prepare dose concentrations of 1000, 100 and 10 µg/mL. Test samples were allowed for some time to evaporate excess of solvent in LFH.

20 mL of autoclaved E- Medium was added to each of the three sterilized beakers and equal volume of the test samples was also added to different beakers labeled as 10 µl, 100 µl and 1000 µl. After thorough examination 10 healthy plants of *Lemna* each with a rosette of three fronds were transferred to the medium and then covered with sterile cotton. In one beaker only medium was added while in another parquet was added which was used as negative control. All the beakers were then transferred to growth chamber for seven days at 30 ^{o}C temperature, 9000 lux light intensity, photoperiod of 12 hours and humidity was adjusted at 60. This experiment was performed in triplicate. On eighth day results were recorded by visual examination of plants for symptoms like colony break ups, chlorosis, necrosis, loss of buoyancy and deformation [252, 254]. Percentage of growth inhibition was determined using the following formula.

$$\text{Growth inhibition (\%)} = 100 - \left(\frac{\text{Number of fronds in sample}}{\text{Number of fronds in control}}\right) \times 100$$

3.2.10.1 Experiment No. 9

The purpose of these experiments was to test the crude extracts of both *Penicillium* and *Aspergillus* species for phytotoxic metabolites using *Lemna* bioassay.

3.2.11 Herbicidal activities

Ethyl acetate extract and *n*-Hexane fractions of *Aspergillus* and *Penicillium* species were screened for herbicidal activities against the test weed *Silybum marianum* L. The experiment was conducted in Petri-dishes using Completely Randomized Design (CRD). For this petriplates were autoclaved and two folds of a sterile tissue paper were carefully placed in each petriplate. Seeds of *Silybum marianum* L. were surface sterilized with 0.1% solution of mercuric chloride and 10 seeds were carefully placed in each petridish on the tissue paper keeping appropriate distance. Different dose concentrations (10, 100, 1000 µg/mL) of the fungal extracts were applied to petridish containing the seeds with the help of a sterile dropper and then water was provided as per requirement each day. Each petridish was replicated three times and a control treatment was kept with each concentration. The seed germination data was recorded on daily basis and the whole experimental setup continued for 14 days. The data was recorded on the parameters such as percentage of seed germination, shoot and root length was measured in mm while shoot and root biomass was measured in grams [286-287].

3.2.11.1 Experiment No. 10

The main aim of experiment 10 was to test the ethyl acetate extract and *n*-Hexane fractions of *Aspergillus* and *Penicillium* species for their herbicidal potential against the test weed *Silybum marianum* L. and to further screen these two fungi for isolation of pure compounds of agrochemical importance.

3.3 Methodology Phase–II

In Phase–II following research objectives were accomplished successfully

- ✓ Fungal compounds were isolated using column chromatography
- ✓ Compounds were further purified using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC)
- ✓ Structural elucidation of the compounds was performed using nuclear magnetic resonance (NMR).

3.3.1 Isolation of fungal metabolites using column chromatography techniques

For isolation of pure compounds from the crude extracts the sample was dissolved in appropriate solvent and allowed for 10 minutes. Using Mortar and pestle a slurry of the sample was prepared in small amount of silica gel of 60 mesh size. Using pestle the sample was thoroughly mixed and allowed to dry completely at room temperature. The glass column was thoroughly washed with water and then with 75% ethanol and allowed to dry. A plug of cotton was placed at the bottom of the column to prevent flow of silica. Then appropriate amount of silica gel was soaked in 100% *n*–Hexane and by continuous mixing it was carefully poured into the column. 100% *n*–Hexane was poured till silica gel got completely settled and formed a clear bed in the column while the solvent was collected in a beaker placed at the bottom of the column. Then the sample was carefully poured on to the silica bed inside the column with the help of a sterile funnel. First of all 100% *n*–Hexane was passed through the column slowly three times to check for the presence of non polar compounds in the sample and then the polarity of the solvent was gradually increased by changing the solvent system (SS). After passing 100% *n*–Hexane the polarity of the SS was gradually increased by the addition of ethyl acetate to *n*–

Hexane solution. During this time TLC was regularly performed to check for isolated compounds. Whenever traces of compounds were observed on TLC the polarity of the SS was increased in fractions to increase the chances for isolation of pure compounds [265-266].

Figure 3.3 Column chromatography

3.3.1.1 Experiment No.11

The main aim of performing experiment number 11 was to isolate pure compounds from the crude extract of both the fungal strains and to further subject it to biological activities which may lead to discovery of a novel bioactive compound.

3.3.2 Thin layer chromatography (TLC)

TLC was performed on pre coated commercially available TLC plates with silica gel 60. For this TLC plates were cut into appropriate size and base line was drawn at the base of plate keeping appropriate distance. A small amount of sample was dissolved in ethyl acetate and about 10 µL of the sample was spotted above the base line on TLC plate. The TLC plate was dipped in a solvent system of appropriate polarity at a depth of five millimeter in a glass tank and was covered with a lid to prevent the evaporation of solvent. As the solvent migrated through the plate the sample also moved by capillary action but at different rates which results in separation of the components of sample. The plate was removed from the tank when the solvent front reached near the top of the plate and was allowed to dry. The bands or spots appeared on TLC plates were then visualized in UV light at wavelength of 254 and 366 nanometer (nm) [263].

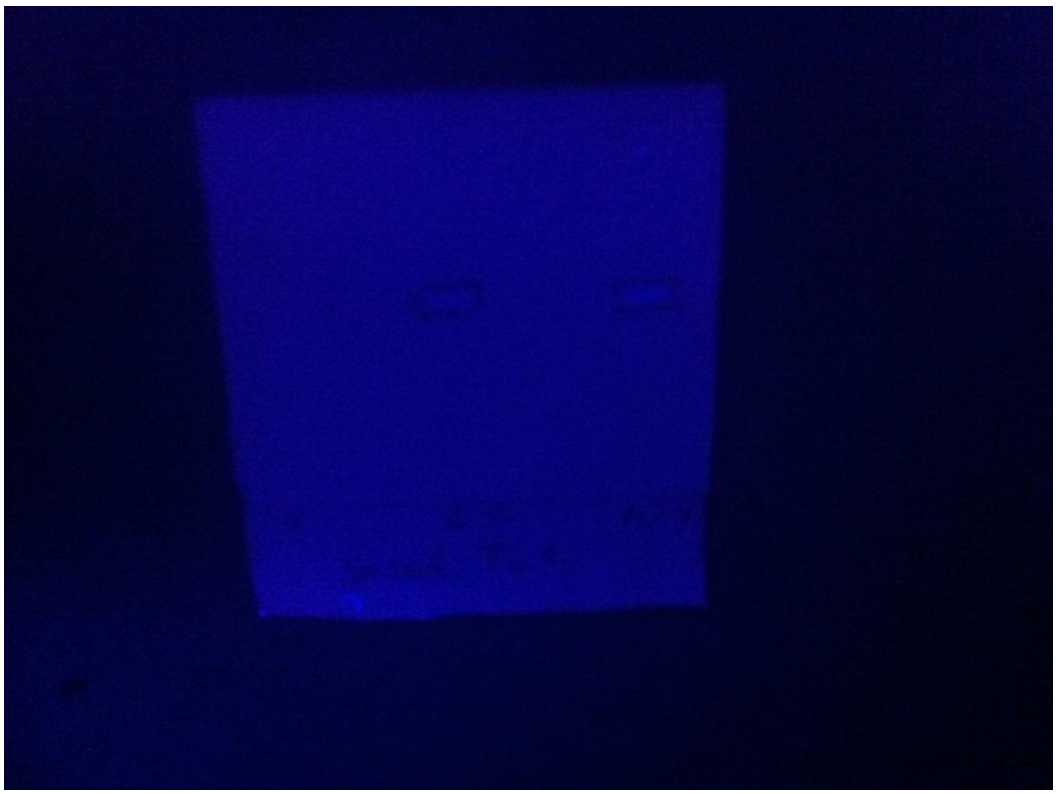

Figure 3.4 TLC plate showing two distinct bands

3.3.2.1 Experiment No.12

This experiment was performed to monitor the identity of each fungal extract and to check the qualitative purity of the compounds isolated through column chromatography. Additionally it also helped to develop the solvent system which was used in column chromatography for isolation of compounds.

3.3.3 Analytical high performance liquid chromatography (HPLC)

The pure compounds were analyzed using analytical HPLC to identify the peaks and also to find out the purity of isolated compounds. For this purpose the pure compounds were vacuum dried and then dissolved in HPLC grade acetonitrile (Sigma-Aldrich). This solution was then filtered with the help of a syringe filter to remove un-dissolved particles and to obtain a clear solution. Then 20 µL of the sample was injected into the injector of HPLC machine (WATERs) and the valve of injector was closed. The solvent system consisted of methanol and water. The analytical run was made for 30 minutes using column with C –18, 5 µ, 100 Å and 250 × 4.6 mm specification while the flow rate of 1mL/minute was maintained in the column with the help of pump. The compounds were detected in UV range between 200 and 400 nm.

Figure 3.5 HPLC used in this study

3.3.3.1 Experiment No. 13

This experiment was performed to further check the compounds for purity and also to find out the peaks of interest.

3.3.4 Structural elucidation of compounds

1D and 2D NMR techniques along with MS techniques were used for structural elucidation of isolated compounds. New and known compounds were recognized using Chapman & Hall Natural Products on CD-ROM (2007) and Chemical Abstracts (Scifinder). Structures were designated as new, if they could not be found in Chemical Abstracts.

3.3.4.1 Nuclear magnetic resonance (NMR) spectroscopy

1D (^{1}H, ^{13}C) and 2D (HSQC, HMBC, COSY) NMR spectra were obtained using 500 MHz Varian NMR machine. Chemical shifts in ppm were referenced to the internal tetra methyl cylane (TMS). The unity of 500 MHz stands 500 for ^{1}H and 125 for ^{13}C NMR.

3.3.4.2 Mass spectrometry (MS)

ESI mass spectra

An Electrospray Ionization (ESI) mass spectrum was measured from an API-150EX mass spectrometer (Applied Biosystems) with a turbo ion spray source.

HR-FTICR-MS spectra

The high resolution positive ion ESI mass spectra were obtained from a Bruker Apex III 70e Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer equipped with a 7.0 T superconducting magnet, an RF-only hexapole ion guide and an external electrospray ion source. The sample solutions were introduced continuously via a syringe pump with a flow rate of 120 µl/h.

CHAPTER 4

RESULTS OF BIOASSAY SCREENINGS

CHAPTER 4 RESULTS OF BIOASSAY SCREENINGS

4.1 Antibacterial activities

The crude extracts of *Aspergillus* and *Penicillium* species were tested against eight pathogenic bacterial strains namely *Xanthomonas oryzae, Klebsiella pneumonia, Bacillus subtilus, Proteus vulgaris, Staphylococcus aureus, Escherichia coli, Salmonella typhi* and *Shigella flexeneri*. The results of antibacterial activities of *n*-Hexane and ethyl acetate fractions of *Aspergillus* and *Penicillium* species are summarized in figures 4.1, 4.2, 4.6 and 4.7. A dose concentration from 1 to 500 µL/mL of the crude extracts was used against pathogenic bacterial strains and the zone of inhibition was measured in millimeters. However, a dose concentrations ranging from 1 to 10 µL/mL showed no clear zone of inhibition.

4.1.1 Antibacterial activities of *Aspergillus* species

For the determination of antibacterial activities, both the *n*-Hexane and ethyl acetate fractions of crude extract of *Aspergillus* species were tested against pathogenic bacteria. First clear zones of inhibition were observed at a concentration of 20 µL/mL of ethyl acetate fraction which was recorded as 5 mm against *S. aureus* and 2 mm each against *B. subtilus* and *K. pneumoniae*. At dose concentration of 50 µL/mL of ethyl acetate fraction, zones of inhibition were recorded as 7.5 mm against *B. subtilus*, 13 mm against *S. aureus,* 5 mm against *K. pneumoniae* and 3 mm against *S. typhi*. A dose concentration of 100 and 250 µL/mL of ethyl acetate fraction of *Aspergillus* species inhibited the growth of all pathogenic bacterial strains except *X. oryzae.* At 100 µL/mL highest zone of inhibition was recorded against *S. aureus* (25.5

mm), *B. subtilus* (15 mm) and *P. vulgaris* (12 mm). A dose concentration of 250 µL/mL of ethyl acetate concentration significantly inhibited the growth of *B. subtilus* (30.5 mm), *S. aureus* (28 mm), *S. flexeneri* (16.5 mm), *K. pneumoniae* (15 mm), *E. coli* (15 mm) and *P. vulgaris* (14 mm). At 250 µL/mL no zone of inhibition was recorded against *X. oryzae* while a zone of 10.5 mm was recorded against *S. typhi.* A dose concentration of 500µL / mL all the bacterial strains were inhibited but the highest zones of inhibition were recorded against *B. subtilus* (47.5 mm) and *S. flexeneri* (45 mm) while lowest zone of inhibition was recorded against *X. oryzae* (9 mm).

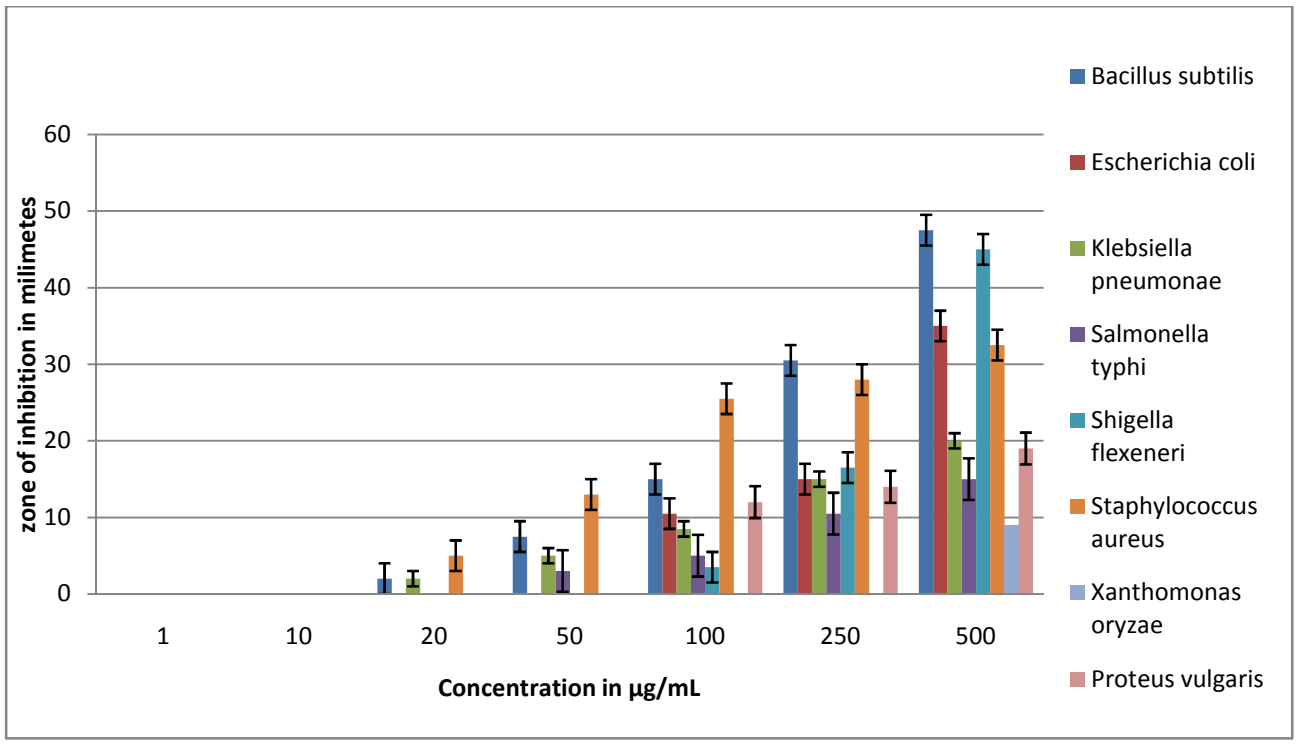

Figure 4.1 Antibacterial activities of ethyl acetate fraction of *Aspergillus* species

The *n-Hexane* fraction of the crude extract of *Aspergillus* species showed limited activity against the pathogenic bacterial strains. A dose concentration of 250 µg/mL inhibited the growth of *S. typhi* (25 mm), *P. vulgaris* (8 mm), *S. flexeneri* (5 mm), *B. subtilus* (5mm) and *E. coli* (4.5 mm) while no zone of inhibition was recorded against *X. oryzae*, *S. aureus* and *K. pneumoniae*. A dose concentration of 500 µg/mL inhibited the growth of all the bacterial strains. Highest

activity was recorded against *S. typhi* (51 mm) while the bacterium that showed least inhibition was *X. oryzae* which showed a 3.5 mm zone of inhibition.

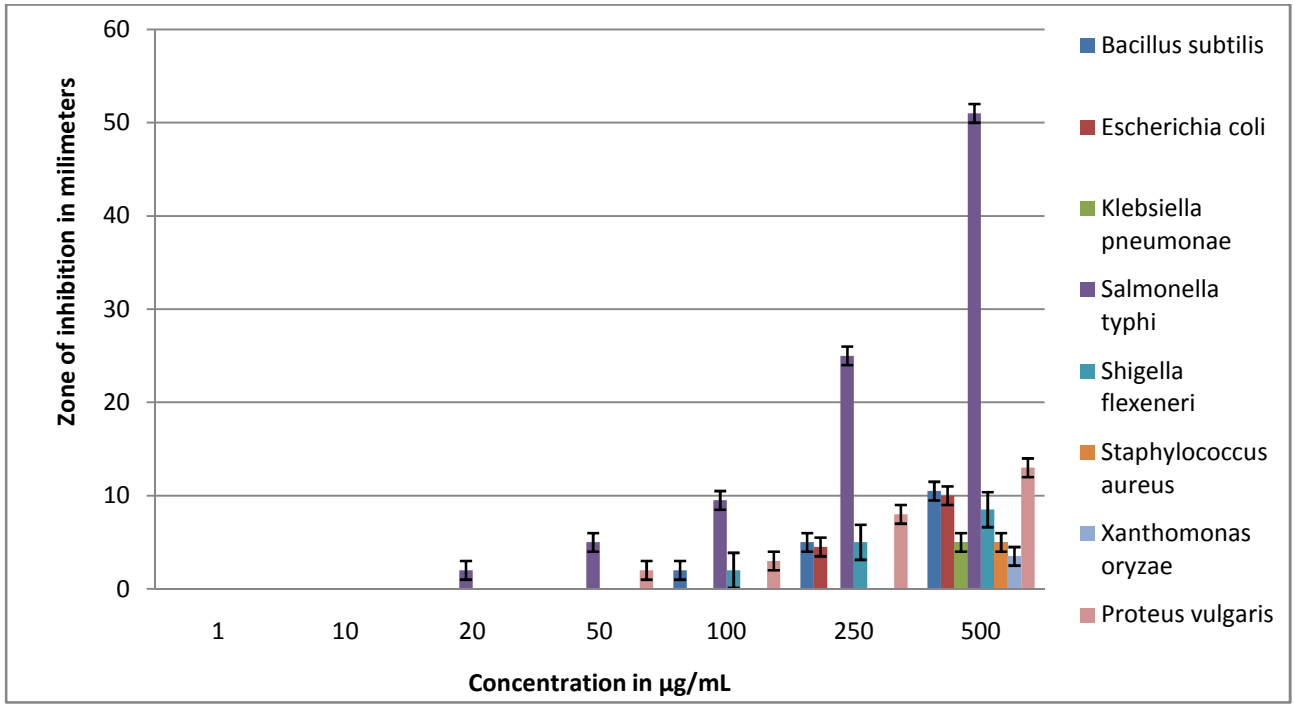

Figure 4.2 Antibacterial activities of *n*-Hexane fraction of *Aspergillus* species

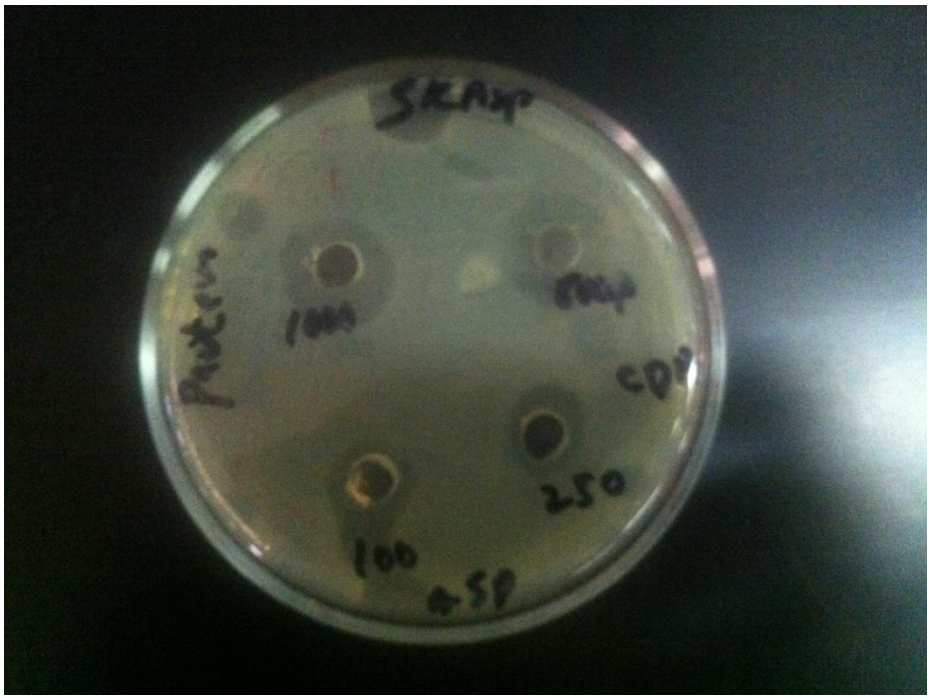

Figure 4.3 Zones of inhibition formed by *Aspergillus* crude extract against *P. vulgaris*

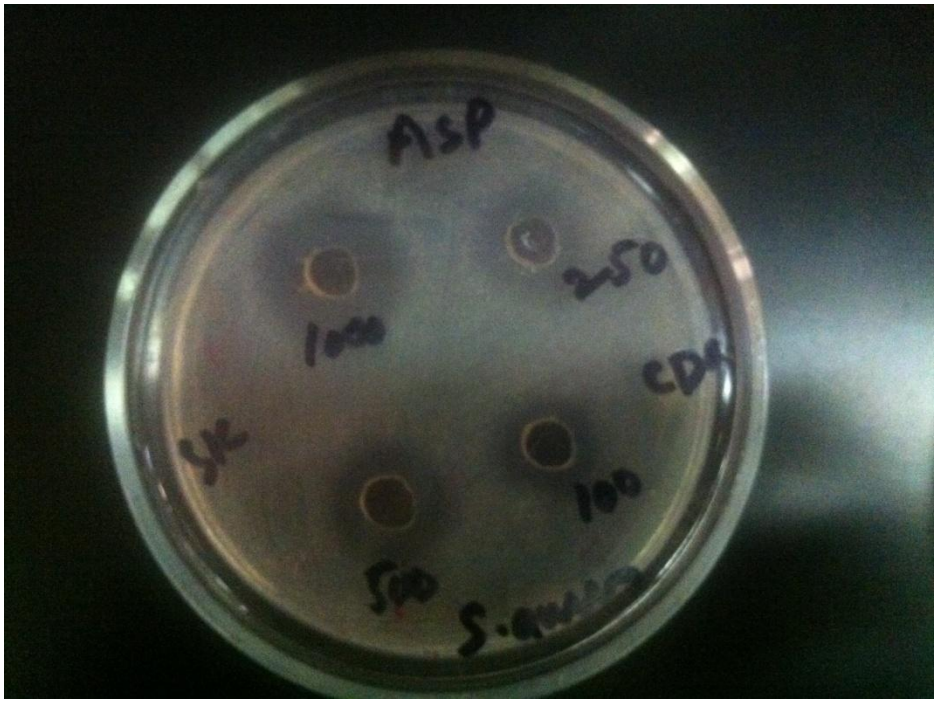

Figure 4.4 Zones of inhibition formed by *Aspergillus* crude extract against *S. aureus*

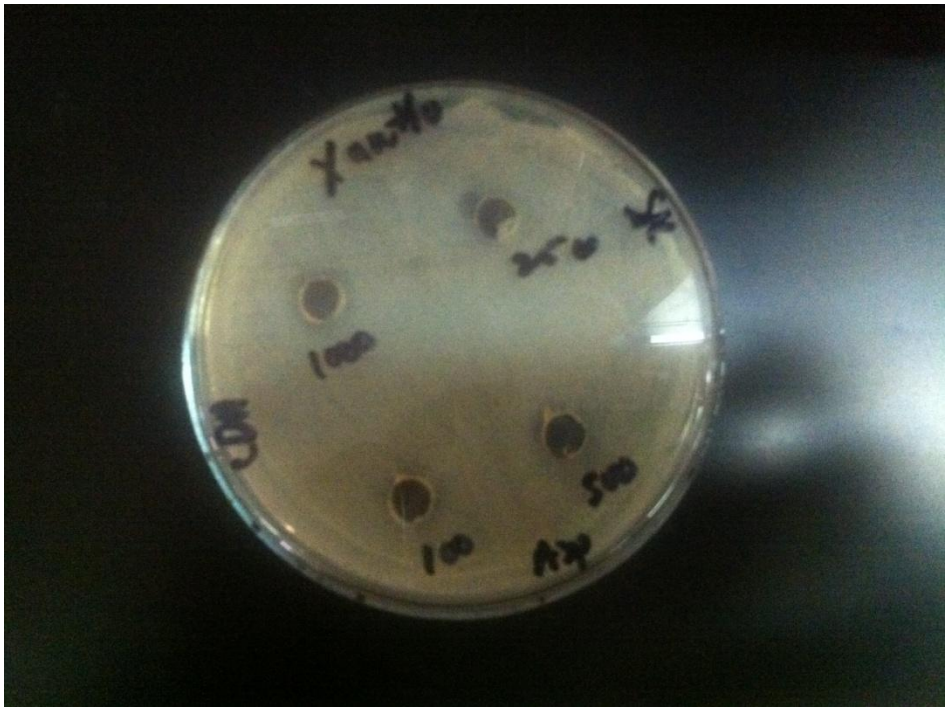

Figure 4.5 Zones of inhibition formed by *Aspergillus* crude extract against *X. oryzae*

4.1.2 Antibacterial activities of *Penicillium* species

Crude extract of *Penicillium* species was tested against eight pathogenic bacterial strains i.e. *Xanthomonas oryzae, Klebsiella pneumonia, Bacillus subtilus, Proteus vulgaris, Staphylococcus aureus, Escherichia coli, Salmonella typhi* and *Shigella flexeneri*. Different dose concentrations of both ethyl acetate and *n*-Hexane fractions of the crude extract were used against the test pathogenic strains which resulted in the formation of different zones of inhibition. First clear zone of inhibition was recorded at 100 µg/mL of ethyl acetate fraction which inhibited the growth of *P. vulgaris* (9 mm), *E. coli* (8 mm), *S. flexeneri* (6 mm) and *K. pneumoniae* (4 mm). At 200 µg/mL all the test organisms showed inhibition except *X. oryzae* against which no zone of inhibition was recorded. However, at this dose concentration highest inhibition was recorded against *E. coli* (18 mm) and *P. vulgaris* (17 mm) while considerable inhibition was noted against *B. subtilus* (10 mm), *S. flexeneri* (10 mm), *S. aureus* (9 mm) and *K. pneumoniae* (7 mm). At 250 µg/mL highest inhibition was recorded against *P. vulgaris* (25 mm) and *E. coli* (21 mm) while *B. subtilus, S. aureus, S. flexeneri* and *K. pneumoniae* formed a zone of 17, 15, 12 and 10 mm respectively. Lowest inhibition was recorded against *S. typhi* and *X. oryzae* with a zone of inhibition of 5 and 6 mm respectively. A higher dose i.e. 500 µg/mL inhibited the growth of *P. vulgaris*, *E. coli, S. aureus, B. subtilus* and *S. flexeneri* by forming a zone of 34, 28, 24, 23 and 21 mm respectively. Lowest zone of inhibition was recorded against *S. typhi* (9.5 mm) while considerable inhibition was noted against *K. pneumoniae* (16 mm) and *X. oryzae* (13 mm).

As compared to ethyl acetate fraction *n*-Hexane fraction of *Penicillium* species proved less effective against the test pathogenic bacterial strains. First considerable activity was noted at 500 µg/mL with a zone of 17.5 mm against *P. vulgaris* and a zone of 9.5, 9 and 7 mm against *S.*

typhi, S. flexeneri and *B. subtilus* respectively. No zone of inhibition was recorded against *K. pneumoniae, E. coli, S. aureus* and *X. oryzae*. Antibacterial activities of the ethyl acetate and *n*-Hexane extract of *Penicillium* species is summarized in figure 4.6 and 4.7.

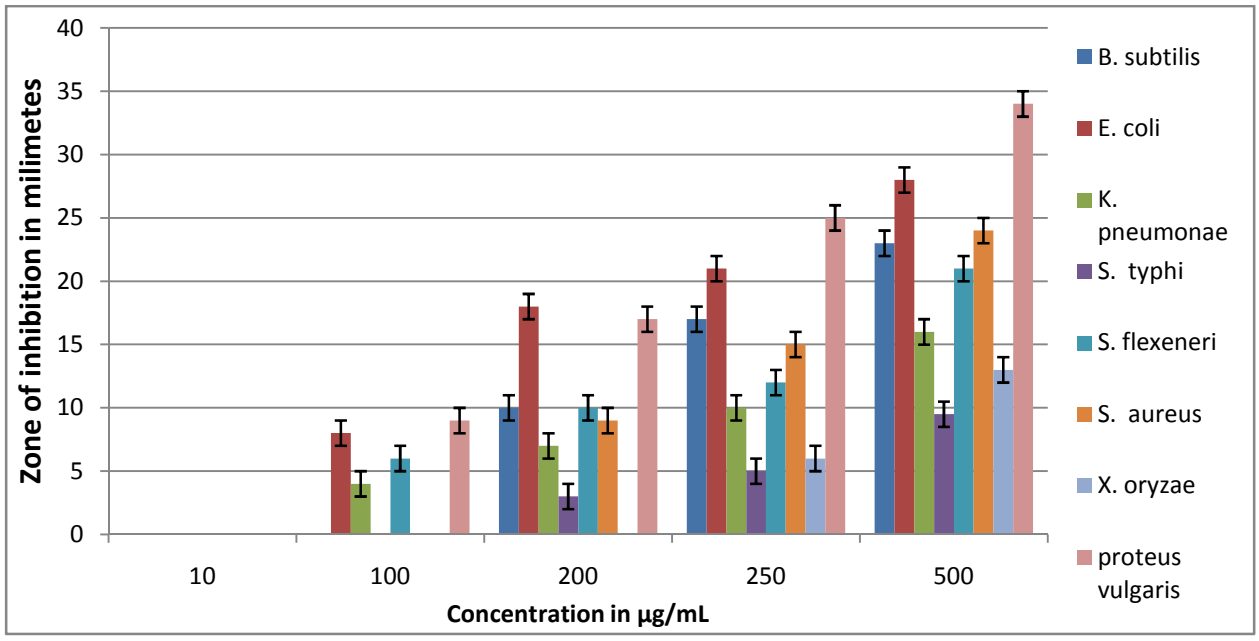

Figure 4.6 Antibacterial activities of ethyl acetate fraction of *Penicillium* species

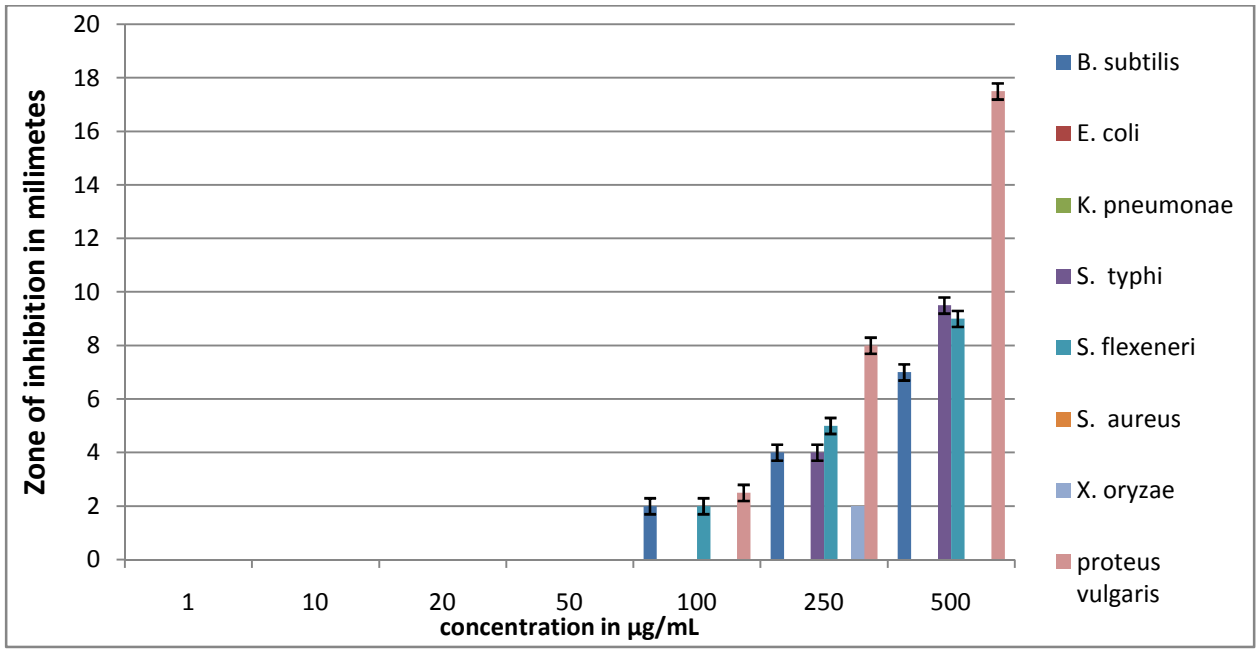

Figure 4.7 Antibacterial activities of *n*-Hexane fraction of *Penicillium* species

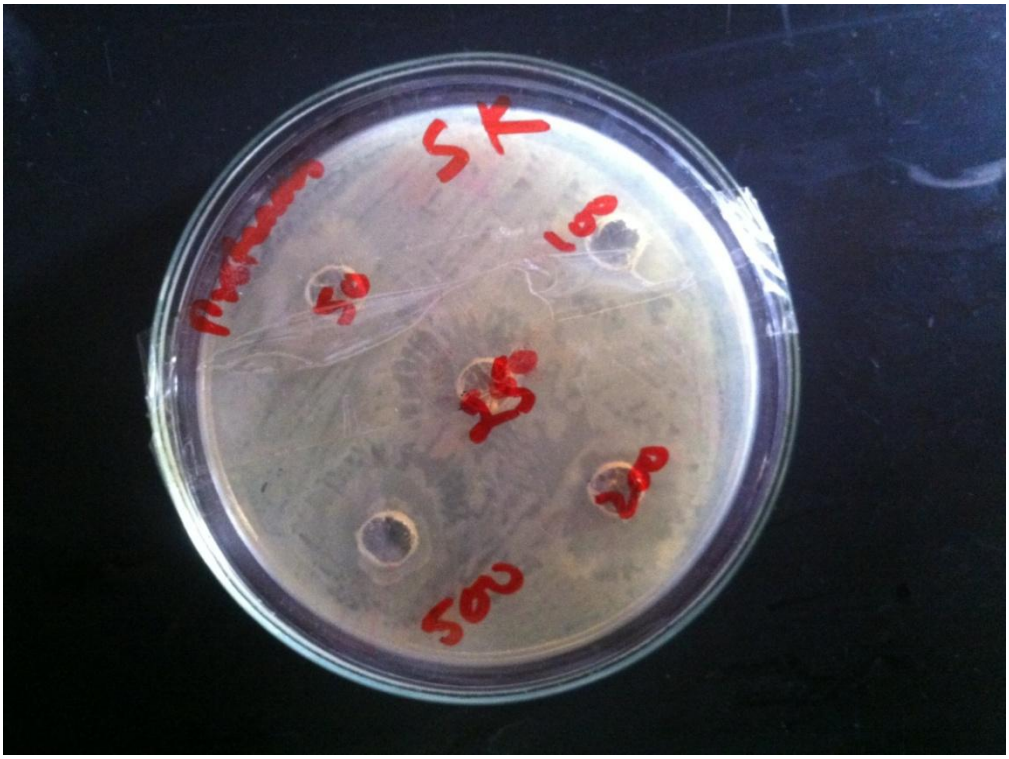

Figure 4.8 Zones of inhibition formed by *Penicillium* crude extract against *P. vulgaris*

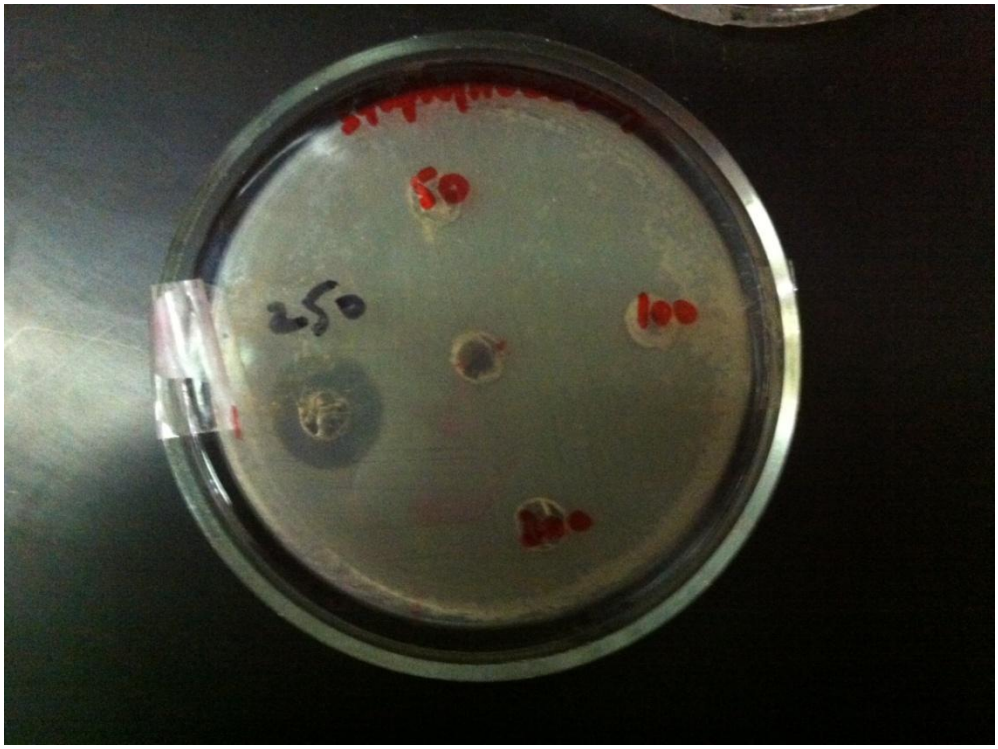

Figure 4.9 Zone of inhibition formed by *Penicillium* crude extract against *S. aureus*

4.2 Antifungal activities

Organic extracts of *Aspergillus* and *Penicillium* species were tested against six different pathogenic fungal strains namely *Fusarium solani, Aspergillus flavus, Microsporium canis, Candida albicans, Candida glabrata, and Trichophyton longifusus*. The results of the antifungal activities of crude *n*- Hexane and ethyl acetate fractions of both the fungal species are summarized in the figures 4.10 to 4.13.

4.2.1 Antifungal activities of *Aspergillus* species

Both ethyl acetate and *n*-Hexane fractions of *Aspergillus* species were tested against six pathogenic fungal strains (*F. solani, A. flavus, M. canis, C. albicans, C. glabrata, and T. longifusus*). Different dose concentrations i.e. from 1 to 1000 µg/mL of both fractions were prepared and tested against pathogenic fungal species. A dose concentration of 1 and 10 µg/mL of ethyl acetate concentration showed no growth inhibition of the fungal strains. First clear growth inhibition was recorded at 50 µg/mL against *M. canis* (9 %), *T. longifusus* (5%) and *C. glabrata* (2%). At 100 µg/mL all fungal strains were inhibited among which highest growth of inhibition was shown by *M. canis* (18%) and *T. longifusus* (13%) while lowest growth of inhibition was recorded against *A. flavus* (2%). A higher dose concentration i.e. 250 µg/mL inhibited the growth of *T. longifusus, M. canis* and *C. albicans* by 27%, 25.5% and 15% respectively while 9.5% linear growth inhibition was recorded each against *C. glabrata* and *F. solani*. At 250 µg/mL lowest growth of inhibition was shown by *A. flavus* (5%). Then a dose of 500 µg/mL was tested which inhibited the growth of all test fungal strains but highest inhibition was recorded against *T. longifusus* (29%), *M. canis* (27.5%) and *C. glabrata* (24.5%). A linear growth inhibition of 16%, 15.5% and15% was recorded against *A. flavus, C. albicans* and *F.*

solani respectively. The highest applied dose concentration i.e. 1000 µg/mL inhibited the growth of *M. canis, C. glabrata* and *A. flavus* by 60.5%, 52% and 44.5% respectively. *C. albicans* and *F. solani* showed 35 and 38.5% growth inhibition while lowest inhibition was recorded against *T. longifusus* i.e. 27.5%. Antifungal activities of ethyl acetate extract of *Aspergillus* species are given in figure 4.10.

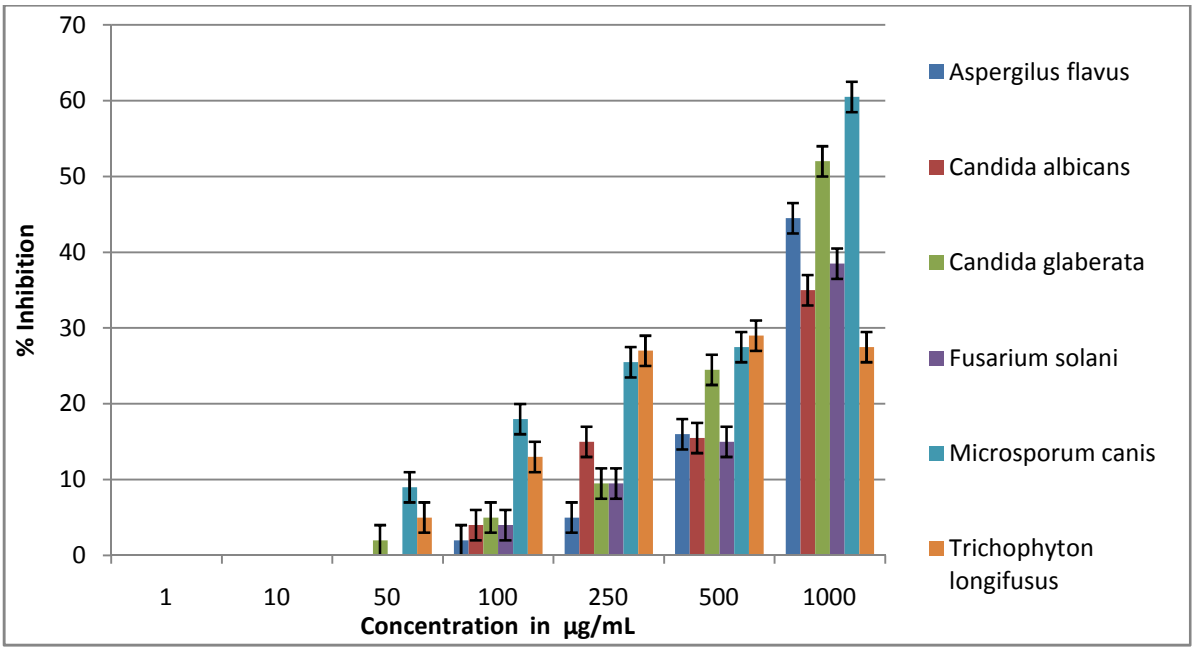

Figure 4.10 Antifungal activities of ethyl acetate extract of *Aspergillus* species

Dose concentrations of 10, 50 and 100 µg/mL of *n*-Hexane extract only inhibited the growth of *F. solani* and *M. canis.* However at 250 µg/mL along with *F. solani* and *M. canis* the growth of *C. albicans* was also inhibited. At 500 µg/mL all fungal strains except *A. flavus* were inhibited. A dose concentration of 500µg/mL inhibited the growth of *F. solani, M. canis, C. glabrata, C. albicans* and *T. longifusus* by 37%, 24%, 18%, 9% and 4% respectively. At 1000 µg/mL all fungal species showed inhibition among which highest inhibition was recorded against *F. solani* (56.5%), *M. canis* (45%), *C. glabrata* (37%) and *C. albicans* (33.5%) while a growth

inhibition of 7.5% and 6% was recorded against *T. longifusus* and *A. flavus* respectively. Antifungal activities of *n*- Hexane extract of *Aspergillus* species are given in figure 4.11.

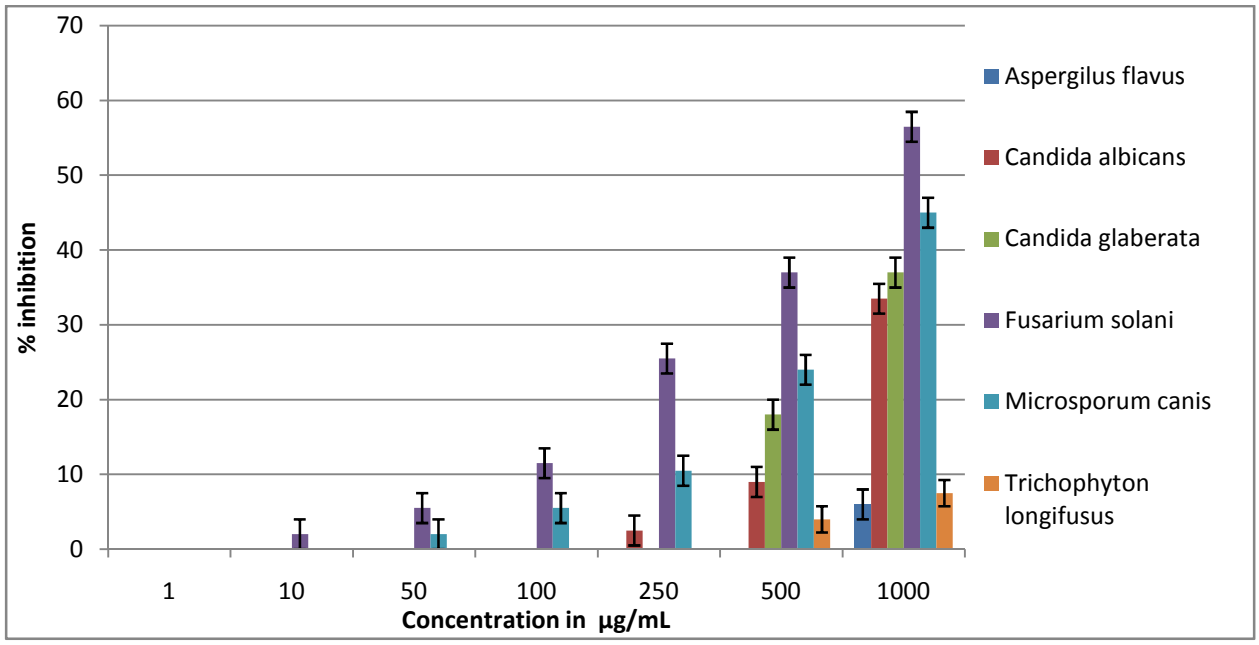

Figure 4.11 Antifungal activities of *n*- Hexane extract of *Aspergillus* species

4.2.2 Antifungal activities of *Penicillium* species

Both ethyl acetate and *n*-Hexane fractions of *Penicillium* species were tested against six pathogenic fungal strains (*F. solani, A. flavus, M. canis, C. albicans, C. glabrata, and T. longifusus*). Different dose concentrations i.e. 1, 10, 100, 250, 500 and 1000 µg/mL were prepared and tested against the pathogenic fungal strains. Results of both ethyl acetate and *n*-Hexane extracts of *Penicillium* species are given in figure 4.12 and 4.13.

Dose concentrations of 1, 10 and 50 µg/mL of ethyl acetate extract of *Penicillium* species showed no activity against the test pathogenic fungal strains. At 100 µg/mL low activity was observed against all pathogenic fungal strains except *A. flavus* against which no activity was found. At this dose concentration growth of *M. canis, T. longifusus, F. solani, C. glabrata* and *C.*

albicans was inhibited by 13%, 12%, 3%, 2% and 1% respectively. At 250 µg/mL *T. longifusus, M. canis* and *C. albicans* showed 29.5%, 19% and 13% inhibition respectively while 7% inhibition was recorded each against *F. solani* and *C. glabrata*. At 250 µg/mL lowest inhibition i.e. 3.5% was recorded against *A. flavus*. At 500 µg/mL highest inhibition was shown by *T. longifusus* (44%) and *M. canis* (31%) while the growth of *C. glabrata, C. albicans, F. solani* and *A. flavus* was inhibited by 18.5%, 11%, 10.5% and 8.5% respectively. A dose of 1000 µg/mL inhibited the growth of all test pathogenic fungal species, however, *T. longifusus, M. canis* and *C. glabrata* showed highest percentage of inhibition i.e. 70%, 63.5% and 52% respectively. At this dose concentration *A. flavus* (44.5%), *F. solani* (38.5%) and *C. albicans* (35%) were also inhibited to greater extent.

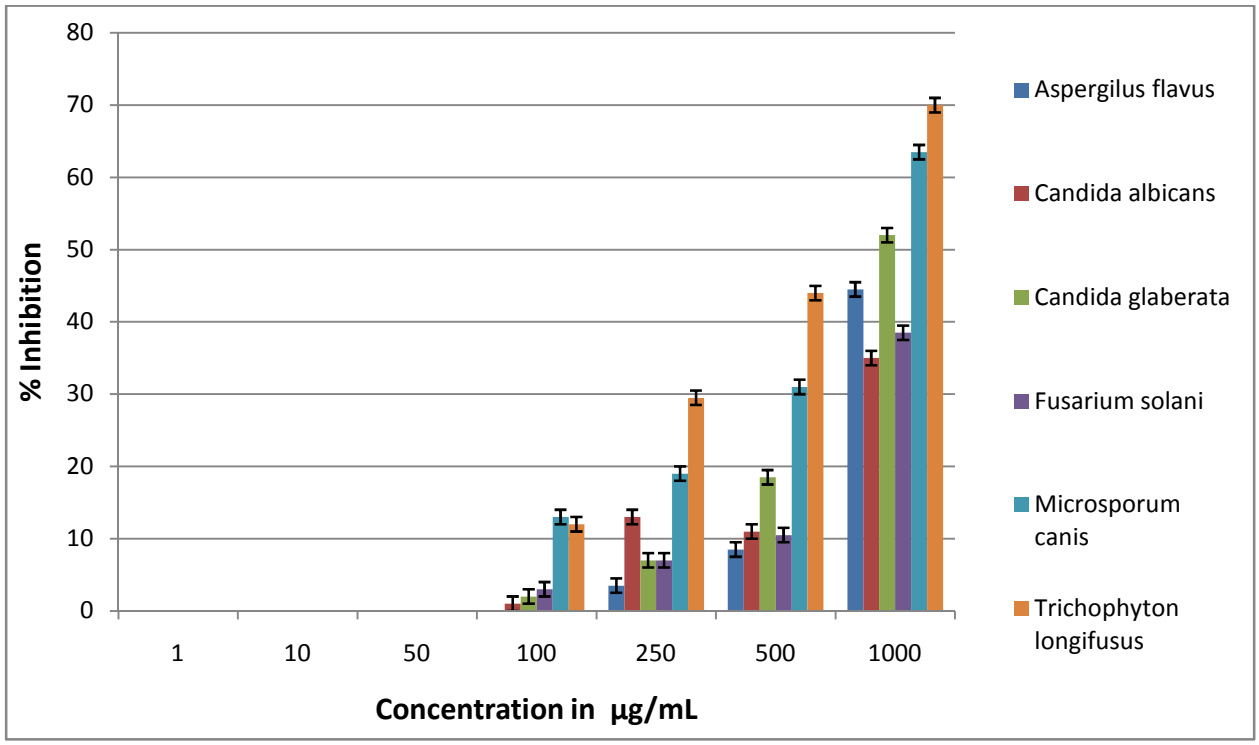

Figure 4.12 Antifungal activities of ethyl acetate extract of *Penicillium* species

Dose concentrations from 1 µg/mL to 250 µg/mL of *n*-Hexane fraction of *Penicillium* species proved ineffective and no growth of inhibition was recorded against any of the test fungal species. However, at a dose of 500 µg/mL of *n*-Hexane extract some activity was recorded against *C. glabrata* (7%), *F. solani* (6%) and *C. albicans* (5%) while *A. flavus*, *M. canis* and *T. longifusus* showed no activity. At higher dose concentration i.e. 1000 µg/mL all test fungal strains except *T. longifusus* were inhibited. At this concentration *C. glabrata, F. solani* and *C. albicans* showed 28.5%, 26.5%, 22.5% inhibition respectively while 6% inhibition was recorded each for *M. canis* and *A. flavus*.

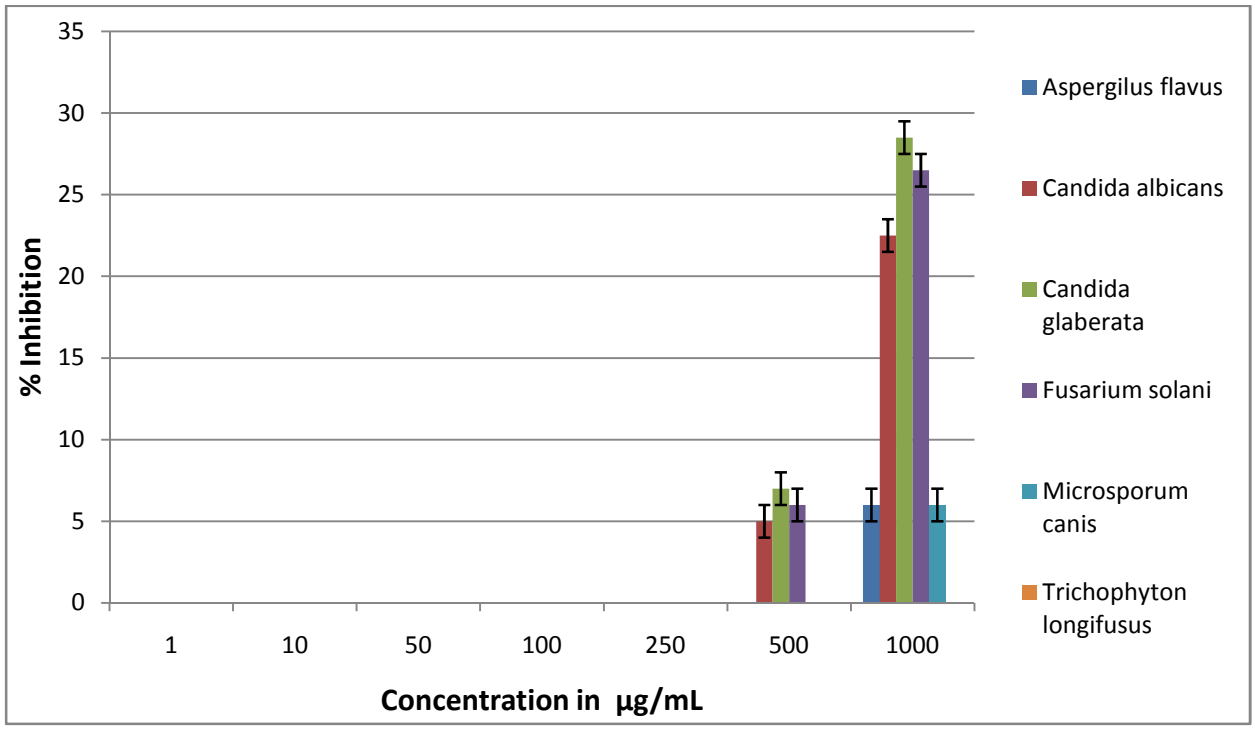

Figure 4.13 Antifungal activities of *n*- Hexane extract of *Penicillium* species

4.3 Cytotoxic activities or brine shrimp lethality test

Crude extract of both fungal species i.e. *Aspergillus* and *Penicillium* species were subjected for cytotoxic activities against brine shrimp (*Artemia salina*). A total of three dose concentrations i.e. 10, 100 and 1000 µg/mL of both ethyl acetate and *n*-Hexane fractions were

used to test the toxicity of fungal metabolites against *A. salina*. The results of cytotoxic activities of crude extracts of both fungal species are summarized in figures 4.14 and 4.15.

4.3.1 Cytotoxic activities of *Aspergillus* species

Dose concentrations of 10, 100 and 1000 µg/mL of ethyl acetate extract of *Aspergillus* species showed mortality percentage of 6.66, 63.33 and 95% respectively while the same dose concentrations of its *n*-Hexane fraction showed 13.33, 81.66 and 98.33% mortality respectively.

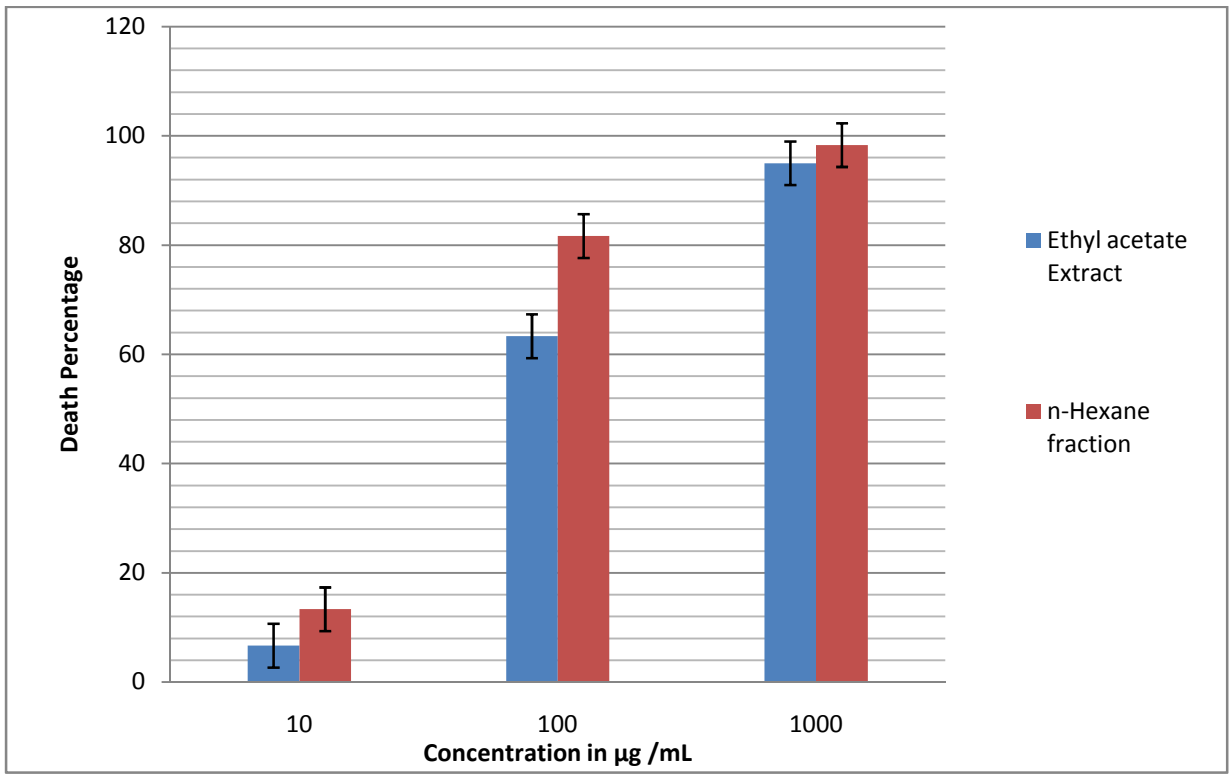

Figure 4.14 Cytotoxic activities of ethyl acetate and *n*-Hexane fraction of *Aspergillus* species

4.3.1 Cytotoxic activities of *Penicillium* species

Three dose concentrations i.e. 10, 100 and 1000 µg/mL of ethyl acetate extract of *Penicillium* species showed mortality percentage of 5, 36.66 and 63.33% respectively while the same dose concentrations of its *n*-Hexane fraction showed 0.00, 30 and 48.33% mortality respectively.

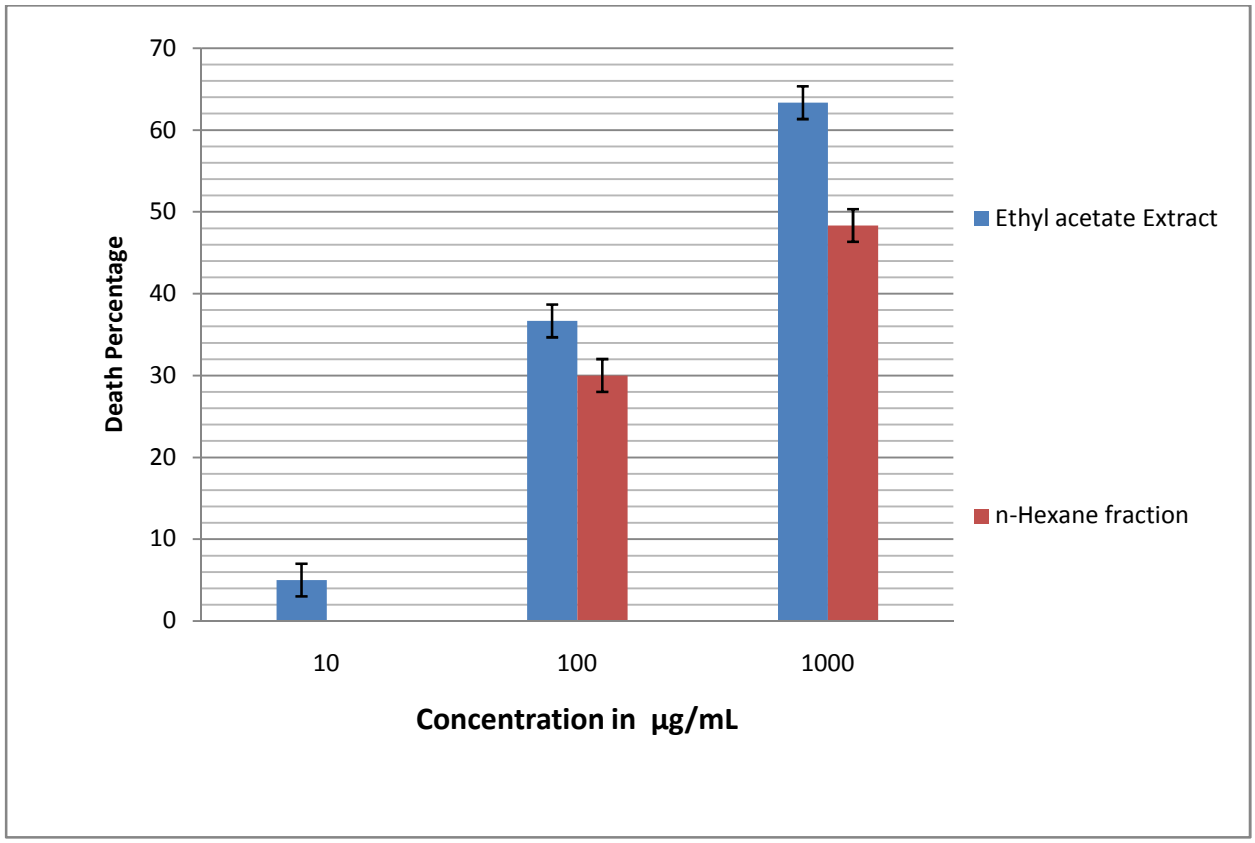

Figure 4.15 Cytotoxic activities of ethyl acetate and *n*-Hexane fraction of *Penicillium* species

4.4 Phytotoxic activities

Crude metabolites of *Penicillium* and *Aspergillus* species were tested against *Lemna* for phytotoxicity. The results are shown in the figure 4.16 and 4.17. Different concentrations of ethyl acetate and *n*-Hexane fractions of both fungal strains showed different degrees of phytotoxicity

on the leaves of *Lemna* plant and different types of necrotic lesions were observed. These lesions varied from reddish brown to dark brown in color. To determine the phytotoxic ability of the crude metabolites a total of three dose concentrations were used i.e. 10, 100, and 1000 µg/mL.

4.4.1 Phytotoxic activities of *Aspergillus* species

A dose concentration of 10, 100, and 1000 µg/mL of ethyl acetate fraction of *Aspergillus* species showed 0, 30 and 65% mortality respectively while the same dose concentration of *n*-Hexane fraction of this fungus showed 6.66, 78.33 and 85% mortality against the *Lemna* plant.

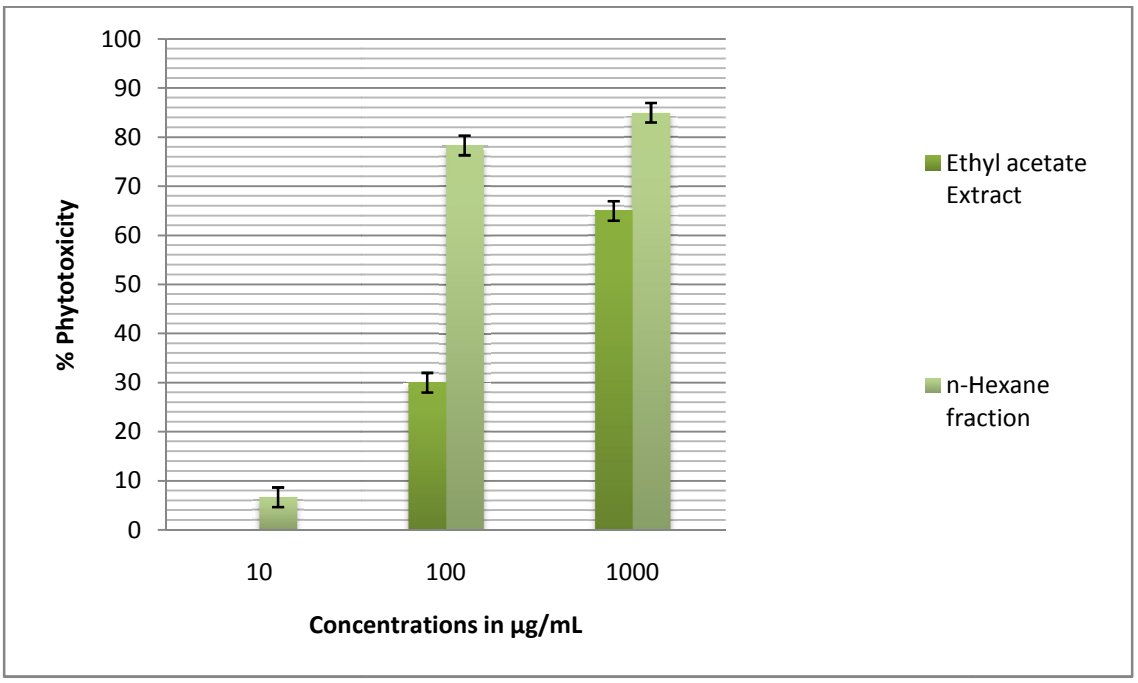

Figure 4.16 Phytotoxic activities of crude extract of *Aspergillus* species

4.4.2 Phytotoxic activities of *Penicillium* species

In comparison to its *n*-Hexane fraction ethyl acetate extract of *Penicillium* species showed more phytotoxicity against *Lemna* plant. Dose concentrations of 10,100 and 1000 µg/mL of crude ethyl acetate fraction of *Penicillium* species showed mortality percentage of 0, 25 and 56.66% respectively whereas the same dose concentrations of its *n*-Hexane fraction showed 0, 11.66 and 30% mortality respectively against *Lemna.*

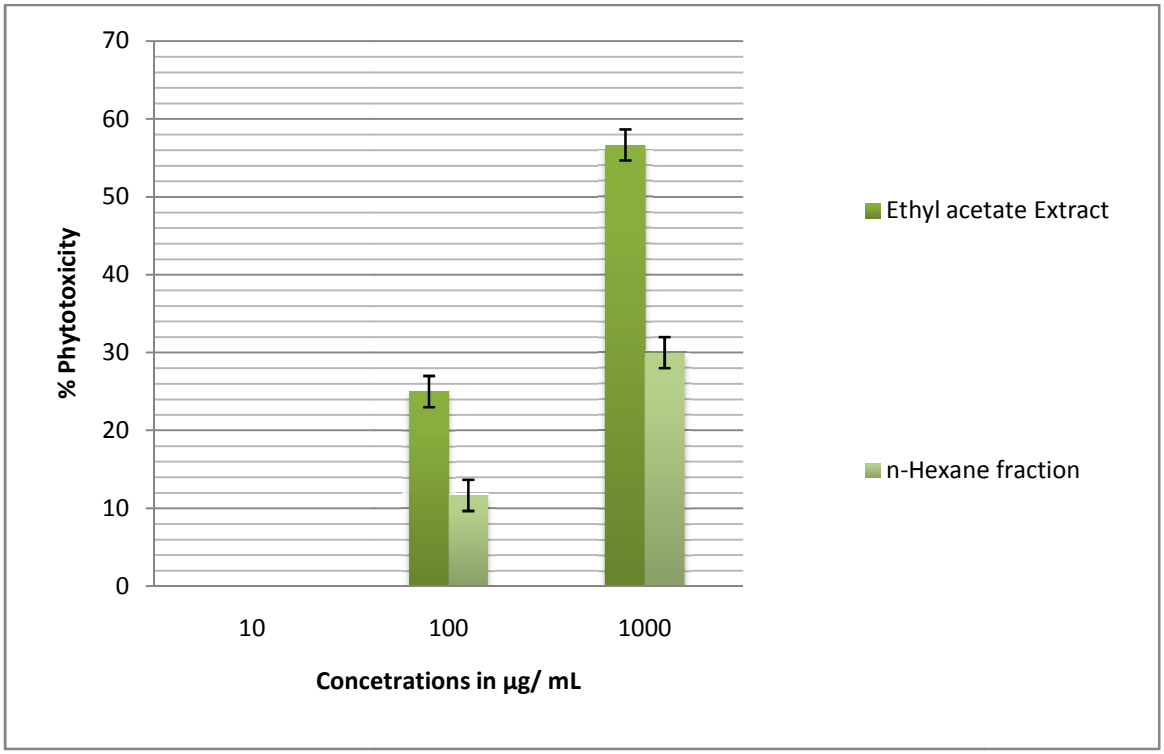

Figure 4.17 Phytotoxic activities of crude extract of *Penicillium* species

Figure 4.18 Phytotoxic activities

4.5 Herbicidal activities

Results showed that crude extracts of both *Aspergillus* and *Penicillium* species with their respective concentrations i.e. (10, 100, 1000 µg/mL) significantly inhibited the growth of *Silybum marianum* L. The percent seed germination data showed that maximum germination was recorded for control plots i.e. (90%) while no germination was observed in other extract applied treatments. Furthermore, the mean data showed that highest shoot length (7.8 cm) and root length (6.3 cm) were observed for control plots while no data was recorded from other extract applied treatments with their respective concentrations. In addition, the mean data showed that maximum shoot weight (0.39 g) and root weight (0.32) were noticed for control treatments while no data was recorded from other extract applied treatments with their respective concentrations. Herbicidal activities of both fungal strains and their respective concentrations are given in the table 4.1.

Table 4.1 Herbicidal activities of *n*-Hexane and ethyl acetate fractions of the crude extracts of *Aspergillus* and *Penicillium* species

Treatments	**Germination (%)**	**Shoot length (cm)**	**Root length (cm)**	**Shoot weight (gm)**	**Root weight (gm)**
Penicillium sp (10, 100, 1000 µ g/mL of both ethyl acetate and *n*- Hexane fractions)	**X**	**X**	**X**	**X**	**X**
Aspergillus sp (10, 100, 1000 µ g/mL of both ethyl acetate and *n*-Hexane fractions)	**X**	**X**	**X**	**X**	**X**
Control	**90**	**7.8**	**6.3**	**0.39**	**0.32**

X = No growth or Zero growth

CHAPTER 5

DESCRIPTION OF METABOLITES

CHAPTER 5 DESCRIPTION OF METABOLITES

After performing bioassay screenings i.e. antibacterial, antifungal, cytotoxic, phytotoxic and herbicidal activities, we developed an idea from the results summarized in chapter 4 of this thesis that both *Aspergillus* and *Penicillium* species can be used for isolation of secondary metabolites. Isolation and purification of compounds was performed using HPLC (Water's).

Analysis of chromatogram of crude extract from *Aspergillus* sp shows (figure 5.1) different peaks corresponding to different secondary metabolites. After simultaneous analysis of the UV, ES^+ and ES^-, four peaks corresponding to A, B, C and D were selected to be isolated using the automated isolation procedure of the above mentioned HPLC.

5.1 Chromatogram of crude extract from *Aspergillus Species*

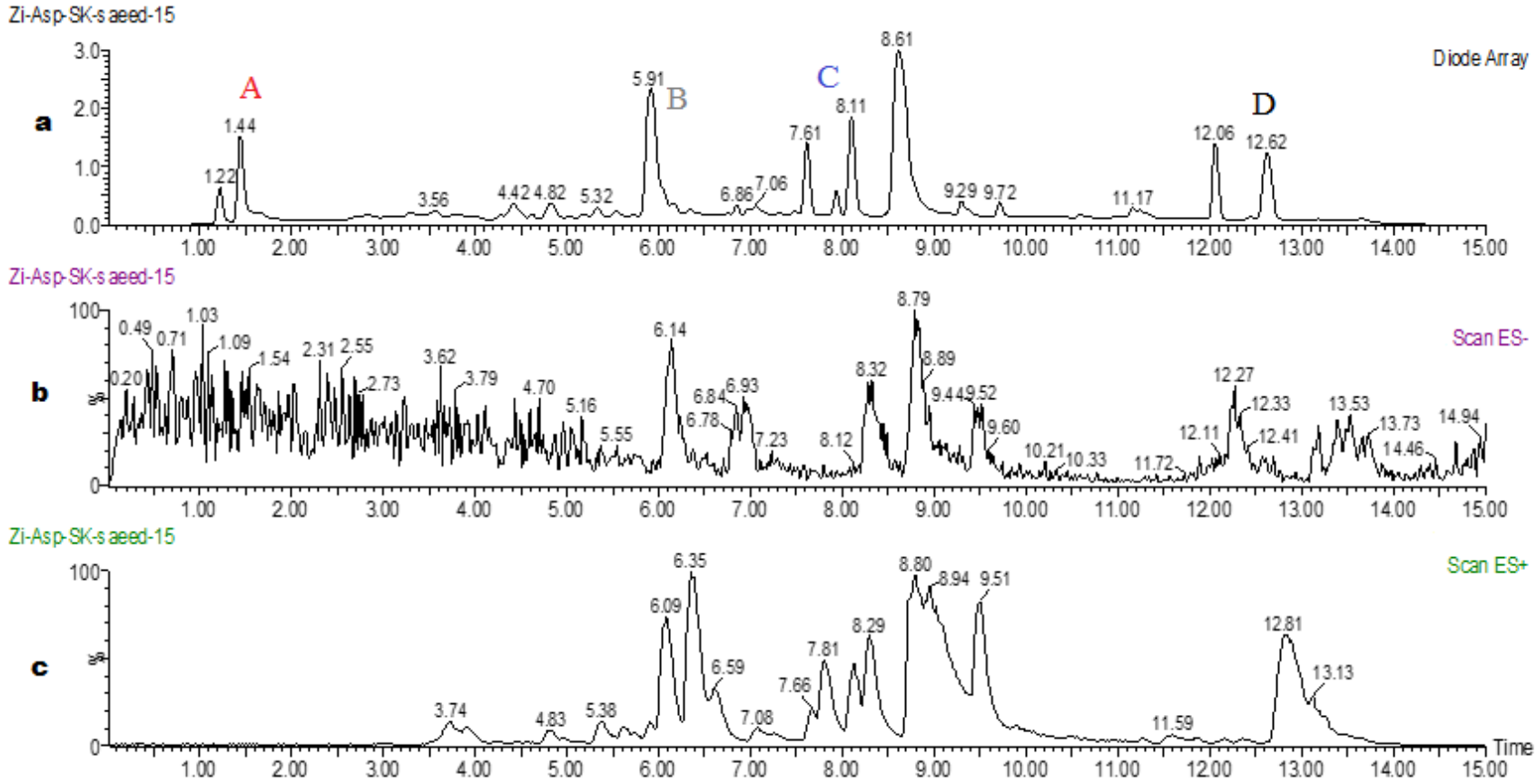

Figure 5.1 LCMS chromatogram of *Aspergillus* sp, showing UV profile of the extract (trace a), ES^-profile of the extract (trace b) and ES^+ profile of the extract (trace c). While the targeted compounds (A) was eluted at 1.44, (B) was eluted at 6.07 (C) was eluted at 8.03 and (D) was eluted at 12.7 rt respectively.

5.2 Chromatogram of known compound (A-142) from *Aspergillus Species*

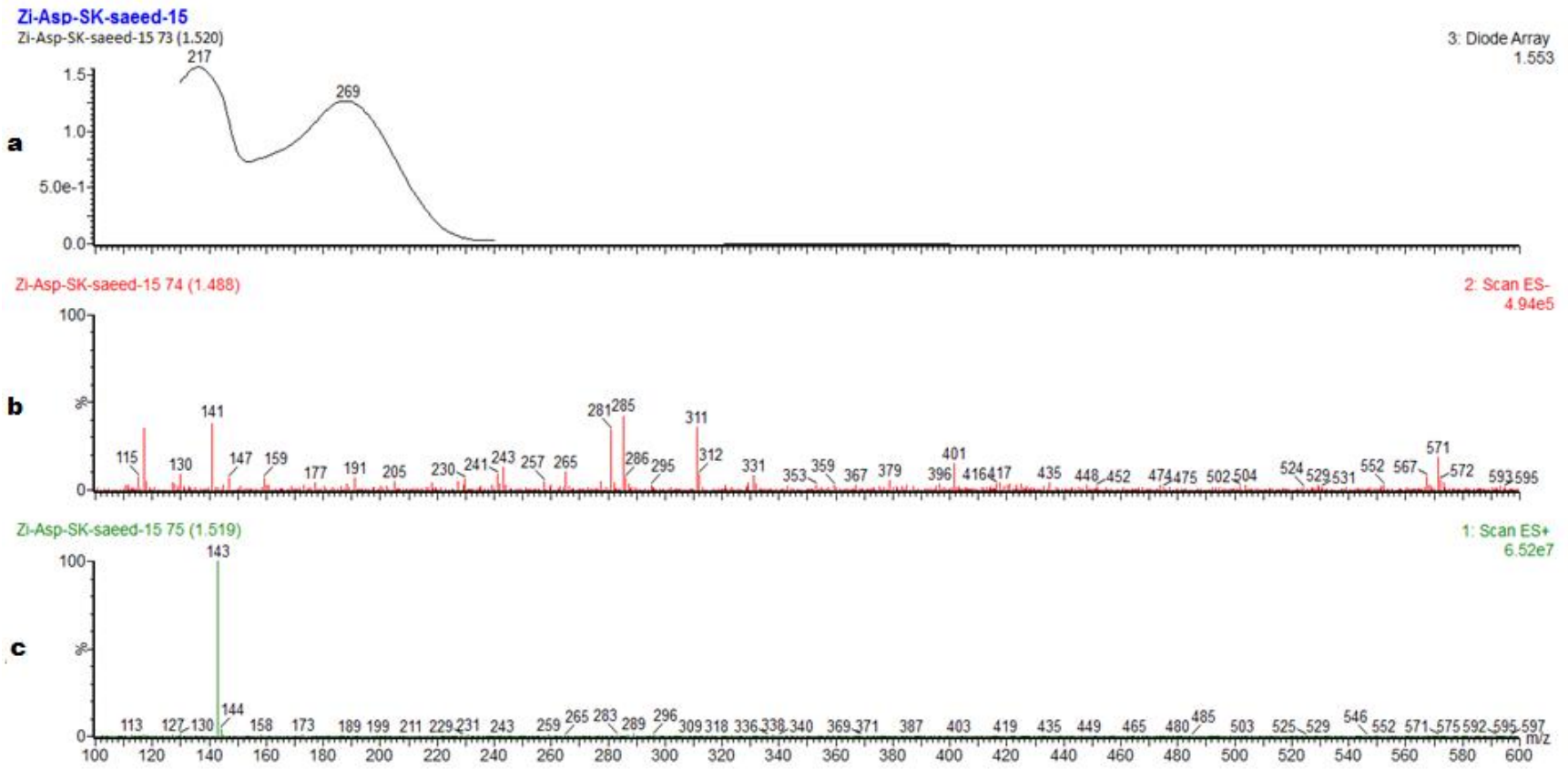

Figure 5.2 LCMS chromatogram of *Aspergillus* sp, showing UV profile of the extract (trace a), ES^- profile of the extract (trace b) and ES^+ profile of the extract (trace c) of compound (142).

Individual estimation of chromatogram of compound A (142) is shown in figure 5.2, showing UV (269), ES^- (141) and ES^+ (143) corresponding to pure compound having mass of 142.

5.2.1 Known compound (142)

The HR–ESI–MS and ^{13}C NMR (Broad Band and DEPT) were utilized to establish the molecular formula of the known compound. HR–ESI–MS showed pseudo molecular ion peaks [M^+] at *m/z* 143.1211 [M]H^+ and 165.1121 [M]Na^+ corresponding to the molecular formula $C_6H_6O_4$ [Calculated as $C_6H_6O_4$ + Na = 165.1121]. The spectrum got from IR spectroscopy showed occurrence of an aromatic ring at 1625 and 1391 cm^{-1} and carbonyl amide at 1660 cm^{-1}. The presence of benzene ring was also confirmed by the absorption maxima in the UV spectrum (221 and 266 nm). For structural elucidation 5 mg of pure compound (142) was mixed in 0.65 mL of deuterated DMSO and a series of one and two dimensional NMR analyses were performed on 500 MHz NMR Spectrometer.

Hydrogen NMR exhibited 23 signals for 31 protons. The one dimension ^{13}C NMR exhibited 6 signals corresponding to 6 carbon skeleton of the compound, exhibiting two quaternary carbons at δ 168.47 and 145.75 respectively while one carbonyl carbon at δ 174. The two dimensional (proton- proton COSY and HMBC) alone with UV and IR were compared with the values in literature [288]. Structure of the known compound is given in figure 5.3 while the NMR data is given in table 5.1.

Table 5.1 Chemical Shifts of Known Compound (142). Data Obtained from 500 MHz Varian NMR

Carbon No.	Multiplicity (DEPT)	^{13}C–NMR	^{1}H (J = Hz)
1	CH_2	60.15	4.33d (J = 5.0 Hz),
2	-C-	167.79	----------
3	CH	110	6.77 s
4	C=O	174.16	----------
5	-C-	146.12	----------
6	CH	138.98	8.43 s

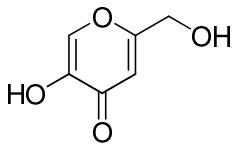

Figure 5.3 Structure of known compound 142

5.3 Chromatogram of known compound (B-262) from *Aspergillus* sp

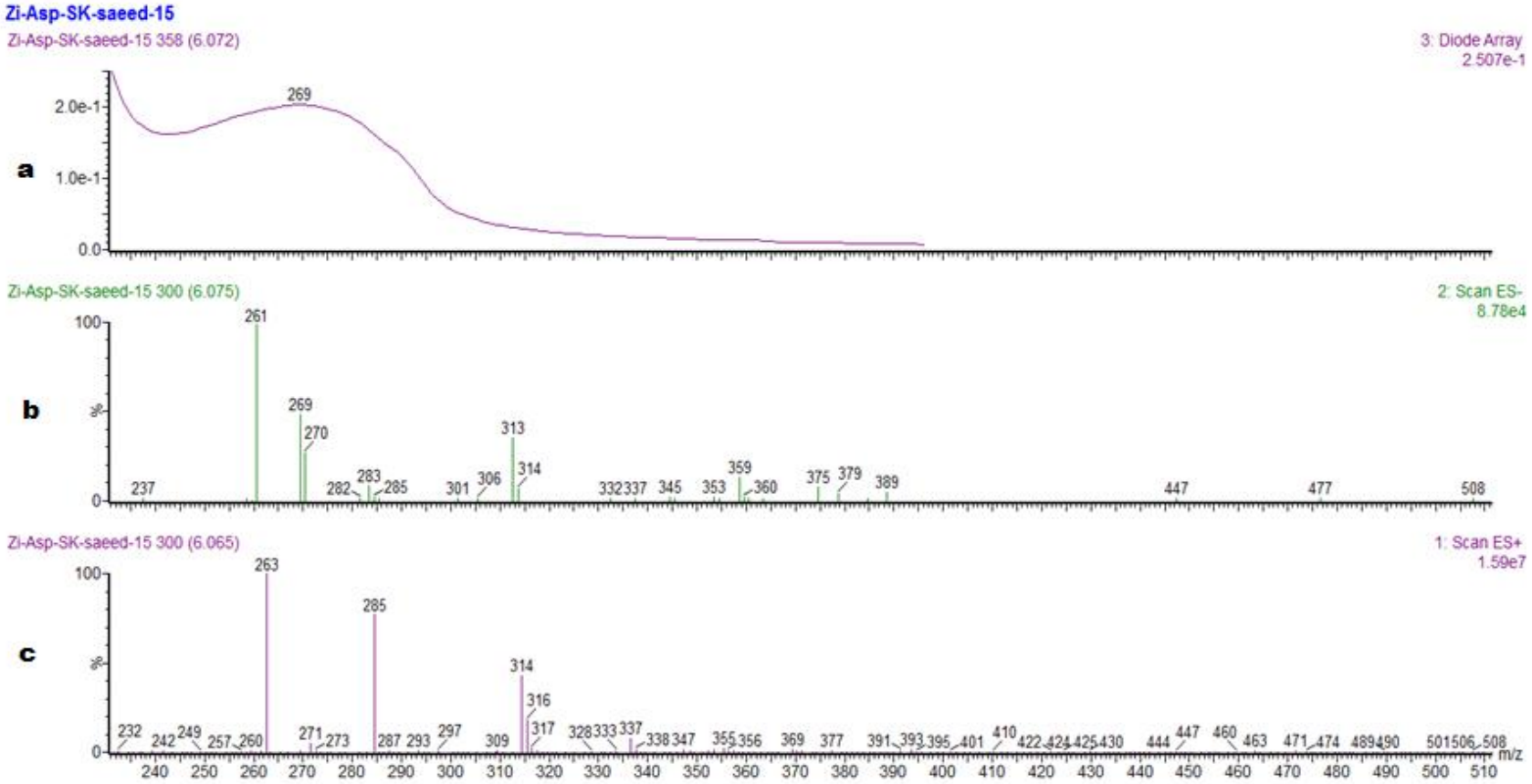

Figure 5.4 LCMS chromatogram of *Aspergillus* sp, showing UV profile of the extract (trace a), ES^- profile of the extract (trace b) and ES^+ profile of the extract (trace c) of compound (262).

Individual estimation of chromatogram of compound B (262) is shown in figure 5.4, showing UV (269), ES^- (261) and ES^+ (263) and (285) corresponding to pure compound having mass of 262.

5.3.1 Known compound (262)

The HR–ESI–MS and ^{13}C NMR (Broad Band and DEPT) were used to establish the molecular formula of the known compound. HR–ESI–MS showed pseudo molecular ion peaks [M^+] at *m/z* 262.1112 [M]H^+ and 284.2121 [M]Na^+ that correspond to molecular formula $C_{14}H_{14}O_5$ [Calculated as $C_{14}H_{14}O_5$ + Na = 284.2121]. The spectrum got from IR spectroscopy showed occurrence of hydroxyl group (3350 cm^{-1}), carbonyl group at (1740 cm^{-1}), and aromatic ring (1635 and 1381cm^{-1}). The absorption maxima in the UV spectrum (320 nm) also showed the occurrence of an aromatic ring.

6 mg of pure compound (262) was mixed in 0.65 mL of deuterated chloroform and a series of one dimensional and two dimension NMR analyses were performed on 500 MHz NMR Spectrometer.

Proton NMR represented eight proton signals. At δ 1.36 and δ 1.39 two signals were found which gave an integration of three protons each that correspond to two CH_3. All the remaining signals represented the integration of single proton each.

The one dimensional ^{13}C NMR gave 14 signals that corresponded to 14 carbons skeleton of the compound, exhibiting 4 quaternary carbons at δ 125.5, δ 161.3,δ 125.5 and δ 138.1while 2 carbonyl carbons at δ 170.2 and 168.9 respectively. The two dimensional HSQC exhibited 8 protonated carbons given in table 5.2. The two dimensional (^1H–^1H COSY and HMBC) represented different correlations and the elucidated structure of the compound is given in figure 5.5. The NMR data of known compound 262 is given in table 5.2.

Table 5.2 Chemical Shifts of Known Compound (262). Data Obtained from 500 MHz Varian NMR

Carbon No.	Multiplicity (DEPT)	^{13}C–NMR	^{1}H (*J* = Hz)
1	C=O	170.2	----------
2	----------	----------	----------
3	CH	83.1	4.56 m
4	CH	38.8	3.87 m
5	CH	120	7.21d (*J* = 10.0 Hz)
6	CH	137.2	7.52d (*J* = 10.0Hz)
7	–C–	125.5	----------
8	–C–	161.2	----------
9	–C–	125.5	----------
10	–C–	138.1	----------
11	CH_3	17.4	1.12d(*J* = 10.0Hz)
12	CH_3	19.7	1.23d(*J* = 10.0Hz)
13	CH	140	6.98d(*J* = 10.0Hz)
14	CH	122	6.5.23d(*J* = 10.0Hz)
15	C=O	168.9	----------

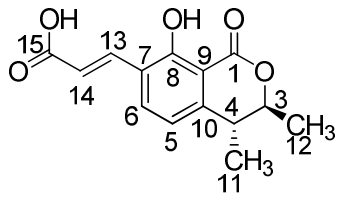

Figure 5.5 Structure of the known compound 262

In $^{1}H–^{1}H$ COSY, the methine proton of H–3 showed correlation with the proton of C–4, also with the methyl protons of C–12, while the H–4 showed correlation with the methyl protons of C–11. The proton H–5 showed correlation the proton of C–6, and the proton H–13 showed correlation the proton of C–14 and vice versa. While in HMBC correlations were compared with the compound already reported in literature. The 2D ($^{1}H–^{1}H$ COSY and HMBC) alone with UV and IR were compared with the values in literature [289].

5.4 Chromatogram of known compound (C-388) from *Aspergillus* sp

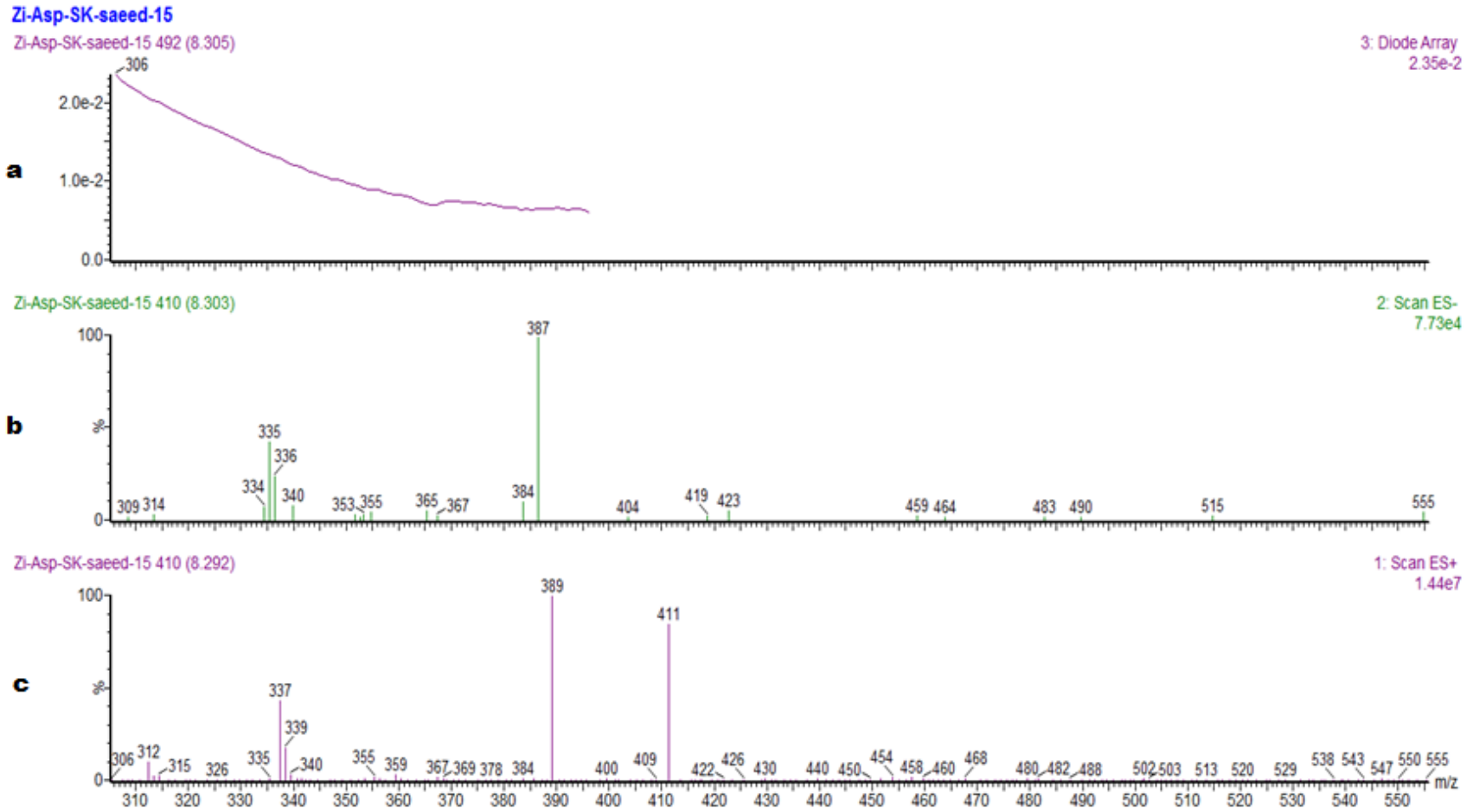

Figure 5.6 LCMS chromatogram of Aspergillus sp. showing UV profile of the extract (trace a), ES– profile of the extract (trace b) and ES+ profile of the extract (trace c) of compound (388).

Individual estimation of chromatogram of compound C (388) is shown in figure 5.6, showing UV (306), ES^- (387) and ES^+ (389) and (411) corresponding to pure compound having mass of 388.

5.4.1 Known compound (388)

The HR–ESI–MS and ^{13}C NMR (Broad Band and DEPT) were used to establish the molecular formula of the known compound. HR–ESI–MS showed pseudo molecular ion peaks [M^+] at *m/z* 389.1111 [M]H^+ and 411.2111 [M]Na^+ corresponding to the molecular formula $C_{20}H_{20}O_8$ [Calculated as $C_{20}H_{20}O_8$ + Na = 411.2111].The spectrum got from IR spectroscopy showed the occurrence of hydroxyl group (3350 cm^{-1}), carbonyl group at (1740 cm^{-1}) and an aromatic ring at (1635 and 1381cm^{-1}). The absorption maxima in the UV spectrum (306 nm) also showed the existence of aromatic rings.

For structure elucidation 5 mg of pure compound (388) was mixed in 0.65 mL of deuterated chloroform and a series of one and two dimension NMR analyses were performed on 500 MHz NMR Spectrometer.

^1H–NMR gave 9 peaks for 20 protons. Three signals were observed which showed integration of 2 protons i.e. one at (δ 3.66, 3.78) and two at δ 1.45 and 3.40 representing three CH_2 groups. All the remaining signals represented the integration of single proton each.

The one dimension ^{13}C NMR exhibited 20 signals representing 20 carbons in the skeleton of the compound, exhibiting 11 quaternary carbons. The two dimensional (^1H–^1H COSY and HMBC) revealed different correlations along with UV and IR were compared with the values in literature [290]. The elucidated structure of the compound is given in figure 5.7 while the NMR data is given in table 5.3.

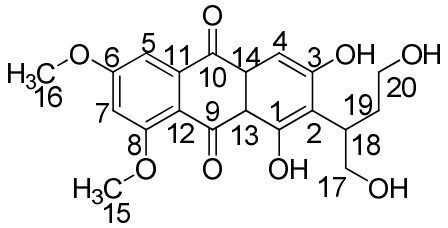

Figure 5. 7 Structure of the known compound 388

Table 5.3 Chemical Shifts of Known Compound (388). Data Obtained from 500 MHz Varian NMR

Carbon No.	Multiplicity (DEPT)	^{13}C–NMR	^{1}H (*J* = Hz)
1	–C–	162.16	----------
2	–C–	124.34	----------
3	–C–	163.00	----------
4	CH	107.78	7.14s
5	CH	105.45	6.99d (*J* = 10.0Hz)2.4
6	–C–	166.60	----------
7	CH	105.35	6.22d(*J* = 10.0Hz)
8	–C–	161.80	----------
9	–C–	186.10	----------
10	–C–	182.85	----------
11	–C–	135.56	----------
12	–C–	115.90	----------
13	–C–	109.45	----------
14	–C–	132.05	----------
15	-O-CH_3	56.53	3.99 s
16	-O-CH_3	56.53	3.99 s

17	CH_2	62.78	3.78 dd(J = 5.0, 10.0Hz) 3.66 dd(J = 5.0, 10.0 Hz)
18	CH	34.30	3.50dt(J = 5.0, 10.0Hz)
19	CH_2	30.65	1.45dd (J = 5.0, 10.0Hz)
20	CH_2	60.50	3.40dd (J = 5.0, 10.0Hz)

The 2D (^1H–^1H COSY and HMBC) alone with UV and IR were compared with the values in literature [290].

5.5 Chromatogram of new compound (D-260) from *Aspergillus* sp.

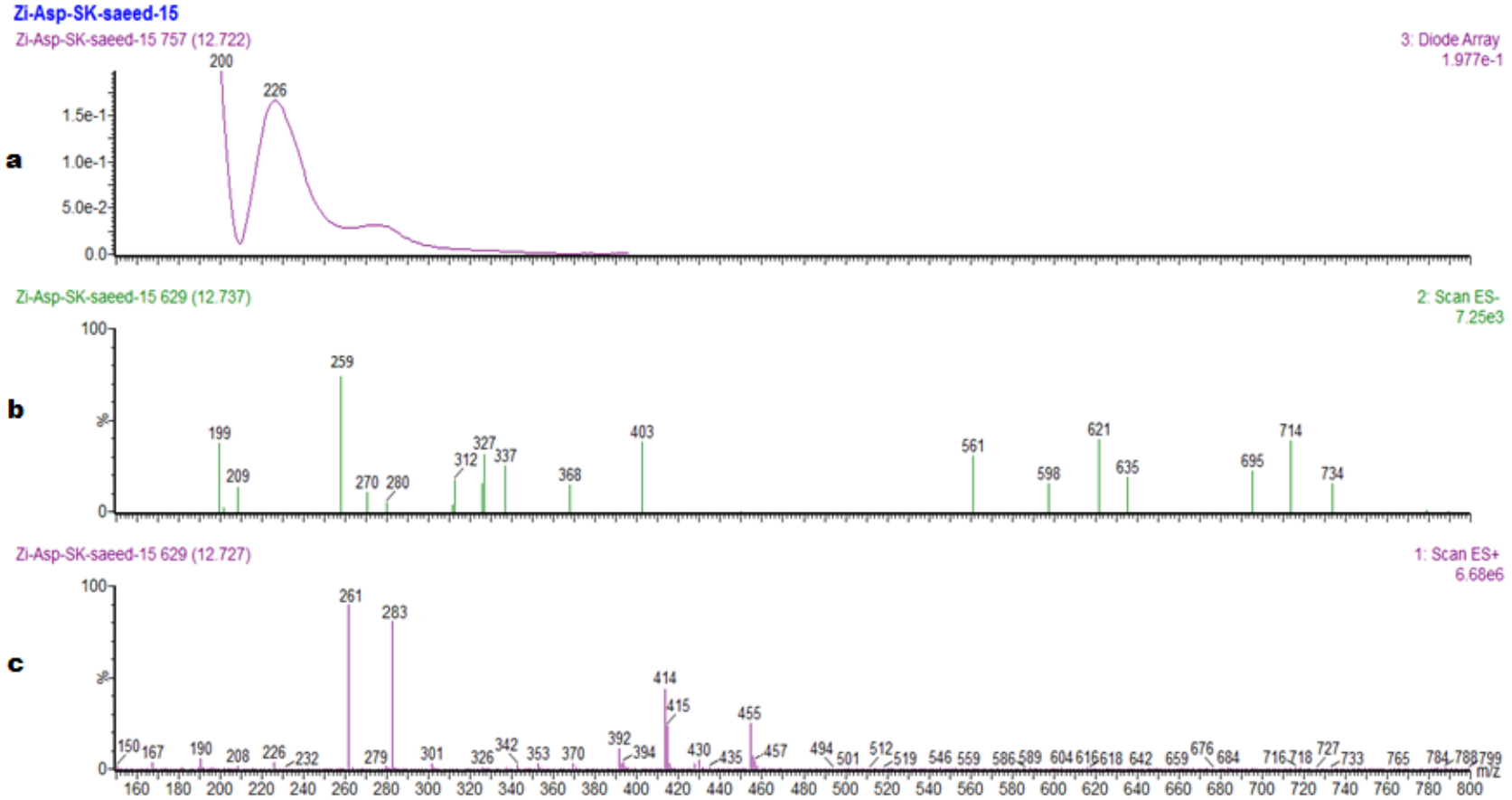

Figure 5.8 LCMS chromatogram of *Aspergillus sp.*, showing UV profile of the extract (trace a), ES^- profile of the extract (trace b) and ES^+ profile of the extract (trace c) of compound (260).

Individual estimation of chromatogram of compound D (260) is shown in figure 5.8, showing UV (226), ES^- (259) and ES^+ (261) and (283) corresponding to pure compound having mass of 260.

5.4.1 New compound (260)

The HR–ESI–MS and ^{13}C NMR (Broad Band and DEPT) were used to establish the molecular formula of the known compound. HR–ESI–MS presented pseudo molecular ion peaks [M^+] at *m/z* 261.1212 [M]H^+ and 283.2131 [M]Na^+ representing the molecular formula $C_{14}H_{16}N_2O_3$ [Calculated as $C_{14}H_{16}N_2O_3$ + Na = 283.2131]. The spectrum got from IR spectroscopy showed the existence of an amide carbonyl (1660 cm^{-1}), and aromatic ring (1635 and 1381cm^{-1}). The absorption maxima in the UV spectrum (278 nm) also showed the existence of an aromatic ring.

For structure elucidation 5 mg of pure compound (260) was mixed in 0.70 mL of deuterated DMSO and a series of one and two dimension NMR analyses were performed on 500 MHz NMR Spectrometer.

Hydrogen NMR (500 MHz) exhibited 12 signals for protons. At δ 3.17 there was a signal which showed integration of 2 protons representing CH_2 group. All the remaining signals represented the integration of single proton each.

The one dimension ^{13}C NMR exhibited 14 signals representing 14 carbons skeleton of the compound, exhibiting 1 quaternary carbon at δ 137.33 while 2 carbonyl carbons at δ 170.91 and 166.90 respectively. The two dimension HSQC showed 10 protonated carbons given in table 5.4. The two dimension (^1H–^1H COSY and HMBC) showed different correlations and the elucidated structure of the compound is given in figure 5.9 and 5.10 while NMR data is given in table 5.4.

Table 5.4 Chemical Shifts of New Compound (260). Data Obtained from 500 MHz Varian NMR

C. No	Multiplicity (DEPT)	^{13}C–NMR	^{1}H (*J* = Hz)
1	CH_2	116.56	6.77d (*J* = 5.0 Hz), 6.79d (*J* = 5.0 Hz)
2	CH	131.89	7.08 m
3	CH_2	38.21	3.17 dd
4	CH	60.08	4.07 m
5	C=O	170.91	----------
6	C=O	166.90	----------
7	CH	57.69	4.45 m
8	CH	38.82	3.56 s
9	–C–	137.33	----------
10	CH	129.45	7.23 s
11	CH	131.04	7.27 s
12	CH	128.07	7.25 s
13	CH	131.04	7027 s
14	CH	129.45	7.23

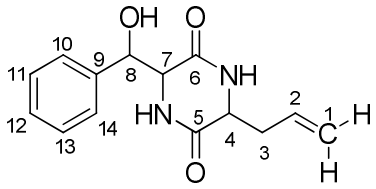

Figure 5.9 Structure of the new compound 260

In $^{1}H–^{1}H$ COSY, the alkene proton of H–2 exhibited correlation with the proton of C–3, also the proton H–3 exhibited correlation with the proton of C–4. The proton H–7 presented correlation with the protons of C–8. While in HMBC, the methylene proton H–1a and H–1b presented triple bond correlation with C–3 and four bonds correlation with C–4. The methylene proton H–2 also exhibited a triple bond correlation with C–4, four bonds correlations with carbonyl carbon at C–5 and weak five bonds correlation with carbonyl carbon at C–6. The protons H–3 represented a triple bond correlation with carbonyl carbon at C–5. The aromatic proton H–10 exhibited triple bond correlation with C–8, and the protons H–8 exhibited double bond correlation with C–7, triple bond correlation with carbonyl carbon at C–6 and weak four bonds correlation with carbonyl carbon at C–5. The two dimension ($^{1}H–^{1}H$ COSY and HMBC) alone with UV and IR revealed that this compound has an aromatic ring attached to six member ring of hetro group and a unique three carbon chain of alkene.

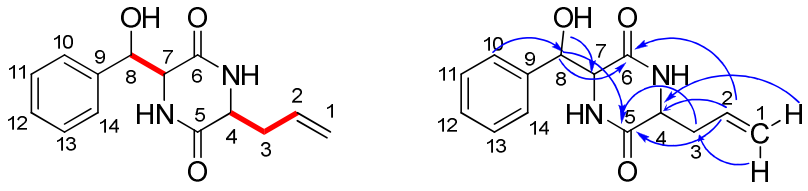

Figure 5. 10 COSY correlations have been shown by red arms, while the HMBC correlations have been shown by blue arrows.

5.6 Metabolites from *Penicillium* species

Analysis of chromatogram of crude extract from *Penicillium* sp. (figure 5.11) shows different peaks corresponding to different secondary metabolites. However, after simultaneous analysis of the UV, ES^+ and ES^-, one peak corresponding to E was selected to be isolated using the automated isolation procedure of the above mentioned HPLC.

5.7 Chromatogram of the crude extract from *Penicillium Species*

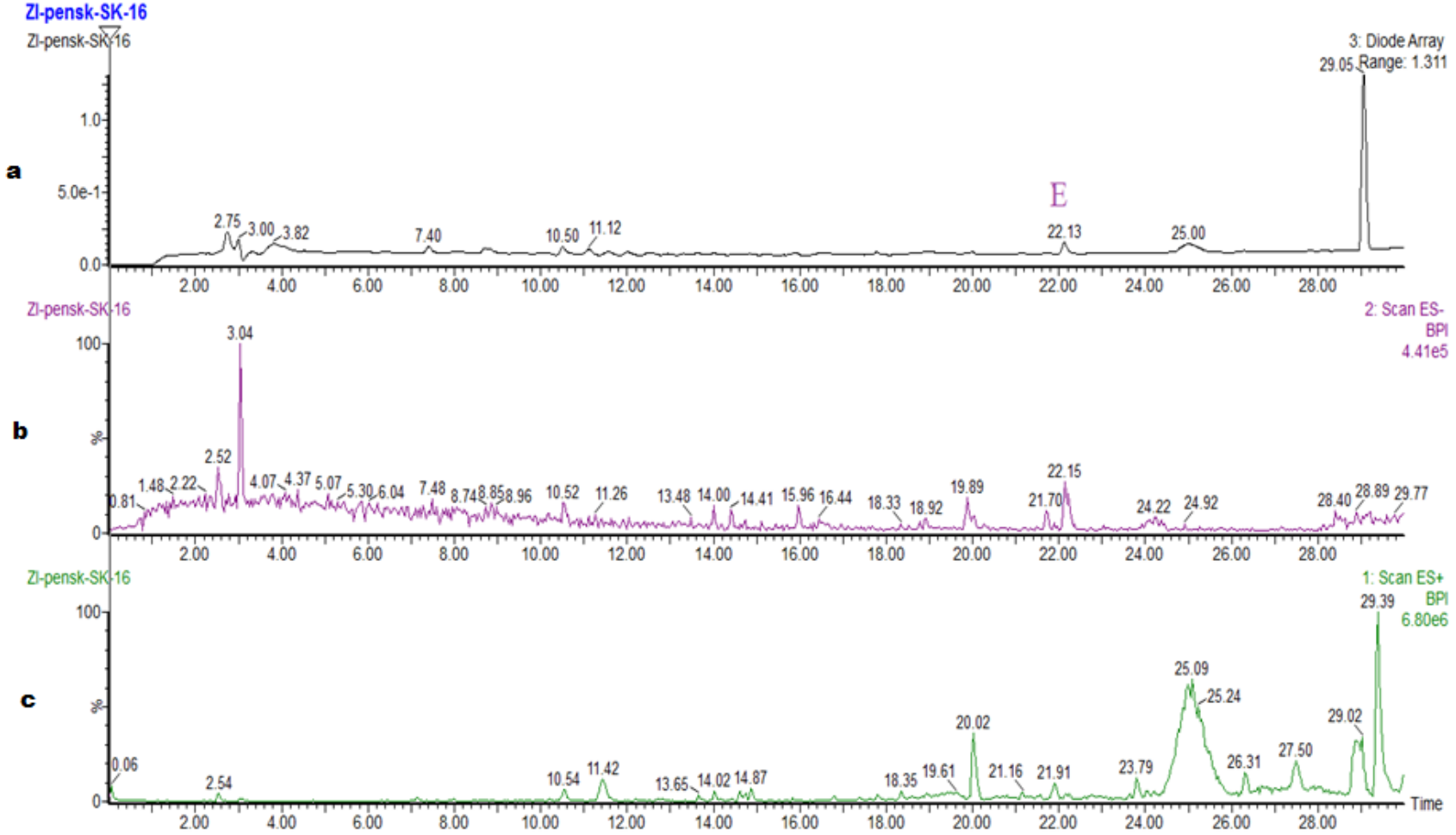

Figure 5.11 LCMS chromatogram of *Penicillium* sp, showing UV profile of the extract (trace a), ES^- profile of the extract (trace b) and ES^+ profile of the extract (trace c). The targeted compound (E) was eluted at 22.100 rt.

5.8 Chromatogram of new compound (E-437) from *Penicillium Species*

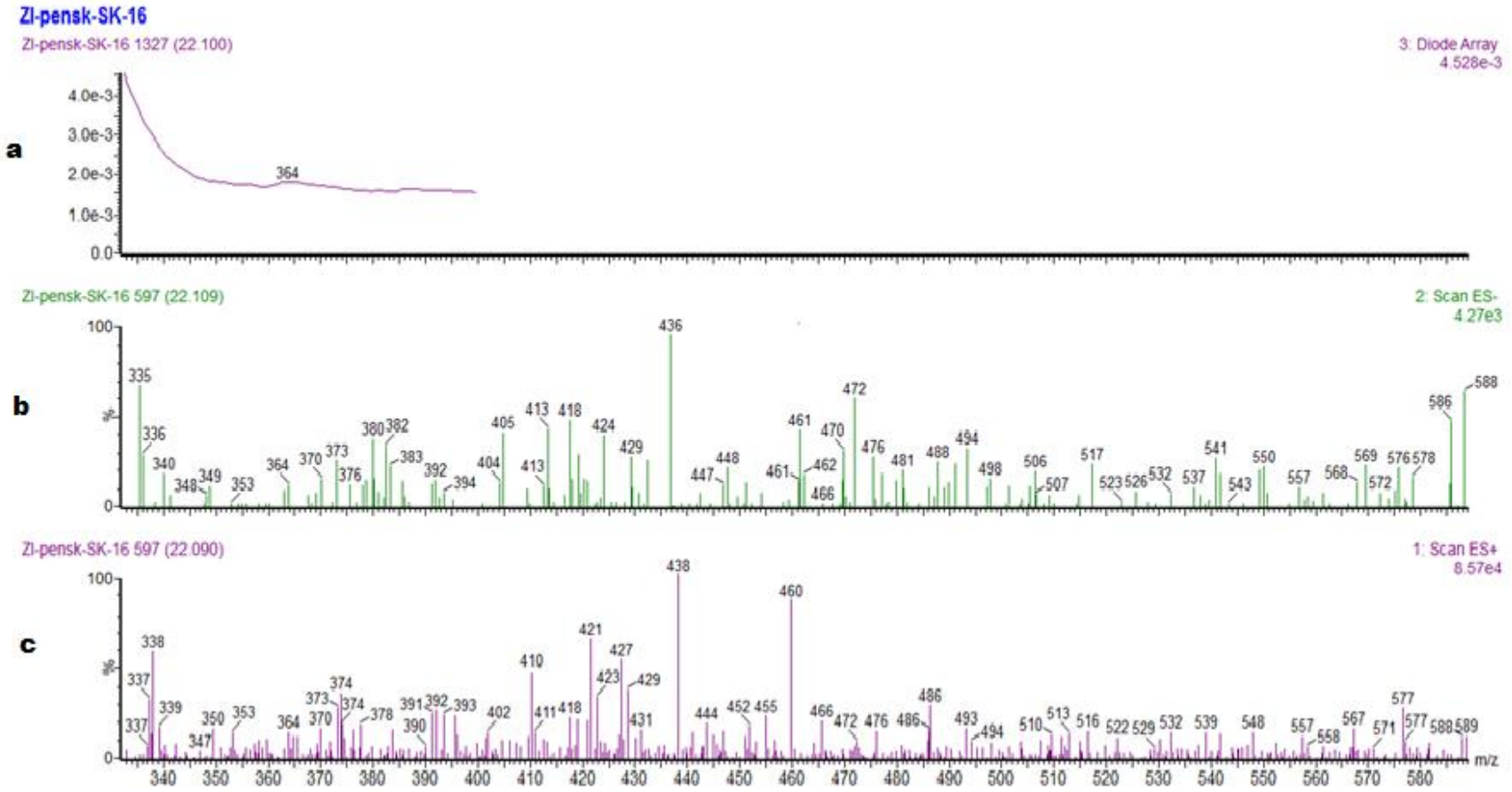

Figure 5.12 LCMS chromatogram of *Penicillium* sp, showing UV profile of the extract (trace a), ES^- profile of the extract (trace b) and ES^+ profile of the extract (trace c) of compound (437).

Individual estimation of chromatogram of compound E (437) is shown in figure 5.12, showing UV (364), ES^- (436) and ES^+ (438) and (460) corresponding to pure compound having mass of 437.

5.8.1 New compound (437)

The HR–ESI–MS and ^{13}C NMR (Broad Band and DEPT) were used to establish the molecular formula of the known compound. HR–ESI–MS presented pseudo molecular ion peaks [M^+] at *m/z* 438.1211 [M]H^+ and 460.1121 [M]Na^+ corresponding to the molecular formula $C_{26}H_{31}NO_5$ [Calculated as $C_{26}H_{31}NO_5$ + Na = 460.1121].The spectrum got from IR spectroscopy showed the existence of an aromatic ring (1635 and 1381cm^{-1}).The absorption maxima in the UV spectrum (364 nm) also confirmed the existence of aromatic ring.

For structure elucidation 5 mg of pure compound (437) was mixed in 0.65 mL of deuterated chloroform and a series of one and two dimension NMR analyses were performed on 500 MHz NMR Spectrometer. ^1H–NMR (500 MHz) exhibited 23 signals for 31 protons.

The one dimension ^{13}C NMR exhibited 26 signals representing 26 carbon skeleton of the compound representing 7 quaternary carbons and one carbonyl carbon at δ 202.47. In ^1H–^1H COSY, the amine proton of H–1 exhibited correlation with the proton of C–2, while the proton H–2 exhibited correlation with the proton of C–3, however, the proton H–3 have shown correlation with the hydroxyl-H of C-4. The H-8 has shown correlation with the H, of C-9 and vice versa. We have further observed the correlation of H-17 with the H, of C-18.

While in HMBC, the aromatic proton H–12 has presented triple bond correlation with C–4 and four bonds correlation with C–5. The proton H–3 also exhibited a triple bond correlation with C–5, four bonds correlations with carbonyl carbon at C–6 and weak five bonds correlation with quaternary carbon at C–7. The protons H–8 represented a triple bond correlation with carbon C–6 and weak five bonds correlation with C-19. The methyl proton H–17 exhibited four bond correlation with C–9 and there bonds correlation with C-19. The methyl proton H–18

exhibited three bond correlations with C–20. Chemical shifts of new compound 437 are given in table 5.5 while structure of compound is given in figure 5.13.

Table 5.5 Chemical Shifts of New Compound (437). Data Obtained from 500 MHz Varian NMR

C. No	Multiplicity (DEPT)	^{13}C–NMR	^{1}H (*J* = Hz)
1	----------	----------	6.77d (*J* = 5.0 Hz),
2	CH	85.23	4.23 m
3	CH	90.10	3.19dd
4	-C-	78.00	----------
5	-C-	115.50	----------
6	-C-	158.15	----------
7	-C-	118.65	----------
8	CH	126.23	7.23 s
9	CH	103.78	6.88 S
10	-C-	139.65	----------
11	-C-	140.55	----------
12	CH	129.43	7.24 s
13	CH	130.33	7.37 s
14	CH	125.46	6.88 s
15	CH	130.33	7.37 s
16	CH	129.43	7.24 s
17	CH	128.50	6.98
18	CH	128.34	6.56
19	-C-	35.98	----------

20	CH_2	50.12	1.77d (J = 10.0 Hz), 1.23d (J = 10.0 Hz)
21	CH	42.00	2.44
22	C=O	202.47	----------
23	CH_2	35.00	1.77d (J = 10.0 Hz), 1.23d (J = 10.0 Hz)
24	CH_2	35.60	2.56d (J = 10.0 Hz), 2.23d (J = 10.0 Hz)
25	CH_3	27.73	1.20
26	CH_3	15.26	0.96
27	CH_3	60.00	3.90

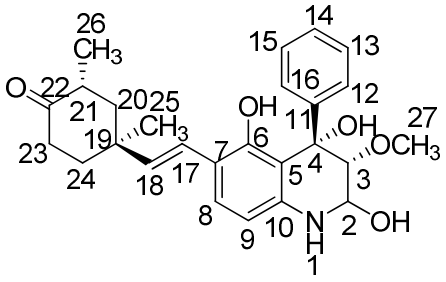

Figure 5. 13 Structure of the new compound 437

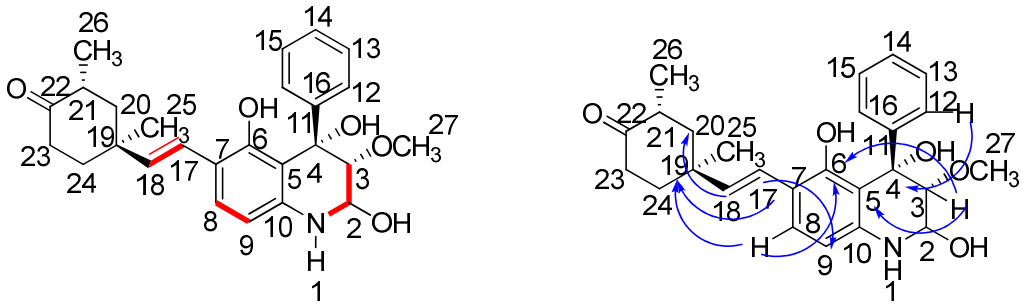

Figure 5.14 COSY correlations have been shown by red arms, while the HMBC correlations have been shown by blue arrows

CHAPTER 6

DISCUSSIONS

CHAPTER 6 DISCUSSIONS

6.1 Background of the problem

Multidrug resistant pathogens have become a serious threat to mankind. Although efforts are being made to synthesize and discover new antibiotics but there are no enough new drugs in pharmaceutical pipeline to compete with the pace at which drug resistant pathogens are evolving. According to the Infectious Disease Society of America 70% bacteria are resistant to at least one commonly used antibiotics [291]. Pharmaceutical companies have also cut back investing on antibiotic production as their use is for short term. Rather they are focusing on chronic drugs which are used for longer time. Health professionals warn that a lack of interest in antibiotic development field can cost humans their health. Already some species of the genus *Acinetobacter* and *Pseudomonas* are resistant to all good antibiotics and many members of *Enterobacteriaceae* are resistant to all penicillins except carbaphenems. New antibiotics are also needed for community acquired infections like tuberculosis and urinary tract infections (UTIs) [292]. The problem of antibiotic resistance is more serious in developing countries including Pakistan because of abundant and misuse of antibiotics. If the research area regarding antibiotic production and development is not strengthened, we will be short of antibiotics. There is a serious threat that because of growing population of antibiotic resistant pathogens many infections will be left untreated.

This scenario shows that there is an urgent and serious medical need for new bioactive compounds to be discovered to find a cure for infections caused by multidrug resistant pathogens. Only the discovery of new and potent antibacterial compounds will lead to the

solution of this problem [177]. So it is the demand of the modern era to explore new sources for the production of antibiotics. In this regard, we have exploited fungi for production of antibiotics and other secondary metabolites. In the present study, the quest for new metabolites of fungal origin led us to test four extracts of two different fungal species and their antifungal, antibacterial, cytotoxic, phytotoxic and herbicidal potential was evaluated. Both the fungal species were grown in broth cultures and their ethyl acetate and *n*-Hexane fractions were obtained. Different concentrations of *n*-Hexane and ethyl acetate fractions were tested against different organisms and results were recorded. These results were then compared with related reported data published in research articles.

6.2 Isolation of fungal strains and their identification

After insects fungi are the second most diverse group of living organisms. Fungi include more than 1.5 million species but less than 5% have been explored [293]. The percentage of isolated fungi may be higher because many studies have reported the isolation of bioactive metabolites from unidentified fungal strains. Still it is a fact that fungi are among the less studied groups of organisms which can act as reservoirs from which a large number of novel secondary metabolites can be isolated. According to Cannon (1997) less knowledge about the fungal species could be attributed to a number of factors such as most of fungal strains are microscopic, some of them produce their fruiting bodies for a very short time which are mostly unclear, species are identified using older techniques like observation of fruiting bodies, pigmentation and colony morphology. However, accurate specie level identification is only possible through utilization of molecular biology techniques [294].

Identification of fungal strains in context of natural products is an important step which often leads to isolation of diverse groups of bioactive compounds especially new compounds. Fungal identification also helps to prevent the repeated isolation of known compounds and new compounds of identified fungal strains could be easily determined based on the available data of natural products. Fungal identification can also proved fruitful in identification of compounds resulted from metabolic engineering.

Fungal species are mostly identified on the basis of their fruiting bodies and spore structure because these structures remain stable and do not show abrupt changes in their form and structure [295]. Mycologists still rely on this method for identification of fungal strains but in modern era this method is complemented by molecular biology techniques to identify fungi. Apart from these, different characteristics like colony and hyphae structure and color, spore arrangement and pigmentation also play important role in fungal identification.

In the present study different fungal strains were isolated from soil and rhizosphere of *Mentha piperita* but on the basis of preliminary antibacterial activities two fungal strains were further processed for isolation of natural products. The two fungal isolates on which this study focused were identified as *Aspergillus* and *Penicillium* species. These fungal strains were grown on different culture media that contained complex carbon and nitrogen source for sporulation. Composition of different media used in the present research work is given in table 3.1 of chapter number 3. However, it was not an easy task to perform species level identification based on morphological and microscopic characteristics as it is very tedious and time consuming process. Ebel (2010) have reviewed that in most cases it is very difficult to identify fungi from their sexual structures and efficient species level identification can only be achieved with the help of molecular biology techniques [296]. As discussed earlier that species level identification of

fungal strains play very important role in isolation of novel natural products to prevent repetitive isolation of known natural products. Due to lack of facility for molecular identification we relied on microscopic identification.

6.3 Fungal growth and nutrient requirements

Fungi are eukaryotic organisms that do not contain chlorophyll and thus exists as heterotrophs in nature [297]. All fungi requires organic carbon source to build their cellular constituents and to carry out life processes. Organic carbon is abundantly present in the cells of all living organisms such as plants, animals and microbes. Carbon can also be found as structural material in living organisms in the form of cellulose, chitin, lignin and keratin. Fungi have the ability to degrade and utilize all forms of carbon for fermentation of different classes of secondary metabolites [298]. As fungi are non-diazotrophs i.e. they cannot fix atmospheric nitrogen so they must be supplied with nitrogen by adding nitrogen containing organic or inorganic ingredients in the medium [299].

In our research, we used PDA and CYA media for the growth of selected fungal species. The media we have used have also been used by other research groups and is reported in literature [300]. Schauer (1987) proposed that for fungal secondary metabolite production complex medium that contain carbon and nitrogen sources and other ingredients should be used [301]. Changes in nutrient composition of the medium for example modification of nitrogen and carbon sources can greatly affect the ability of fungal strain to produce biologically active secondary metabolites. For example, Kansoh (2010) stated that addition of 2% starch and 0.24% potassium nitrate which were used as carbon and nitrogen sources greatly increased the production of antimicrobial compounds by *Penicillium viridicatum* [302]. We observed that PDA

and CYA were suitable for growth of *Penicillium* sp. isolated from soil and *Aspergillus* sp. isolated from rhizosphere while CYB was found suitable for both fungal growth and secondary metabolites production. CYB is a complex medium in which saccharose acts as carbon source while yeast extract used as source of nitrogen. Apart from carbon and nitrogen sources different types of macro and micronutrients are also added in the form of three solutions i.e. A, B and C. Composition of these three solutions are given in section 3.1 of this thesis. This complex nature and composition of CYB makes it a suitable medium for fungal secondary metabolites production. In our research work we observed that CYB actively supported the growth of both fungal species and enough quantity of organic extract also was recovered.

6.4 Culture conditions for production of metabolites

In this study different culture conditions were used to exploit the fungal strains to produce bioactive natural products. However, different inductions did not always proved fruitful in production of bioactive natural products. Miller (1986) reported that due to less understanding of natural products of filamentous fungi quantitative production of secondary metabolites in laboratory is very difficult because many conditions are required for production of fungal natural products [303]. In present study it is noted that some factors such as nature of the fungal strain, type of substrate, stage of development, pH of the medium, incubation temperature, period of incubation and speed of rotation all play important role for fungi to produce biologically active secondary metabolites.

Both terrestrial and marine species of *Aspergillus* and *Penicillium* genera have been largely used for production of biologically active secondary metabolites. These species are continuously reported to produce novel bioactive metabolites. Romer-Rassing and Gurtler (2002)

have reported that *Aspergillus* and *Penicillium* are two fungal genera which have the highest index of creativity in the fungal Kingdom with respect to production of biologically active secondary metabolites [304]. Some recent literatures which have reported isolation of novel bioactive compounds from *Aspergillus* and *Penicillium* species also support this statement [26, 75, 305-306].

6.4.1 Static vs. shaking culture conditions

It has been observed that as compared to static cultures fungal strains grown in shaking culture system produce more bioactive secondary metabolites [307]. But fungi cultivated in stated cultures have also been reported to produce bioactive natural products. When fungi are grown in static cultures they face two different conditions. At one end the surface layer of the fungus is continuously in contact with oxygen but at the same time this layer has poor contact with the medium. On the other hand the underside of the fungal culture although it is in contact with the medium but thick fungal growth makes it anaerobic which may result in growth inhibition and production of bioactive natural products [308]. But it also depends upon the characteristics of the fungus that whether it should be grown in static or shaking conditions. As most of the literature has recommended shaking culture conditions for fungal metabolites production and based on the experience of previous studies in our group, in the present study we have utilized shaking culture conditions by growing the fungal strains at 150 rpm at 28 ^{o}C. However, in future a comparison of static and shaking cultures is necessary to find out which type of culture result in production novel bioactive compounds.

6.4.2 pH of the medium

pH of the medium is one of the important factors which must be kept in mind while growing terrestrial and marine fungal strains is the pH of the medium. Modification of pH is comparatively easy by addition of dilute acid or alkali solution. Marine fungal strains grow in salt water so a high salt concentration is added to the medium while growing marine fungal species [309]. However, terrestrial fungal strains do not require high salt concentration for their growth. In our study both fungal strains showed better growth when cultured in medium with slightly acidic pH i.e. at 6.5. pH of medium was adjusted by adding few drops of HCl or NaOH solution as per requirement. After 2 to 3 days of cultivation, when fungal strains started to grow the pH of the medium further decreased. Kreisel and Schauer (1987) and Papagianni (2005) reported that this decrease in pH of the medium is an indication of fungal growth because they start to consume salts present in the medium [301, 310]. During the exponential growth phase fungi also release H^+ ions from their mycelia which decreases the pH of the medium [301]. We observed that this decrease in pH of the medium can also be due to production of acidic metabolites by fungi. In our study kojic acid have been isolated from *Penicillium* sp. which is an acidic metabolite produced by fungi.

6.4.3 Fermentation period

Production of secondary metabolites in fungi depends upon the growth of fungi i.e. fungi will start the production of secondary metabolites when its normal growth and development is completed. Fungi actively grow during exponential phase while secondary metabolites production usually starts during the lag phase of growth. As different fungal species grow at different rates, fluctuation has been observed in production of secondary metabolites during

fermentation period. Usually short fermentation period results in production of fewer amounts of secondary metabolites. Sometimes prolong cultivation of fungi for production of bioactive compounds may result in less antibiotic concentrations [311]. It has also been reported that bioactive secondary metabolites can harm the producing microorganisms if not secreted for longer times. There are some mechanisms through which the producer organisms prevent suicide which may result because of its own secondary metabolites. These mechanisms are generally known as self defense mechanisms. Some important self defense mechanisms in fungi include production of antibiotic inactivating enzymes and metabolic shielding. In the later mechanism antibiotics are produced as prodrugs which remain inert inside the cell of fungi but subsequently activated during secretion. The resistance of producer organisms towards antibiotic compounds is less at the early stage of development (trophophase) but later it increases in idiophase. During this phase active production of antimicrobial metabolites occurs but the producer organism remain unaffected as these metabolites are secreted out words through efficient efflux mechanisms [312].

We observed that both fungal strains produced bioactive secondary metabolites during normal fermentation period i.e. the fermentation period was not too short neither too long. As some marine species are have short fermentation period while endophytic fungi usually require longer fermentation periods for production of metabolites. Fermentation period at which both fungal strains produced bioactive natural products was from 12 to 14 days. During most of this period the fungi showed active growth rate. It was observed that growth of fungal strains slow down after 10^{th} day of cultivation which may be due to different factors such as nutrient and oxygen deprivation in the medium. In attempt to obtain different variety of natural products, fermentation period was extended for both fungal species but it did not greatly affected the yield

of organic extract. Walker and White (2005) stated that fungi mostly produce bioactive compounds when they enter into stationary phase. As stationary phase comes after exponential phase during which most of the nutrients are depleted from the medium. If this stationary phase is prolonged some physiological factors such as accumulation of toxic substances in the medium, high CO_2 concentration, low pH and insufficient O_2 concentration may decrease the ability of fungi to produce desirable bioactive secondary metabolites [313].

6.5 Bioassay screenings

In the quest for bioactive fungal secondary metabolites, the fungal extracts were tested for antibacterial, antifungal, cytotoxic, phytotoxic and herbicidal activities. The organic extracts from both *Aspergillus* and *Penicillium* sp. were fractionated into ethyl acetate and *n*-Hexane (1:1). Both these fractions were tested for above mentioned bioassays and as compared to *n*-Hexane, ethyl acetate fraction was observed more active. It was observed that the organic extract from *Aspergillus* showed more activity than *Penicillium*. Although activities of ethyl acetate extract were higher but in some cases *n*-Hexane fraction of *Aspergillus* sp. also showed very good activities against some bacterial and fungal strains as shown in figure 4.2 and 4.10 in section 4.1 and 4.2 of this thesis. The available literature on fungal secondary metabolites is also in compliance to our findings because organic extracts of fungi contain biologically active metabolites that can inhibit growth of both fungi and bacteria [314-315].

It is reported that marine and endophytic fungi are rich sources of bioactive metabolites [29, 316-318]. From our results we conclude that plant associated fungi are more active as compared to soil inhabiting fungi as plant associated *Aspergillus* sp. proved more active as compared to soil borne *Penicillium* sp. However, both *Penicillium* and *Aspergillus* sp. have

been reported to produce metabolites with antibacterial and antifungal properties. Onyegeme-Okerenta (2009) isolated antimicrobial metabolite from *Penicillium* which showed high level of antimicrobial activities against some pathogenic bacterial strains and actively inhibited the growth of *E. coli* and *B. subtilus*. Muhammad (2013) also reported similar compound from *A. niger* and *P. notatum* with antimicrobial activities against another set of pathogenic bacteria (*Staphylococcus aureus* and *P. multocida*). In the present study crude extract of both *Aspergillus* and *Penicillium* actively inhibited the growth of pathogenic bacteria i.e. *S. typhi*, *E. coli and S. aureus* by forming active zones of inhibition as shown in figure 4.1 and 4.6 of chapter number 4.

Aspergillus sp. isolated from the rhizosphere of *Justicia adathoda*, was assessed by Prabavathy (2012) for antimicrobial activities. The extract was found to posses activity against a set of pathogenic bacteria and fungi (*E. coli, K. pneumonia*, *P. aeruginosa* and *Candida albicans*). Bioassay guided isolation lead to a compound with antimicrobial activities [315]. *Aspergillus* species used in the present study was also isolated from rhizosphere of *Mentha piperita.* Ethyl acetate extract of *Aspergillus* sp. we obtained proved more effective as compared to *Aspergillus* sp. reported by Prabavathy (2012) as in our case it not only inhibited the growth of *Candida albicans* but also inhibited the growth of five other fungi i.e. *Aspergillus flavus, Candida glabrata, Fusarium solani, Microsporium canis and Trichophyton longifusus* as shown in figure 4.9 of chapter number 4. *Aspergillus* species used in present study also exhibited strong antimicrobial activities against most of the tested bacterial strains as shown in figure 4.1 of chapter number 4. Among the tested bacterial strains most resistance was shown by *X. oryzae* which formed a zone of 9 mm at highest tested dose concentration of *Aspergillus* extract while no zone of inhibition was recorded even at highest tested dose concentration of *Penicillium* extract against this bacterium.

Aspergillus sp. from plant rhizosphere and soil borne *Penicillium* sp have been reported by many researchers as producers of antimicrobial metabolites [208, 319]. Based on the biological activities of crude extracts of both the species used in the present study, it is concluded that both fungal strains could be a good source of biologically active secondary metabolites upon further exploitation. Therefore, it is suggested to exploite these species for the isolation of antibacterial and antifungal compounds.

Members of the genus *Aspergillus* have immense chemical diversity and produce variety of metabolites that includes polyketides, terpenoids, alkaloids and xanthones. These metabolites along with antifungal and antibacterial properties also have shown strong cytotoxic activities [318, 320]. These studies are in compliance to the findings of our research work because *Aspergillus* sp. used in present study along with antibacterial and antifungal properties also showed a high degree of cytotoxic activities against brine shrimp eggs. A higher degree of cytotoxicity was recorded for crude extract of *Aspergillus* sp. in comparison to *Penicillium* sp. as at highest dose concentration i.e. at 1000 µg/mL of ethyl acetate extract 95% mortality rate was recorded for *Aspergillus* sp. while 63.33% mortality was observed for *Penicillium* sp. It has been reported that high degree of cytotoxicity of *Aspergillus* metabolites may be due to the production of mycotoxins in the medium [321]. Cytotoxic metabolites of fungi have frequently been reported as potential anticancer drug leads. One of the major areas of fungal natural products has been devoted to the discovery of new anticancer drugs [199].

Since ancient times, plants have been traditionally used for their allelopathic potential. For example, flavonoids reported from many different plants are considered as important allelochemicals produced by plants which can inhibit the growth of other plants [322]. However, alternative to plants fungi have been recently utilized as producers of allelochemicals for

application in agriculture [250]. The fact that comparative to plants fungal strains can easily be grown and maintain under laboratory conditions attracted the attention of scientists towards utilization of fungi for production of bioactive secondary metabolites of agrochemical importance. Antonio and coworkers (2000) tested the crude extract of mycoherbicidal fungus *Ascochyta caulina* against different plants of monocot and dicot families and some weeds. Bioassay guided isolation lead toward the isolation of a new compound with phytotoxic and herbicidal potential. With the help of 1D and 2D NMR techniques the compound was named as trans-4-aminoproline [323]. An endophytic *Helminthosporium* sp. has been reported to produce important compounds of biological interest under laboratory conditions. A total of six compounds were isolated which were named Ophiobolin A, 6-Epiophiobolin A, 3-Anhydroophiobolin A, 3-Anhydro-6-epiophiobolin A, Ophiobolin B, and Ophiobolin I. Among the isolated compounds ophiobolin A has shown stronger phytotoxic properties by inhibiting the growth of eight different plant species in phytotoxicity assays [324].

In present study *n*-Hexane and ethyl acetate fractions of both *Aspergillus* and *Penicillium* species inhibited growth of *Lemna minor* and also prevented germination of *Silybum marianum* L. seeds. These experiments have confirmed the presence of some active phytotoxic components in the extract. The *n*-Hexane fraction of *Aspergillus* sp. inhibited the growth of *Lemna* by 85% while its ethyl acetate fraction inhibited the growth by 65%, which shows that the non polar components of crude extract of *Aspergillus* sp. are more toxic as compared to the ethyl acetate fraction because the *n*-Hexane fraction mostly contain maximum of non polar and/or oily components. However, ethyl acetate fraction of *Penicillium* sp. proved more phytotoxic as it inhibited the growth of *Lemna minor* by 56.6 % while its *n*-Hexane fraction inhibited the growth of *Lemna* by 30%. Although different fractions from crude extract of both fungal strains have

presented good phytotoxic and herbicidal activities but it is important to further investigate which specific metabolite is responsible for phytotoxicity. While working with *Aspergillus* sp. there is always a possibility that the activity may be due to release of toxins by the fungus. But it is encouraging that *Penicillium* sp. has also shown good activities both against *Lemna minor* and *Silybum marianum* L.

Fungi have been reported to produce variety of compounds with herbicidal potential. Nakajima and coworkers (1991) exploited a newly discovered fungus *Paecilomyces variotii* SANK21086 for production of bioactive metabolites with herbicidal activities. Bioassay guided isolation was performed with lead to the isolation of a novel compound. This compound showed good herbicidal activities against weeds of both monocot and dicot families with limited effect on corn plants. This compound was named as Cornexistin which belonged to nonaride group of compounds [325]. Akbar and Javaid (2012) used petriplate method to test extracts of four *Drechslera sp* on two species of weeds i.e. *Chenopodium album L*. that belong to dicot family of plants and *Avena fatua L.* which is an important weed of the wheat crop. According to their results the fungus filtrate effected the germination of *C. album* by 28 -50%, shoot length was reduced by 54 to 91% and shoot biomass decreased by 58 to 81%. Root length and biomass also showed significant decrease as it was reduced by 66 to 88%. For *A. fatua* germination decreased by 28-54% because of different filtrate concentrations. Roots of *A. fatua* proved more susceptible as compared to shoots. The fungal filtrate reduced shoot length of *A. fatua* by 27 to 67% , shoot biomass was reduced by 27 to 57%, root length and root biomass was reduced by 55 to 86% and 47 to 77% respectively as different dose concentrations were applied [326]. In our study we also tested extract of both *Aspergillus* and *Penicillium* sp. on test weed *Silybum marianum* L. seeds using petriplate method. In control treatments seed germination percentage was recorded as 90%,

shoot length was recorded as 7.8 cm, root length was recorded as 6.3 cm, and shoot weight was recorded as 0.39g and root weight was recorded as 0.32g. However, no growth or seed germination was observed in other extract applied treatments. This experiment was performed in triplicate but similar findings were observed each time. These findings shows that extract from *Aspergillus* and *Penicillium* sp. have shown toxicity against *Silybum marianum* L. by strongly inhibiting its seed germination and inhibition of root and shoot initiation.

From the present investigations we conclude that crude extracts of both *Aspergillus* and *Penicillium* sp. contain potential bioactive metabolites which have antibacterial, antifungal, cytotoxic activities. Along with these activities the crude extracts were found to have the potential of inhibiting the growth of Lemna, inhibited seed germination and roots and shoots initiation of *Silybum marianum L.* Based on these results the crude extract of both fungal species was further investigated to find out exact metabolite responsible for inhibition. Work towards identification of these interesting metabolites resulted in isolation of 5 different compounds.

6.6 Bioactive secondary metabolites of fungi

In the present study, 5 different compounds were isolated from ethyl acetate extracts of *Aspergillus* and *Penicillium* species. Among these 5 compounds, three were known compounds while 2 were new compounds.

Both terrestrial and marine species of *Aspergillus* and *Penicillium* species have been largely used for production of biologically active secondary metabolites and these fungal genera have the highest index of creativity in the fungal Kingdom with respect to production of biologically active secondary metabolites [304] Some recent literatures which have reported isolation of novel bioactive compounds from *Aspergillus* and *Penicillium* species also support

this statement [26, 75, 305-306]. *Aspergillus* sp. from marine origin are mostly reported for production of novel bioactive metabolites e.g. alkaloids, chrome derivatives, unsaturated fatty acids, anthraquinone and sesquiterpenes etc. However, in addition to specie variation, different habitats can influence the ability of fungi to produce bioactive natural products [327-328]. Schulz *et al* (1995) also reported that production of secondary metabolites by fungi is often associated with their role in environment. They concluded that plant associated fungi produce a larger proportion of secondary metabolites as compared to soil isolates [221].

These findings are in accordance to our study as in the present study we worked on *Aspergillus* sp. which was isolated from the rhizosphere of *Mentha Piperita* while *Penicillium* sp. was a soil isolate. As compared to *Penicillium sp*. the crude extract of *Aspergillus sp*. proved more active and also showed more chemical diversity during purification of compounds. Based on literature available on *Aspergillus* sp. it was not surprising that *Aspergillus* strain used in the present study proved more bioactive and showed chemical diversity (Chapter 5). The four compounds isolated from *Aspergillus* sp. that include one new and three known compounds are described in chapter 5 of this study. The molecular masses of isolated compounds from *Aspergillus* sp. were determined by LCMS as 260 for new compound and 388, 142 and 262 for known compounds respectively.

Previous data shows that compounds isolated from *Aspergillus* sp. showed a wide range of biological activities including antifungal [329], antibacterial [330] and anticancer [331]. Apart from these, secondary metabolites of *Aspergillus* have been reported to reduce obesity by lowering appetite [332]. Considering these properties of *Aspergillus* compounds it is important to further investigate the biological properties of purified compounds. But for this it is necessary to obtain these compounds in greater quantities.

The genus *Penicillium* is one of the most diverse genera of kingdom fungi and its chemical diversity has also been greatly explored. However, both *Penicillium* and *Aspergillus* continue to be the sources of novel secondary metabolites [208, 333]. Our findings further support this statement as along with *Aspergillus* sp. one new compound has been isolated from *Penicillium* sp. which has been described in section 5.7 of this thesis. The molecular mass of isolated compound from *Penicillium* sp. was determined by LCMS as 437. One of the known compounds isolated during this study was kojic acid isolated from *Aspergillus* sp. which is a well known commercially available compound produced by many different fungi. Because of this reason while exploring the chemical diversity of fungi, scientists generally avoid common fungal genera like *Penicillium* and *Aspergillus* to minimize the chances of rediscovery of the same compounds.

6.7 Summary and conclusions

In this study different fungal strains were isolated from soil and rhizosphere of *Mentha piperita* using potato dextrose agar medium. However, based on preliminary antimicrobial testing for bioactivity two fungal strains were selected for further investigations. The two fungal strains were identified on the basis of colony morphology, pigmentation and structure of fruiting bodies as *Penicillium* and *Aspergillus* sp. Czapec yeast broth medium was used for production of metabolites.

The fungal crude extracts were subjected for bioactivity testing using different bioassays like antibacterial, antifungal, phytotoxic, herbicidal and cytotoxic assays. Bioassay guided isolation of the compounds was performed using column chromatography and HPLC techniques. Mass of the isolated compounds was determined using LCMS and structural elucidation of

compounds was performed using NMR techniques. A total of 5 compounds were isolated from extract of both fungal species that included two new and three known compounds. The two new compounds were 3-allyl-6-(hydroxy (phenyl) methyl) piperazine-2,5-dione and (2*R*, 4*S*)-2, 4-dimethyl-4-((*E)*-2- ((3*S*, 4*S*)-2, 4, 5-trihydroxy-3-methoxy-4-phenyl-1, 2, 3, 4-tetrahydroquinolin-6-yl) vinyl) cyclohexanone while the three known compounds were (*E*)-3-((3*S*,4*R*)-8-hydroxy-3,4-dimethyl-1-oxoisochroman-7-yl) acrylic acid, 2-(1, 4-dihydroxybutan-2-yl)-1,3-dihydroxy-6, 8-dimethoxyanthracene-9, 10 (4a*H*, 9a*H*)- dione and 5-hydroxy-2-(hydroxymethyl)- 4*H*-pyran-4-one.

From the results of the present study it is concluded that fungi are rich source of natural secondary metabolites with interesting properties and bioactivities. As compared to soil inhabiting fungi, plant associated fungal strains can exhibit more chemical diversity. In our research work we observed that in comparison to *Penicillium* sp. *Aspergillus* sp. produced more bioactive compounds. Along with other bioassay screenings fungal extract tested for herbicidal activities showed very good results. As compared to *Penicillium* sp. the organic extract of *Aspergillus* sp. showed more cytotoxicity which may be due to production of mycotoxins. Because of fungal diversity for production of bioactive secondary metabolites, and increasing resistance for the available drugs, it is necessary to further explore more fungal genera so as to meet the modern challenges.

FUTURE DIRECTIONS

FUTURE DIRECTIONS

The ultimate aim of present research work is the search for bioactive natural products of fungal origin. However, it is recommended that production of bioactive natural products could be more fruitful if the following aspects are studied more extensively in future.

- ✓ To test the isolated pure compounds for side effects on animals, humans and plants.
- ✓ Fungal natural products can inhibit growth and seed germination of different weeds. So it is necessary that natural herbicides of fungal origin should be tested.
- ✓ Optimization of fermentation conditions is necessary to further enhance the yield of bioactive secondary metabolites synthesized by different fungal species.
- ✓ To minimize the toxicity and/or to increase the antimicrobial activities of the purified compounds by modification in their structures by its derivatization using fungi as tools. This will help to increase the efficacy and selective toxicity of the purified compounds towards microorganisms.
- ✓ Feeding experiments should be performed using labeled isotopic precursors for investigation of metabolic pathways for production of novel secondary metabolites.
- ✓ Strain improvement using biotechnology and genetic engineering techniques can be used to further enhance the production of bioactive natural products of interest.
- ✓ Identification and confirmation of regulatory genes involved in the synthesis of bioactive compounds is necessary for which gene knockout experiments could be performed.

Although, a large number of bioactive compounds have been isolated from fungi but only a small proportion of the total fungal species have been investigated so far. Therefore, the research based on fungal natural products is a wide area which can offer great opportunities to discover novel bioactive compounds that could be utilized as effective clinical drugs and herbicides in future.

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