# EXPLOITATION OF *ASPERGILLUS* AND *CLADOSPORIUM* SPECIES FOR BIOLOGICALLY ACTIVE SECONDARY METABOLITES

**Ph. D Thesis**

**By**

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**CENTRE FOR BIOTECHNOLOGY AND MICROBIOLOGY UNIVERSITY OF PESHAWAR**

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# EXPLOITATION OF *ASPERGILLUS* AND *CLADOSPORIUM* SPECIES FOR BIOLOGICALLY ACTIVE SECONDARY METABOLITES


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

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**Abid Ali Khan**

## AUTHOR'S DECLARATION

I solemnly declare that the research work presented in this thesis was carried out in accordance with the requirements of the University of Peshawar's regulations for Research Degree Programs. The author has not been submitted this work for any other academic award. The work is original and of author's own data. While work done in collaboration with, or without the assistance of, others, is indicated as such. The views expressed in the thesis, belongs to the authors.

15–August–2013

Date_____ Signature_____

## DEDICATION

I DEDICATE THIS HUMBLE WORK TO
MY

## PARENTS

AND TO COMMEMORATE
MY LATE

## GRANDFATHER

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## LIST OF ABBRIVATIONS

| | |
|---|---|
| $^{o}C$ | Degree Celsius |
| AAA | Aminuteo acyl adenylate |
| ACE1 | Avirulence conferring enzyme1 |
| ACP | Acyl Carrier Protein |
| AD | Adenylation Domain |
| ART | antisense RNA technique |
| AT | Acyl Transferase |
| ATP | Adenosine Triphosphates |
| CD OR CZD | Czapek Dox |
| CD | Condensation Domain |
| CMeT | C–methyltransferase |
| COSY | Correlation Spectroscopy |
| DCC | Decarboxylative Claisen Condensations |
| DH | Dehydratase |
| DPY | Dextrose Polypeptone Yeast–extract Media |
| ER | Enoyl Reductase |
| ESI | Electrospray Ionisation |
| FAS | Fatty Acid Synthase |
| GET | Genetic Engineering Technology |
| HMBC | Heteronuclear Multiple Bond Correlation |
| HPLC | High Performance Liquid Chromatography |
| HR | Highly Reducing |
| HRc | Homologous Recombination |
| HSQC | Heteronuclear Single Quantum Coherence |

| | |
|---|---|
| IR | Infrared |
| KR | β–ketoreductase |
| KS | β–ketosynthase |
| LB | Luria Bertani Medium |
| LC | Liquid Chromatography |
| m/z | Mass to Charge Ratio |
| MEA | Malt Extract Agar |
| MS | Mass Spectrometry |
| MSB | Magnetic Stirring Bars |
| NHEJ | Non–Homologous End Joining |
| NMR | Nuclear Magnetic Resonance |
| NPC | Natural Product Chemistry |
| NR | Non–reducing |
| NRPS | Non–ribosomal Peptide Synthetases |
| OSS | Organic Synthetic Scientists |
| PDB | Potato Dextrose Broth |
| PKS | Polyketide Synthase |
| PKS–NRPS | Polyketide Synthase Nonribosomal Peptide Synthetases |
| TD | Thiolation Domain |
| UV | Ultra Violet |
| WT | Wild Type |

# ABSTRACT

*Aspergillus carbonarius* (NRRL–369) and *Aspergillus oryzae* from *Aspergillus* genus as well as *Cladosporium carrionii* and *Cladosporium resinae* (NRRL–6437) from *Cladosporium* genus were selected for the present study. Nutrient media were optimized for the growth and production of secondary metabolites. Out of five different media used, *A. carbonarius* and *A. oryzae* produced relatively more metabolites in Czapek–dox (Glucose and Starch) broth media (CGSB). Whereas; *C. carrionii* and *C. resinae* produced relatively more metabolites in Czapek yeast extracts broth (CYB). To further increase secondary metabolites productivity, two additional chemical compounds (suberoyl anilide hydroxamic acid; SAHA and 5–azacytidine; 5–AZA) were also used as chemical inducers for all fungi except *C. carrionii.* A dose of 10 µM/100 mL of SAHA resulted in higher secondary metabolites production from *Aspergillus* species and 15 µM/100 mL of SAHA resulted in higher secondary metabolites production from *C. resinae*. While a dose 15 µM/100 mL of 5–AZA resulted in higher secondary metabolites production from all the species.

Secondary metabolites produced were then studied for its respective biological activities. In antibacterial assay a dose of 500 µg/mL of ethyl acetate extracted from *A. carbonarius* inhibited the growth of *B. subtilis* (64.5%), while for antifungal testing a dose of 1000 µg/mL ethyl acetate extract inhibited the linear growth of C. glabrata (58.5%). Whereas, in cytotoxic activities, dose of 1000 µg/mL of ethyl acetate extract showed 94% mortality against brine shrimps, while for phytotoxic activities, a dose 1000 µg/mL showed 90% mortality against Lemna.

A dose of 500 µg/mL of ethyl acetate extracted from *A. oryzae* inhibited the growth of B. subtilis (94%), while for antifungal testing, a dose of 1000 µg/mL of

ethyl acetate extract inhibited the linear growth of *M. Canis* (84%). Whereas, in cytotoxic activities a dose of 1000 µg/mL of ethyl acetate extract showed 52% mortality against brine shrimps, while for phytotoxic activities, a dose of 1000 µg/mL of ethyl acetate extract showed 67% mortality against Lemna.

Furthermore, during the antibacterial assay a dose of 500 µg/mL of ethyl acetate extracted from *C. carrionii* inhibited the growth of B. subtilis (66%), while for antifungal testing a dose of 1000 µg/mL ethyl acetate extract inhibited the growth of *C. albicans* (60%). Whereas, in cytotoxic activities a dose of 1000 µg/mL of ethyl acetate extract showed 87% mortality against brine shrimps, while for phytotoxic activities, a dose of 1000 µg/mL ethyl acetate extract showed 80% mortality against Lemna.

Finally during the antibacterial assay a dose of 500 µg/mL of ethyl acetate extracted from *C. resinae* inhibited the growth of *S. aureus* (81%), while for antifungal testing a dose of 1000 µg/mL of ethyl acetate extract inhibited the growth of *A. flavus* (15%), while in cytotoxic activities a dose of 1000 µg/mL of ethyl acetate showed 93% mortality against brine shrimps, while for phytotoxic activities, a dose of 1000 µg/mL of ethyl acetate showed 80% mortality against Lemna.

The biological activities indicates that, the extracts from *A. oryzae* and *C. carrionii* inhibited the growth of experimental organisms with greater extent as compared to *A. carbonarius* and *C. resinae*; therefore, *A. oryzae* and *C. carrionii* were further selected for the isolation of pure metabolites. A total of three new and four known metabolites were isolated. Two new metabolites were isolated from *A. oryzae* while one new and four known metabolites were isolated from *C. carrionii* using preparative High Performance Liquid Chromatography (HPLC) and column chromatography techniques. The structures of all the compounds isolated were

elucidated using (1D and 2D) NMR, IR and HR–MS techniques. The new metabolites were 6–butyl–3–methylene–2–oxotetrahydro–2H–pyran–4–carboxylic acid (**A–41**), 6–butyl–3–methylene–2–oxo–3,6–dihydro–2H–pyran–4–carboxylic acid (**A–42**) and (3S,6S)–3–allyl–6–benzylpiperazine–2,5–dione (**D–44**) whereas, the known metabolites were 5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one (**C–43**), 6–(3–methylbut–2–enyl)–1H–indole–3–carboxylic acid (**45**), 2-(4,6-dihydroxy-3-oxo-1,3-dihydroisobenzofuran-1-yl) acetic acid (**46**) and 2-(4-hydroxy-1,3-dihydroisobenzofuran-1-yl) acetic acid (**47**).

The two new metabolites (**A–41** and **B–42**) from *A. oryzae* were selected for the determination of their biosynthetic pathways using [1–$^{13}$C] labelled acetate. The [1–$^{13}$C] labelled acetate was added to the media on 4$^{th}$, 5$^{th}$ and 6$^{th}$ days respectively. After the feeding of isotopic [1–$^{13}$C] labelled acetate as precursor, the labelled metabolites were isolated using HPLC and the pattern of their incorporation were determined using high field NMR.

The basic idea of the present work was to isolate biologically active secondary metabolite(s) from fungi and to produce good quality of antibiotics for the welfare of the society.

# CHAPTER 1

# INTRODUCTION

# 1 INTRODUCTION

## 1.1 Introduction to Fungi

Fungi are living–organisms which are also called *Eumycota* (*true fungi* or *Eumycetes*). Their existence is most common in water, land/soil, in air, and also in/on animals and plants. Fungi are of great importance because they are uniformly dispersed in the environment and also offer a vast biodiversity. It has been estimated that there are about seventy thousand (70,000) known fungal species, which is approximately 4.66% of the total fungal population [1].

Fungi are very famous for the production of biologically active chemical compounds/secondary metabolites [2]. These biologically active secondary metabolites are classified into four groups including alkaloids, isoprenoids, non–ribosomal peptides and polyketides [3]. Fungi are mysterious organisms that even a single species can produce different secondary metabolites depending on the availability of nutrients and environmental conditions [4].

Endophytic fungi are the fundamental resource of producing bioactive (both beneficial and harmful) secondary metabolites, which have a potential use in agriculture, food and in pharmaceutical industry. Over the last three decade, a number of important bioactive compounds with anticancer (doxorubicin) [5], antimicrobial (penicillin–G) [6], cytotoxic and insecticidal activities have been successfully isolated [7]. The endophytic fungi is known to be the main source for the production of biologically active secondary metabolites [8-9], which have been developed as a new and standard pharmaceutical agents [10-11].

Not only the soil endophytic fungi but marine organisms are known to be good source of new bioactive metabolites which are identified to be a capable and potential source for agricultural, environmental, industrial and pharmaceutical uses [12] and

some of them serves as valuable source for the production of clinically important anti–cancer compounds [13].

### 1.1.1 *Aspergillus* species

*Aspergillus species* have shown in figure 1.1, are known to be a good producer of bioactive compounds with minimum number of cytotoxic compounds in their extracts therefore, it is convenient to isolate potent compounds from them [14], *Aspergillus versicolor* is a good example for the production of polyketides [15]. Ochratoxin–A is a secondary metabolite isolated from *Aspergillus species*, its main function is to protect fungi from insect predation [16].

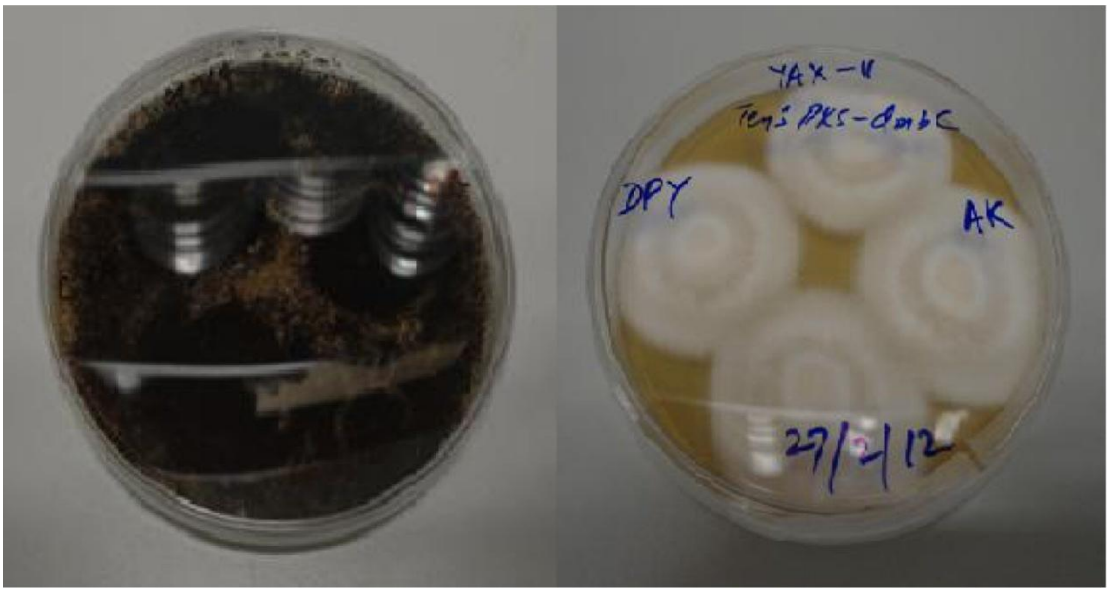



**Figure 1.1 *Aspergillus carbonarius* (NRRL–369) to the left, whereas, *Aspergillus oryzae* (tenS.PKS/dmbC) to the right.**

### 1.1.2 *Cladosporium* species

*Cladosporium species* have shown in figure 1.2, are widespread fungi, and are of different types, especially endophytic fungi. These fungi are consisting with a wide range of properties e.g., *fungicolous*, are the human pathogenic as well as phyto–pathogenic [17]. Most of the *Cladosporium species* are non–pathogenic to humans, however; some species are found to cause skin infections, sinusitis, toenails and pulmonary infections. If pulmonary infections were left untreated, they may lead to a complicated disease (pneumonia) [18]. *Cladosporium carrionii* is a causative agent for several types of chromoblastomycosis and asthma [19].


**Figure 1.2 *Cladosporium resinae* (NRRL–6437) to the left while to the right it is *Cladosporium carrionii* F–2.**

Fungi is a tremendous source for the production of bioactive compounds e.g. penicillin from *Penicillium notatum*. Bacteria is also a fundamental source for the production of modern medicine e.g., bacitracin from *Bacillus subtilis*, beside this another important chemotherapeutic agent i.e. taxol have been produced by an endophytic fungi [20-21]. It is clear that bacteria and fungi are the producers of

antibiotics and approximately about twenty two percent (22%) of the available and recognized antibiotics have been isolated from filamentous fungi [22-23].

Among the four types of natural products non–ribosomal peptide and polyketides are the two major and important groups of metabolites produced by fungi through different type of biosynthetic pathway, i.e. non–ribosomal peptides are synthesized by a cluster of enzymes called non–ribosomal peptide synthetases (NRPS) and polyketides by a step wise reactions catalyzed by a group of modular enzymes called polyketide synthases (PKSs) [24-26].

In this study *Aspergillus* and *Cladosporium* species were exploited to laboratory conditions for the production of some new biologically active secondary metabolites, because they have shown good results against the pathogenic bacteria in their preliminary biological screening.

## 1.2 Aims

The aim of this study is to explore biologically active secondary metabolites from fungi. For discovery of new drugs to combat prevailing ailments, development of robust methodologies and their utilization is important. As some of the fungi are toxic to human therefore, very limited research regarding, the isolation of biologically active secondary metabolites have been done so far.

The unauthentic practice and frequent use of antibiotic in our country has changed the dimension and lead to the development of resistant pathogenic species to some of the current available medicines. Therefore the search for new drugs is predominantly important. Therefore it is of great interest to isolate secondary metabolites from fungi with bioactive properties.

## 1.3 Objectives

- To isolate the fungi from soil samples
- To study the antibacterial activities of the secondary metabolites.
- To characterize the biologically active secondary metabolites.
- To understand the biosynthetic pathways of biologically active secondary metabolites by feeding acetate.

## 1.4 Study benefits

The plants have been used as medicine for long time but the recent trend of using fungi for that specific purpose will clearly classify the fungi on the basis of their medicinal importance [27].

- This will offer a scientific foundation of exploiting fungi for bioactive compounds in Pakistan.
- The discovery of new bioactive compounds may result in the development of new pharmaceutical agents.
- The new pharmaceutical agents will reduce the imports of raw material for the pharmaceutical industries.
- This can build up a connection among the research organization and pharmaceutical industries and will result in the accessibility of standard drugs at reasonable prices.
- This will provide an excellent opportunity for the opening of new jobs in the country Pakistan [28].

Almost all the medicines have been initiated from the natural resources (plants/micro–organisms), which are beneficial in one way or other. The exploration of fungi for new biologically active compound(s) could be a new source for the

pharmaceutical industries. While the estimation or identification of their biosynthetic pathways will help the Organic Synthetic Scientists (OSS) to synthesize these compound(s) and/or their derivatives in laboratories by various chemical reactions. Therefore, this project is a land mark for the use of "may be useless microbes for the production of high class antibiotics". The production of antibiotics will improve the socio–economic situation of our country Pakistan [27].

# CHAPTER 2

# LITERATURE REVIEW

# 2 LITERATURE REVIEW

## 2.1 Fungi

Fungi survives in a wide–ranging habitats, including water, land/soil, in air, and also as in/on animals and plants, including both terrestrial and marine environments [29-30]. However, majority of them are terrestrial, living in/on soil or surviving on dead bodies of the multi–cellular organisms including both plants and animals. They are also contributing to the natural recycling of the dead bodies in to organic compounds. Besides this many of the terrestrial fungi are pathogenic to animals and plants and causes potentially hard to cure diseases to them [29-31].

The chemistry of fungi is a little more complex because they are structurally different from animals and plants however; they have some of the features similar animals and some to plants. For example they decompose their foods as an extra–cellular and then absorb the nutrients like animals, whereas, they follow the same biosynthetic pathways for the synthesis of terpenes and polyketides secondary metabolites using similar starting units as the plants do [29, 32].

By knowing the feature that fungi possess the same biosynthetic pathways like plants; they became more important for bio-organic scientists. Because they have been widely used as an experimental model for the reason that they can be grown easily in the laboratory conditions i.e., yeast [33-34].

## 2.2 Fungi as a source of natural products

Natural products are organic compounds which are produced as secondary metabolites by living organisms and almost all the secondary metabolites possess biological importance. The secondary metabolites have shown different varieties of the structurally and functionally diverse group of natural products. The diversity has

led them biologically active against various chronic diseases rendering them important and valuable for the human being [35].

Natural products are the main source for the development of drugs. According to one of the survey conducted in 2003; about 61% (535 out of 877) of the drugs have been developed from the natural isolated chemical compounds in the entire world in twenty two years (from 1981 to 2002). Seventy eight percent (78%) of antibacterial and seventy four percent (74%) of anticancer compounds are from natural products [36]. Thus natural products offers a remarkable platforms for the development of front–line medicines [37].

Significant improvement and variation in the discovery of microbial natural products is confined to the management of nutrient and environmental factors which encourages the biosynthesis of secondary metabolites. While the small changes in nutrient and/or environment have the ability to affect both the quantity and diversity of the secondary metabolites as fermentation products [38].

The natural products isolated from any source have been classified as Alkaloids, Isoprenoids, Non–ribosomal peptides and Polyketides. All the groups are different from one another in their structures, functions and even in biosynthetic pathways.

### 2.2.1 Alkaloids

Alkaloids are the natural organic compounds mainly of plants origin having at least one basic nitrogen heterocyclic ring which possess remarkable physiological activities in human. Alkaloids are divers in their function, because some stimulates central nerves system (CNS) in human, some as pain relieving, while some are toxic and others cause paralysis. Most of the alkaloids are colourless crystalline solids and

a few are liquids. They are lipids soluble except that of the liquid ones are aqueous soluble. Coniine (**1**) [39], Graminutee (**2**) [40], Papaverine (**3**) [41] and Quinine (**4**) [42] are some of the common alkaloids figure 2.1.

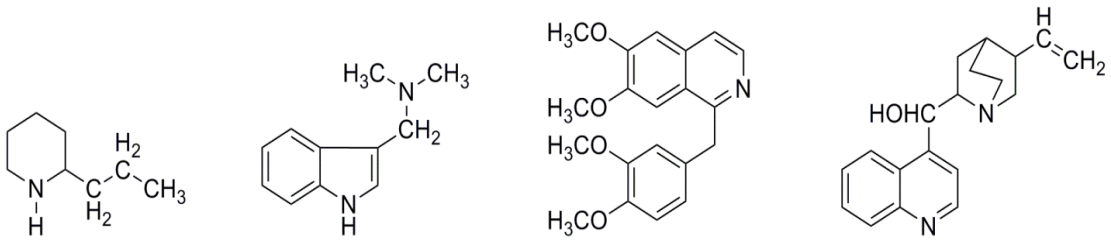



**Figure 2. 1 Structures of some common Alkaloids [39-42].**

### 2.2.2 Isoprenoids

Isoprenoids (also called as terpenes or terpenoids) are natural products that give proper odour or flavour to the secondary metabolites of microorganisms (bacteria, fungi and plants). Isoprenoids are the oils from plants that consist of a mixture of hydrocarbons (polyene) and their oxygenated derivatives. Isoprenoids is one of the largest group of natural products with approximately 25,000 known compounds [43-44].

Otto Wallach received Noble Prize in 1910 by working with isoprenoids and therefore established a rule, which is so called isoprene–rule. According to the rule, all the molecules of isoprenoids are synthesized from two or more than two isoprene (**6**) [45] units joining into head to tail fashion. Some of the common and well known isoprenoids from plants are Camphor (**5**) [46]**,** Isoprene (**6**) [45]**,** Limonene (**7**) [47]**,** Myrcene (**8**) [48] and Vitaminute–A (**9**) [49], whereas, microorganisms may also

synthesized some of the important isoprenoids such as aristolochene (**10**) [50] and gibberellin–$GA_4$ (**11**) [51] figure 2.2.

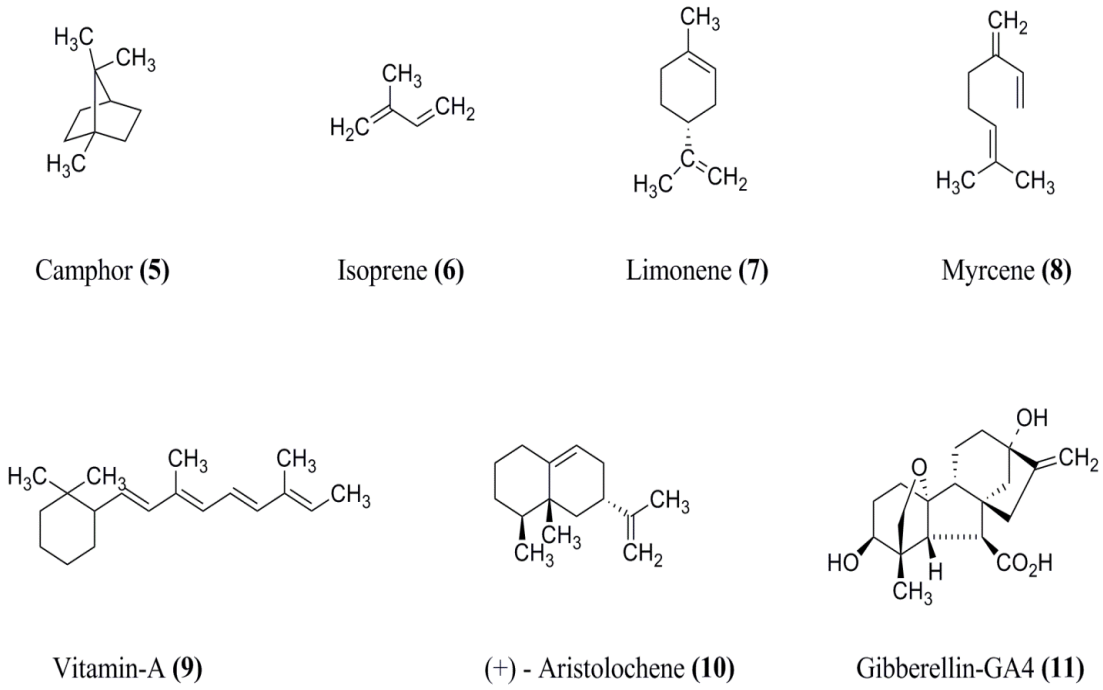
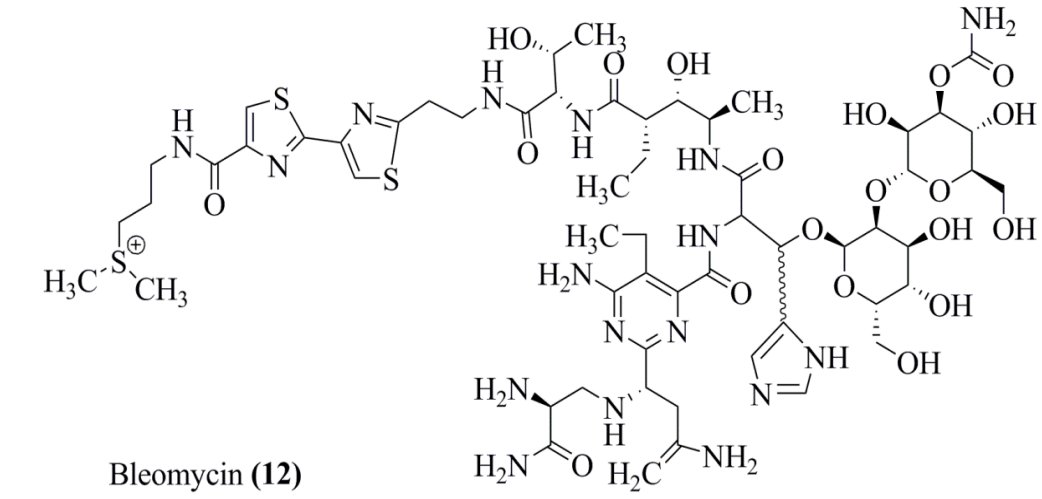


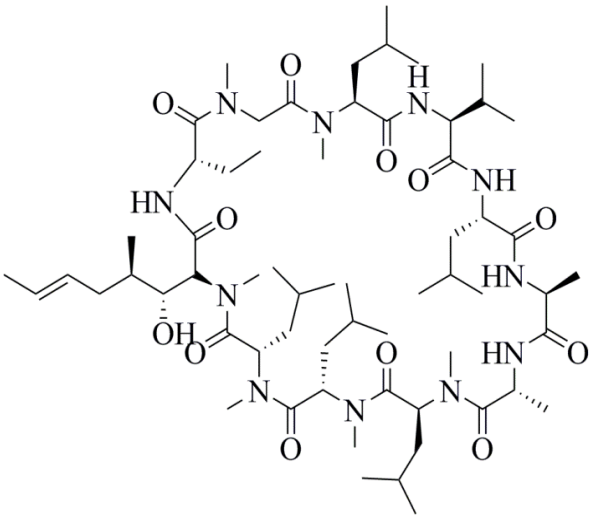

**Figure 2.2 Structures of some common isoprenoids [45-51].**

### 2.2.3 Non–ribosomal peptides

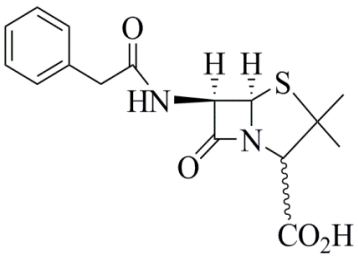
Non–ribosomal peptides are the natural products; which are important class of secondary metabolites, mainly produced by micro–organisms including actinomycetes, bacteria and fungi. Non–ribosomal peptides are produced by multi–domain, multi–modular enzyme called as Non–ribosomal peptides synthetases (NRPSs) [52]. These secondary metabolites are bio–synthesized in the cytoplasm outside the ribosome by cystolic protein [53-54]. Some common NRPS are Bleomycin (**12**) [55], Cyclosporin–A (**13**) [56] and Penicillin–G (**14**) [6] figure 2.3.



Bleomycin **(12)**



Cyclosporin A **(13)**

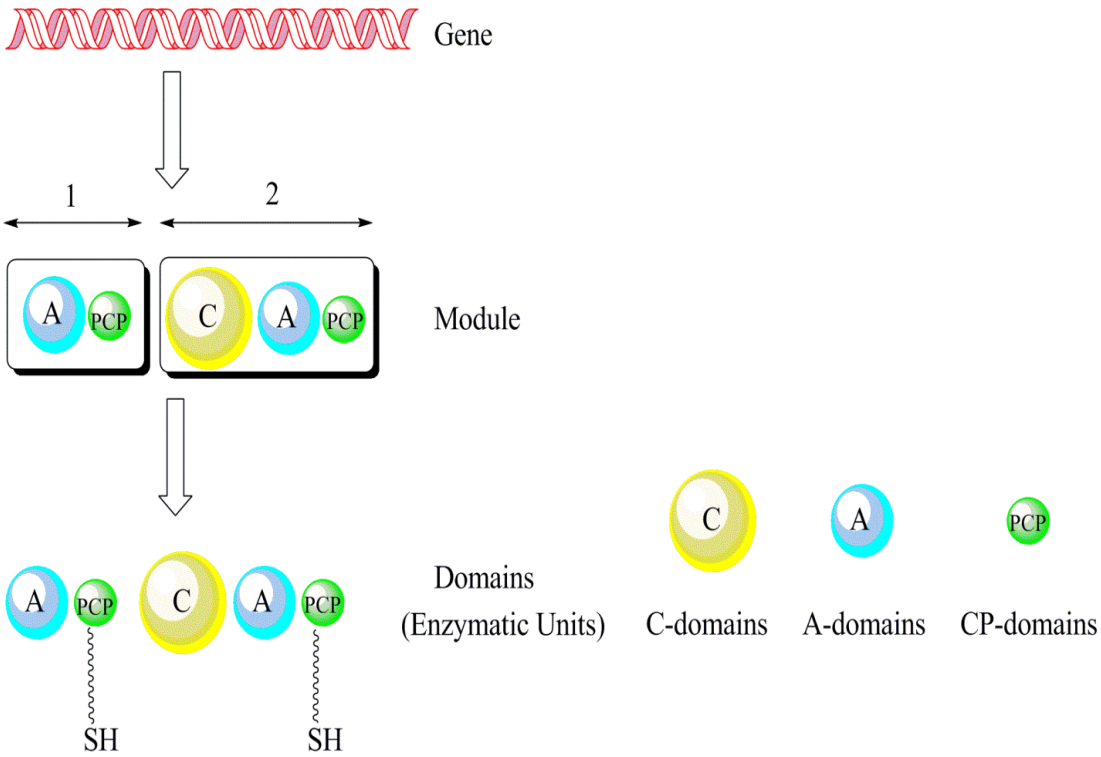


Penicillin G **(14)**

**Figure 2. 3 Structures of some common Non–ribosomal peptides [6, 55-56].**

Non–ribosomal peptides are large multi–domain protein consisting of several modules (a group of domain or segments of the NRPS's polypeptide chain) which has the potential to join the building blocks (amino acid) together forming a long peptide chain. The mechanism, involved in the bio–synthesis of polypeptide chain is the selection of amino acid, then activation and finally the condensation of amino acids

together. As the module is a group of domains, therefore, the chemistry of domain is very much essential for the function of the module. The major domains that tie up the amino acids together are the adenylation domain (A–domain); that serves as catalyst for the activation of substrate. The peptide carrier protein domain (PCP–domain) or otherwise called thiolation domain (T–domain), this is an important domain because it is always activated by 4–phosphopantetheinyl–transferases (4–PPTases) to start its function. This domain is a bonding domain which links the substrate through covalent bond. Finally the condensation domain (C–domain) is held responsible for the peptide bond [52].




**Scheme 2. 1 Gene is differentiated into modules which can be subdivided further into domains. Domains are the enzymatic units that tie up the amino acids together to form polypeptide chain [52].**

The NRPSs are simultaneously used as template and biosynthetic equipment, because the module will select the amino acids and also tie up all the catalytic functions. This is often a quality of the whole mechanistic that fungi synthesized a complete metabolite from a single NRPS.

#### 2.2.3.1 Adenylation–domain

The adenylation domain (A–domain) consists of approximately 550 amino acids; the main function of this domain is to select the amino acid for making the protein and also controls the primary sequence of protein. A–domains are responsible for activation of amino or carboxylic acid substrate as amino acyl adenylate (AAA), whereas, during the activation process adenosine tri phosphate (ATP) is consumed [57-58].

So far, two crystalline structures of A–domains have been properly characterized after their purification. The crystal structure of the phenylalanine–activating the A–domain of the gramicidin S–synthetase A, (GrsA) were isolated from *Bacillus brevis* that explains the role of those amino acids residues which are involved in the co–ordination of the substrate [52, 59].

#### 2.2.3.2 Condensation–domain

The condensation domain (C–domain) consists of approximately 450 amino acids; the main function of this domain is to connect amino acyl substrates with peptide carrier proteins of the adjoining modules through peptide bond. The C–domains are the essential unit of non–ribosomal peptide synthesis, [52, 60].

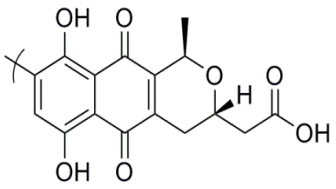
#### 2.2.3.3 Peptide carrier protein

The peptide carrier protein (PCP) domain is relatively small domain that consists of approximately in the range 80–100 amino acids, which is a small domain as compare to the other domains. This domain is selective and stands for the transfer of a specific unit which may have the ability to accept the activated amino acid otherwise it will not function. The PCP domain is covalently bounded to its 4PP–cofactor as thioester. The 4PP–cofactor is then transferred to a conserved serine residue of the carrier protein (CP). Which, acts like a flexible arm and hence permit the travel of bounded amino acyl and peptidyl substrate between different catalytic centres [52, 61].

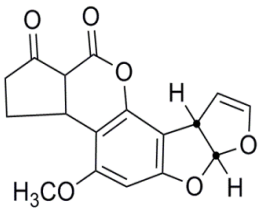
### 2.2.4 Polyketides

Polyketides are natural products that have been considered the most valuable classes of secondary metabolites. They are produced by bacteria, fungi, marine organisms, as well as plants [30]. Polyketides are structurally and functionally diverse class of natural products that exhibit a variety of biological activities. Among the polyketides, aromatic polyketides have more biological potential against the microorganisms and cancer cells, for example aspergiolide–A, a novel anticancer compound, produced as a secondary metabolite by fungus [62].

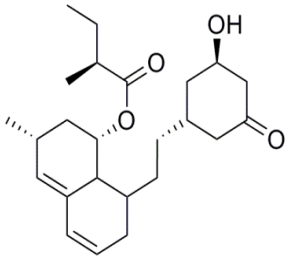
Some of the important biological active polyketides are actinorhodin (**15**), aflatoxin B1 (**16**), lovastatin (**17**) and 6–methylsalicylic acid (**18**) [63-64] figure 2.4. However, structurally and functionally different polyketides even have the same pattern of their assembly–by the decarboxylative claisen condensations (DCC) between an acyl thioester (**19**) and malanoyal thiolester (**20**) [30] scheme 2.2.



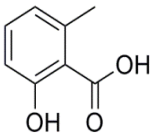
Actinorhodin **(15)**
Antibiotic



Aflatoxin B1 **(16)**
Carcinogen



Lovastatin **(17)**
Anti-cholesterol

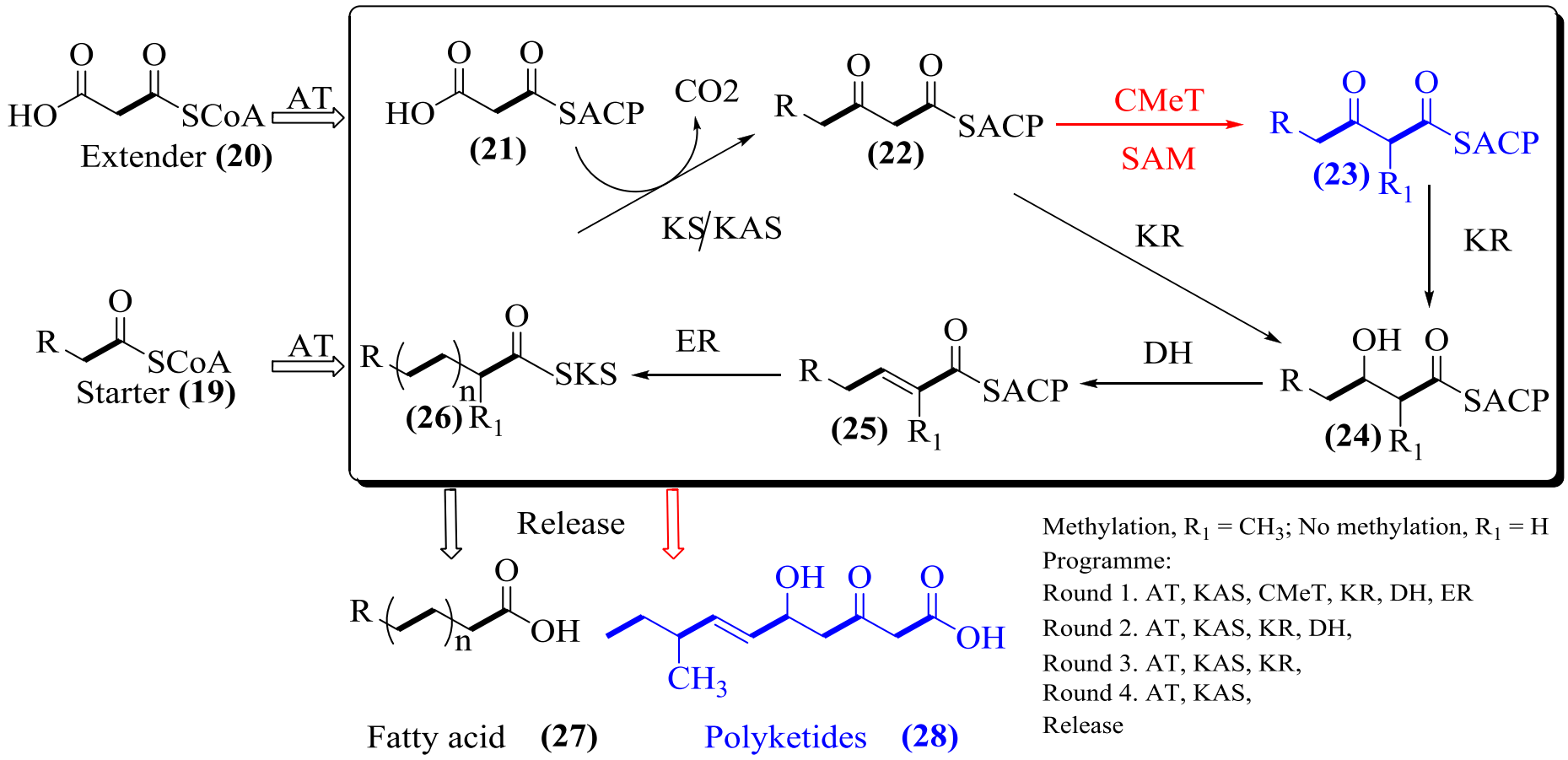


6-Methylsalicylic acid **(18)**
Antibiotic

**Figure 2. 4 Some examples of the polyketides [64].**

Enzymes are involved to catalyze the condensations between the starter and extender units. They are called as polyketide synthases (PKSs) based on the enzymes involved in the biosynthesis of fatty acids. Because of the mechanism of the polyketide biosynthesis are similar to the fatty acid biosynthesis. Therefore polyketide synthases (PKSs) have been classified and characterized using the pattern of nomenclature for fatty acid synthases with little modifications [5, 64-65].

The major catalytic domains like acyltransferase (AT), β–ketoacylsynthase or β–ketosynthase (KAS or KS) and acyl carrier protein (ACP) are found in all Fatty acid synthase (FAS) and in polyketides synthase (PKS) [30, 66-67]. They also have β–ketoreductase (KR), dehydratase (DH) and enoylreductase (ER) domains, whereas, the (PKS) has an additional important domain i.e. C–methyltransferase (CMeT) which is responsible for the methylation of polyketides [63, 66]. A very simple and straight forward elucidation and explanations of both the biosynthetic pathways have been shown in scheme 2.3.

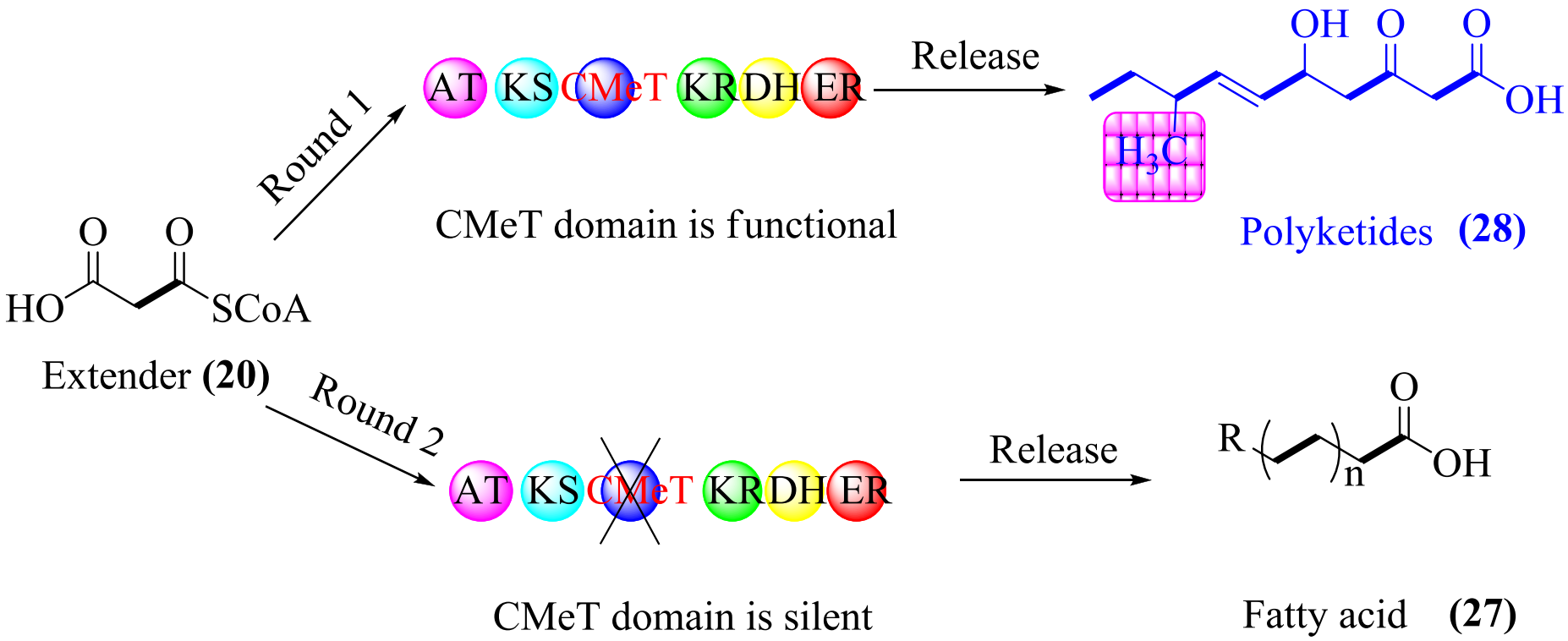



**Scheme 2.2 Different enzymatic catalyzed steps in the biosynthesis of Fatty acid and Polyketides, bold bonds indicates the pattern of incorporation of the labeled acetate units [30].**

In **round 1**, the CMeT is functional therefore, it results in the formation of Polyketides (**28**) while its silencing in **round 2** results in the Fatty acid production (**27**) even by using the same raw material malonate (**20**).

Acyl carrier protein (ACP) is used by the Fatty acid synthase (FAS) that carries the malanoyal thiolester or malonate (**20**) unit and rapidly attached with the acyl chain, this feature has a very clear homology to Polyketide synthases (PKS).

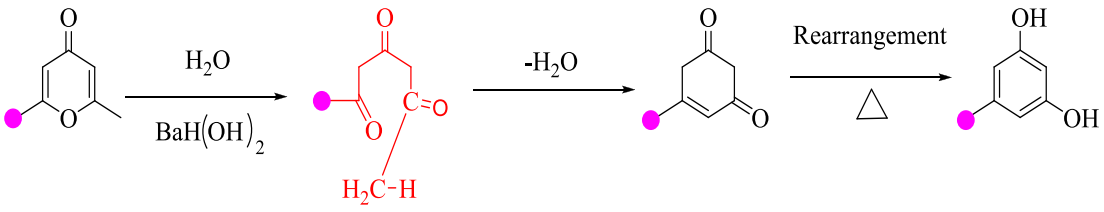
Most of FAS and PKS proteins also require an acyltransferase (AT) enzyme to transfer acyl groups from CoA onto the KS and ACP components. During the biosynthesis of fatty acid, the newly formed β–ketothiolester (**21**) is further proceeded for chemical reactions, while it is attached to the terminal thiol of the ACP: first of all it is reduced to secondary alcohols by a β–ketoacylreductase (KR) (**24**); this then undergoes dehydration reaction catalyzed by dehydratase (DH) for the formation of an unsaturated thiolester (**25**); and finally the enoyl reduction (ER) results a fully saturated thiolester (**26**). Fungal PKS has the ability to deploy all these chemical reactions; furthermore whenever the chain is methylated, then the methyl group is provided from S–adenosylmethionine (SAM). This probably occurs after KS/KAS, giving a methyl β–ketothiolester (**23**).




**Scheme 2.3** **All the domain in the module are the same, while only the CMeT domain is silent in round 2, results in the production of fatty acid. It shows that CMeT is the only domain which can differentiate the polyketide pathways from fatty acid pathways.**

### 2.2.4.1 History of polyketides biosynthesis

In 1893 James Collie at London University, obtained a product Orcinol (**32**), ever first polyketides, by the chemical reaction of de–hydro–acetic acid with barium hydroxide. This simple aromatic compound became challenge for the scientists because the mechanism was based on the key polyketone intermediate (**30**) [62] figure 2.5. By knowing the chemistry and resolving the mystery of polyketone intermediate (**30**) after the pioneer work of collie, which hypothesized that acetate is the precursor of almost all polyketides. Therefore, the field of polyketides were developed. Latter on the bacteria and fungi were engineered by cloning their enzymes to accomplish the task of the hidden pathways of polyketides [67-68].




Dehydroacetic acid **(29)** Octane-2,4,6-trione **(30)** 5-methylcyclohexa-4-ene-1,3-dione **(31)** Orcinol **(32)**

Note ● = **Methyl group**

**Figure 2. 5 Collie's un–predictive synthesis of Orcinol (32) from dehydro–acetic acid(29)[68].**

### 2.2.4.2 Radioactive isotopic ($^{14}C$) in polyketides biosynthesis

The major interest in the field of polyketides came from impetus of Arthur Birch's in 1950. He spent much of his time in Robinson's laboratory at Oxford, which was a famous laboratory for the research in bio–organic chemistry. Birch's contribution to the field of polyketides was important for two reasons. First, he

suggested that polyketones (polyketides) could be produced by the repeated condensation reactions of acetate units (staring units), and second, he tested his suggestions by feeding the isotopically labelled acetate units to an organism for the production of suitable polyketides [68]. Birch selected 6–methylsalicylicacid (6–MSA), an aromatic polyketide (**37**) for the confirmation of his idea, which is involved in the biosynthetic pathways of the toxin patulin (**38**) and also has some biological importance [69] figure 2.6.

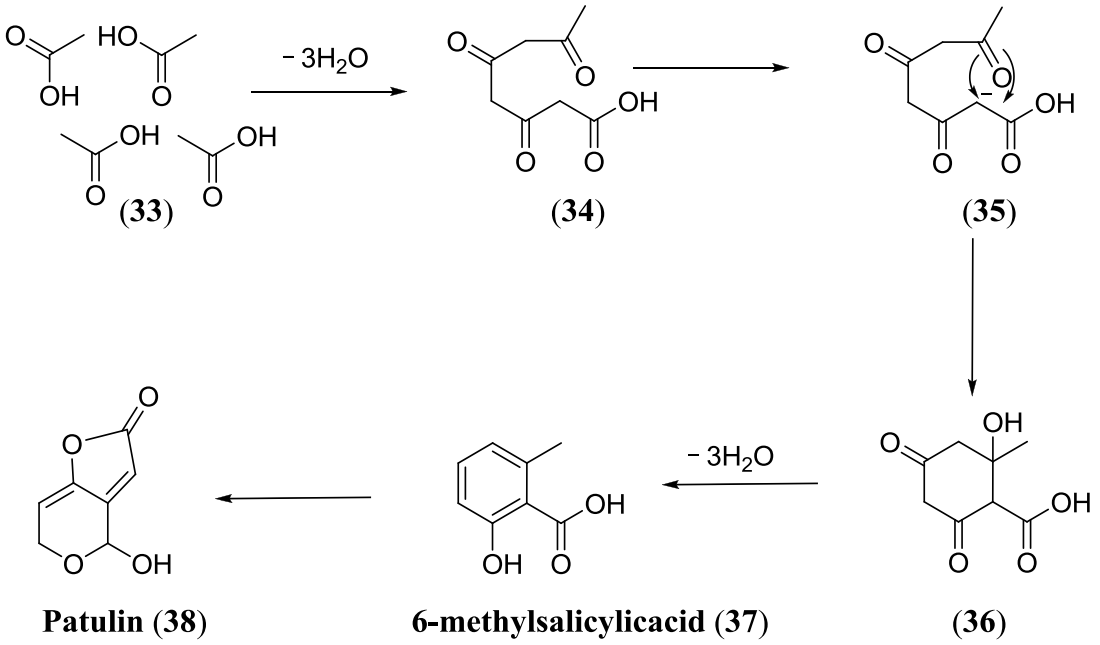



**Figure 2.6 Sequence of reactions in the biosynthesis of 6–methylsalicylic acid and patulin [68].**

Four acetate units (**33**) are linking or bounding to each other by 'head–to–tail' manner and produced a triketo acid (**34**), and then one of the keto groups in (**34**) is reduced to hydroxy group. Then different mechanistic reactions, including the formation of carbanion at the β–keto residue in (**35**) would then allow an aldol condensation to form a six–membered carbocyle (**36**). Finally, reasonable reactions

like dehydration and enolisation reactions will results the formation of aromatic natural products (**37** and **38**) [62].

Birch, further tested the ideas by feeding acetate labelled with [1–$^{14}$C] to *Penicillium patulum* the producer of (6–MSA) [68] in figure 2.7, because that was the only available technique. However, for the confirmation of the idea, it was necessary to understand the pattern of labelling in the compound. The incorporated sites in the compound (6MSA) were predicted by the degradation methods. Then the fragments were correlated with specific sites in the natural product. The three products were isolated by the degradation and then were exposed to radioactivity measurement for the determination of their 'relative molar activity.' These results were the same as predicted by Birchs' [68].

Although radiolabels are useful tracers in the incorporation studies, however, they are time consuming, tedious and difficult to handle. Therefore radiolabels have no longer been used in microbial systems for labelling studies.

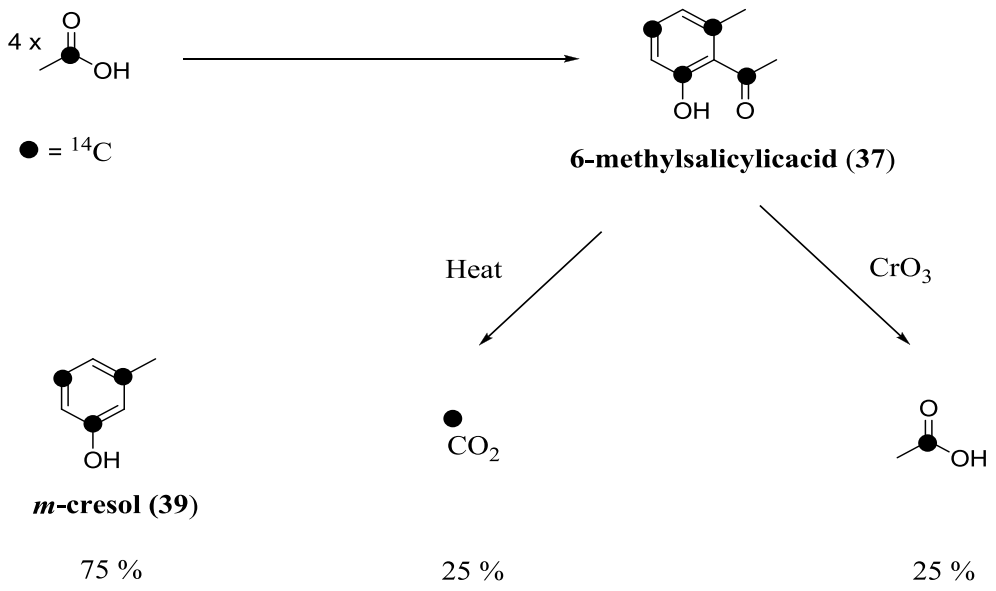



**Figure 2.7 Birch's verifications that 6–methysalicylic acid is assembled from four acetate units [68].**

### 2.2.4.3 Stable isotopic ($^{13}$C) in polyketides biosynthesis

The emergence of the nuclear magnetic resonance (NMR) spectrophotometer resulted in the growth and development in the field of feeding isotopically labelled precursors. Until1960, the natural product chemists depended on the degradation phenomenon to produce recognisable fragments for the structure elucidations. They brought all the fragments together on paper to create an idea of the structure of compounds. That was really a very tiring job but yet the scientists developed some standard and well–tried methods of degradation and structural determination [70-71]. However, the NMR solved all these challenges at once or almost overnight. Because the stable isotopic labels ($^{13}$C) were open to direct detection by NMR spectroscopy. It was significant to use the isotope $^{13}$C, because it has a suitable nuclear spin for NMR observation like $^{1}$H [63]. Therefore, the chemists turned to stable isotope ($^{13}$C) instead of the radioactive isotope ($^{14}$C).

Isotopically labelled precursors usually [1–$^{13}$C] acetate is administered in the standard way after the time course production for the desire metabolites and then the metabolites will be re–isolated for the estimation of incorporations [72]. It is then a tricky job to establish the sites of isotopic enrichment by the measurement of $^{13}$C from NMR spectrum. In successful experiment, the incorporated isotope will give even an increase of (1%) over the natural abundance, however for the reliable results it is better to look for higher increase in the signal size. Luckily, maximum of the polyketides are produced by the micro–organisms especially fungi, therefore, they often takes up labelled substrates [63].

In ideal situation, each pair of coupled $^{13}$C nuclei will have a unique coupling constant which helps in the confirmation of $^{13}$C NMR spectrum. Labelled $^{13}$C nuclei that have replaced their original carbon, the signal appears as an enriched singlet. It is

essential for interpreting this easy pattern of signals to dilute the labelled precursor by unlabelled acetate otherwise, more than one doubly–labelled isotopic precursor (acetate) will incorporate into a single molecule and this will result a complicated spectrum by inter–unit $^{13}C$–$^{13}C$ couplings.

Additional estimation of the mechanisms of biosynthetic pathways can be obtained by studying the probability of hydrogen atoms in intermediates molecules. Different experiments have been developed to study the effect of $^{2}H$ or $^{3}H$, either by the direct observation of $^{2}H$ or $^{3}H$ by NMR spectroscopy or indirect detection of $^{2}H$ or $^{3}H$ isotope attached on the adjacent neighbour $^{13}C$ label in the precursor. The use of such technologies, then α shift is sufficient to demonstrate extra information regarding the path of hydrogen atoms involved in the biosynthetic pathways.

In this technology, the hydrogen atoms are replaced by deuterium or tritium and the carbons are replaced with $^{13}C$ to which they are attached. Then the chemistry is very simple because one deuterium in the molecules results the shifting of $^{13}C$ signal to upfield by about 0.3 ppm and it appears as a 1:1:1 triplet with ($J$ ~ 20 Hz) due to the coupling $^{2}H$–$^{13}C$. Now for each additional deuterium with $^{13}C$, there will be further upfield shift of the carbon and a corresponding increase in the multiplicity of the signal have shown in figure 2. [73].

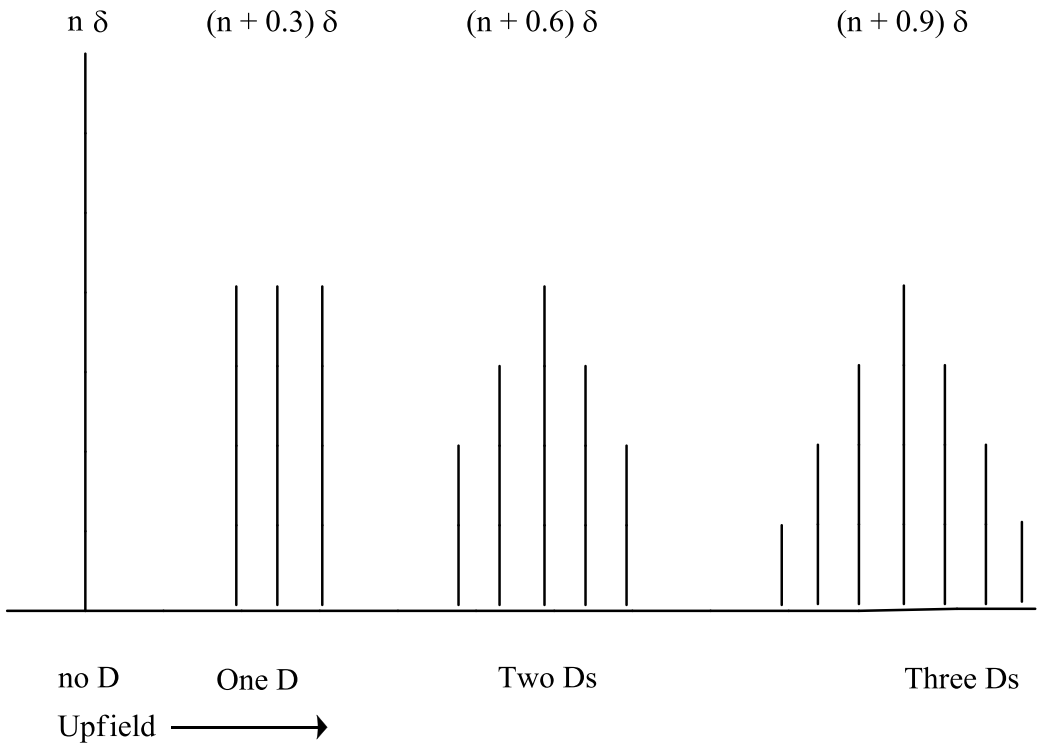



**Figure 2. 8 Imaginary $^{13}C$ NMR spectrum demonstrating the α–effect. While "D" stands for deuterium. Each additional deuterium shifts the signal of carbon to upfield by 0.3 ppm along with the increases its multiplicity.**

With a high field NMR spectrometer, therefore, it is obvious to determine the extent of deuterium labelling in considerable detail. It will be sufficient to say that, with the developments of various elegant scientific methods, the bio–organic chemists published a lot of data, upon which they have built a detailed story of the types of biosynthetic processes employed in polyketides pathways.

## 2.3 Fungi and Biotechnology

Fungi have been used as folk medicines, i.e. a very common fungi *Agaricus campestris* Linn (field mashroom), when used about 3 to 6 gm for 2 to 3 times a day will serves as tonic and is used against anti–inflammation, sinusitis and tuberculosis

[71]. Beside this *Laricifomes officinalis* were used against diarrhoea, night sweating, *Inonotus obliquus* were used for chronic gastritis, early tumours and ulcer, *Daedaleopsis flavida* can cure jaundice dramatically by reducing the level of bilirubin and biliviridin, whereas, *Ficus religiosa* Linn is used for all kind of kidney disorders [72].

It is clear that fungi are the significant source for new and/or biologically active secondary metabolites. During the period of 1981 to 2002, 40% of the total drugs and 14% of the antifungal drugs launched were of natural products or biologically modified natural products [36]. However in agriculture, it is estimated that only about 10% of the natural products are available in the market for the protection of crops [74]. However, for the development of new drug, it is necessary to test their adverse action to humans, but for some critical illness some extent of toxicity could be accepted i.e. in case high fungal infections the use of standard antifungal drugs like amphotericin–B, may cause severe kidney infections [75].

Although; the investigation of new microorganism from the un–investigated areas will result many new biologically active secondary metabolites, while only a small fraction (0.1–1%) of all microorganisms have been exploited in laboratory conditions [76]. Because there is a huge genetic diversity that is a challenging job to understand, but simply we can approach our target towards metabolites by the modifications in growth conditions [77] or by genetic engineering to make a transformant organisms by inserting the biosynthesis genes of uncultivable microorganisms [78-79].

Genetic approaches could be helpful techniques for estimating the biosynthetic potential of microorganisms. That can be evaluated for known biosynthesis genes and then possible suggestions will be made that, which strain can produce what kind of

compounds. This approach has been remained successfully for the gene (s) involved in the synthesis of polyketides [80-81].

Nevertheless, it is not necessary that in–depth search for micro–organisms will results novel bioactive components but some time a very easily accessible micro–organisms may produce broad spectrum of diverse metabolites depending on the culturing conditions and also some additives, or chemical modifiers. Single strain may results an increase in metabolites and even a new compounds, this approach is called one strain many compounds (OSMAC) [82-83].

### 2.3.1 Fungal metabolites available commercially

From the very time, natural products have been used by man and the plants were the most important source of medicine used as composts/ mixture [84]. Due to their abundant variety and structural diversity, natural products are of great significance in biotechnology and pharmacology. They can also be used as a model for synthesis by knowing their characteristics [85-86]. The best known examples of natural metabolites available commercially are antibiotics, especially from the date of discovery of penicillin by Alexander Fleming in 1928 and its production in the market by Chainand Florey in 1940 [87].

Natural resources, in particular fungi, are known a best factory for their metabolic capacity to produce a broad diversity of bioactive metabolites. These can be extremely toxic, e.g., mycotoxins, or be rather useful because they can be used as drugs for various diseases [88]. Fungi produce a vast range of secondary metabolites. Some of the metabolites are high–value products with pharmaceutical applications such as penicillins, a group of structurally related β–lactam antibiotics isolated from *Penicillium chrysogenum*. Several non–β–lactam antibiotics are also produced by

fungi such as griseofulvin. Griseofulvin which is isolated from *Penicillium griseofulvum* has been used for several years to treat dermatophyte infections of the skin, nails and hair of humans. Some common secondary metabolites of fungal origin are listed in table 2.1.

**Table 2.1 Some common secondary metabolites produced commercially from fungi [89].**

| Metabolites | Fungal source | Application |
|---|---|---|
| Cephalosporins | *Acremonium chrysogenum* | Antibacterial |
| Ciclosporins | *Tolypocladium* spp | Immunosuppressants |
| Fusidin | *Fusidiumcoccineum* | Antibacterial |
| Gibberellins | *Gibberellafujikuroi* | Plant hormone |
| Griseofulvin | *Penicillium griseofulvum* | Antifungal |
| Penicillins | *Penicillium chrysogenum* | Antibacterial |
| Zearalenone | *Gibberellazeae* | Cattle growth promoter |

In recent years, marine fungi have been explored more deeply to obtain novel and biologically active compounds, because they are still less explored. Nevertheless, successful stories in marine fungi are quite significant. Cephalosporin–C which was originally isolated the first time from *Cephalosporium acremonium* isolated from a sewage outlet off the Sardinian coast have played a key role to the reduction of infectious diseases and suffering of people throughout the world since last three

decades [90]. However, it was about incidental discovery and it took another 30 years until marine–derived fungi were investigated more systematically [91].

## 2.4 Genetic Engineering in Fungi

Genetic engineering is defined as any change in the natural genetic code of an organism for a specific function. It may be a single base pair change or a complete synthesis of a genome of an organism [92]. This work was started in early 1920 to 1940 by Hermann Joseph Muller and Charlotte Auerbach, it was only a modification of gene by radiations and chemicals [93-94]. However, it was developed by Paul Bergin 1972 by the achievement of first recombinant DNA [95]. Latter on genetic engineering reached to its high level of success by achieving genetically engineered human insulin through cloning and expression of the gene in *E. coli* by Genetic engineering technology (GET) in 1978 [96], this was an ever first successful targeted achievement.

Transformation is a core method for attaining the genetic modification in fungi [97-98]. One of the most important and useful method for transporting the genetic mattering into fungi is the protoplast mediated transformation (PMT) [98-99]. In this method the cell wall is removed with the help of enzyme from young mycelia while leaving the protoplast covered by cell membrane. Whereas the use of calcium ion ($Ca^{+2}$) enhance the penetration of DNA into the cell membrane.

However some time some of the fungal strains do not develop their cell wall around their cell membrane [97]. Therefore, other methods like *Agrobacterium* mediated transformation (AMT), biolistic, electroporation and lithium acetate mediated transformation methods were also developed in last decades for the solution of the problems happened by PMT [97-98], AMT depends on using a carrier

organism, *Agrobacterium tumefasciens* to transport the genetic material into the host [100].

Naturally, it is the bacterium that infects the whole fungal or plant's cell and then starts the genetic alteration by means of integration a part of certain plasmid, so called Ti, into their genome. By knowing this phenomenon, the gene of interest is usually incorporated on the Ti plasmid after some modification in it. Finally, the recombined plasmid is transferred into bacterium and the result of this transformation is generally a single integration on the fungal genome [100-101].

There are two types of methods or mechanisms for the integration to transfer the genetic materials onto the receiver fungal genome [102-103]. The first method is homologous recombination (HRc) [103]. This leads to the integration of the familiarized DNA sequence on to a homologous target on genomic locus, which is catalysed by the Rad 52 epistasis proteins group. The second method is called non–homologous end joining (NHEJ) [103]. This results to the ligation of the transformed DNA sequence to the recipient genome without homology. This leads to ectopic integration of several copies on variable genomic sites. Approaches for the genetic engineering of fungi and other micro–organisms were developed during last decades, these includes: gene knock–out, gene silencing and gene over expression to confirm their link with metabolites.

### 2.4.1 Gene knock out approach

Gene knockout approach is a technique of genetic engineering in which one desired gene of an organism is made inoperative, *Agrobacterium* mediated transformation (AMT) was successfully used to disorder the genes hypothesised to be linked to radicicol biosynthesis from *Chaetomium chiversii*. It was also noted that the

use of AMT has achieved better homologous recombination in *A. awamori* [102]. It is not necessary that all the fungi will obey the procedure of AMT, because, some fungal species are disobedient to AMT like *Sclerotinia sclerotiorum* and *Aspergillus niger* [102]. In addition, it was found that some genetic loci and some fungal species, are resistant to homologous recombination [104]. This could be greatly enhanced through knocking out genes expressing system. On the other hand PMT usually results in numerous ectopic integrations which make it beneficial for both heterologous and homologous overexpression [105]. Hence, from the mentioned study, one can conclude that there is no single method for transformation which could deal with all the genetic modification approaches.

### 2.4.2 Gene silencing approach

Recently the gene silencing techniques have been introduced and the scientists are increasingly using for the confirmation gene involved in the production of metabolites. They depend on down–regulation of gene expression. These techniques do not influence gene transcriptional process. However it shows its effect by decreasing the level of expressed RNA [106]. As a result the corresponding protein level turns down and silencing of gene function succeeded. However in some cases complete blockage of expression has been achieved [107]. Gene silencing techniques are proven to be more proficient than gene knock out [108], because of the fact that these techniques didn't need homologous recombination which could be inappropriate to some of the fungi [109].

Among gene silencing techniques two types of methods are the most famous. The first is the antisense RNA technique (ART) which depends on the incorporation and expression of a DNA sequence in the antisense direction to the target gene.

Therefore, both the native mRNA and the antisense RNA overlaps with each other leading toward the translation blockage [110].

The second method used is RNA interference (RNAi or si RNA) [108]. Its function is the expression of short homologous double stranded RNA that starts up the mechanism for degrading the native RNA. It is a set of proteins called dicer proteins, RNA–dependent RNA polymerase (RdRP), and the RNA induced silencing complex (RISC) mediate RNAi responsible for the gene silencing but these are absent in some fungi [111].

### 2.4.3 Over expression of gene

The gene(s) over expression is a term that mainly depends on upgrading the level of gene(s) expression. When it is done in its original organism, then it is called homologous over expression, while when it is done in the other organism, then it is called heterologous over expression. The basic theme of gene(s) over expression is achieved by the discoveries of novel and biologically active secondary metabolites from fungi, and these discoveries are the achievements of the applied biotechnology [26, 112].

#### 2.4.3.1 Homologous over expression

In the process of homologous over expression, the gene is cloned and over expressed in the native organisms under the control of native or non–native promoter and terminator; the promoter may either be constitutive or inducible. This process is different from the process of homologous recombination that is generally occurs during gene knock out experiments [113]. While in gene knock out experiments, it is proposed that the gene or parts of the gene are cloned to recombine with the targeted

gene in the chromosome to disrupt its expression. However, homologous over expression is mainly accomplished by ectopic integration on different location(s) within the chromosome from its native copy [114-115].

#### 2.4.3.2 Heterologous over expression

In the process of heterologous expression, the gene is cloned and over expressed in the non–native organisms under the control of suitable promoter and terminator; the promoter may either be constitutive or inducible. This process is performed to provide the proposed or desired protein in suitable quantities which will be able for detection and application [116-117]. Heterologous expression can be performed in wide range; starting from simple bacteria like *E. coli* to complex eukaryotic organisms like animals and plants [118-119]. while the choice of the host depends on the extent of the knowledge about the capacity of the host to pick up express the foreign gene effectively and also the availability of suitable substrate molecules [119-121].

# CHAPTER 3

# MATERIALS AND METHODS

# 3 MATERIALS AND METHODS

## 3.1 Culture media used

Fungi is the experimental organisms in this project, therefore, thirteen different nutrient media were used for the growth of fungi. Whereas; de–ionized water was used for all types of media and/or solution preparations.

### 3.1.1 Bannerot synthetic broth (BSB)

For preparation of Bannerot Synthetic Broth 2.8 gm of Glucose, 1.3 gm of $MgSO_4.7H_2O$, 2.7 gm of $KH_2PO_4$, 2 gm Pancreatic Peptones (PP) and 10 mg of Yeast extract in 1000 mL (qsp) de–ionized water, then autoclaved at 121 °C temperature for 15 minutes on 15 psi pressure [122].

### 3.1.2 Czapek yeast extract agar (CYA)

Czapek Yeast Extract Agar (CYA) Nutrient media for the growth of Fungi, to prepare CYA medium we need to prepare three solutions in advanced i.e. solution **A, B,** and **C**. We need 30 gm of saccharose, 5 gm Yeast extract, 15–20 gm agar, 50 mL of solution (**A**), 50 mL of solution (**B**) and 1 mL of solution (**C**), in 1000 mL (qsp) de–ionized water, autoclaved at 121 °C temperature for 15 minutes on 15 psi pressure.

The composition of solution (**A**); dissolved 20 gm of $NaNO_3$, 5 gm of KCl, 5 gm of $MgSO_4.7H_2O$ and 0.1 gm $FeSO_4.7\ H_2O$, in 500 mL (qsp) de–ionized water.

The composition of solution (**B**); dissolved only 10 gm $K_2HPO_4$ in 500 mL (qsp) de–ionized water. However, the composition of solution (**C**); dissolved only 1 gm of $ZnSO_4.7H_2O$ and 0.5 gm of $CuSO_4.5H_2O$ in 100 mL (qsp) de–ionized water [123].

### 3.1.3 Czapek yeast extract broth (CYB)

Composition of CYB is the same as that of CYA, but without agar [122].

### 3.1.4 Czapek–dox agar (CDA or CZDA agar)

For the preparation of CDA media, 10 gm peptone, 30 gm sucrose, 3gm $NaNO_3$, 0.5 gm of $MgSO_4.7H_2O$, 0.5 gm of KCl, 0.01 gm, $FeSO_4.7H_2O$, 1 gm $K_2HPO_4$ and ~15 gm agar in 1000 mL of de–ionized water. The medium was then autoclaved at 121 $^oC$ temperature for 15 minutes on 15 psi pressure. The CDA media were used as a selective media for growth of *Aspergillus oryzae* (tenS.PKS/dmbC) because the wild type *A. oryzae* M–2–3 were not able to grow in CD agar [123].

### 3.1.5 Czapek–dox minimal media (CDM or CZDM)

For the preparation of CDM media, 10 gm peptone, (20 gm glucose, 30 gm sucrose, 30 gm maltose or 20 gm starch), 3 gm of $NaNO_3$, 0.5 gm of $MgSO_4.7H_2O$, 0.5 gm of KCl, 0.01 gm $FeSO_4.7H_2O$ and 1 gm $K_2HPO_4$ in 1000 mL of de–ionized water. The medium was then autoclaved at 121 $^oC$ temperature for 15 minutes on 15 psi pressure. When the media contain glucose and sucrose then it was used for growth of *A. oryzae* transformants, whereas; we used sucrose and starch in addition to the previous components were used for induction and metabolite production from those of *A. oryzae* transformants [123-124].

### 3.1.6 Dextrin polypeptone yeast extract agar medium (DPY)

For preparation of DPY media, 20 gm of dextrin, 10 gm of polypeptone, 5 gm of yeast extract, 5 gm of $KH_2PO_4$, 0.5 gm of $MgSO_4$, and 15–20 gm of agar were dissolved in 1000 mL of de–ionized water. The medium was then autoclaved at 121

$^{o}$C temperature for 15 minutes on 15 psi pressure. DPY were used for the development of spores of fungi [123].

### 3.1.7 E. medium

For preparation of E. Medium, 0.86 gm of $KH_2PO_4$, 1.515 gm of $KNO_3$, 1.18 gm of $Ca(NO_3)_2.4H_2O$, 0.492 gm of $MgSO_4.7H_2O$, 0.00286 gm of $H_3BO_3$, 0.00362 gm of $MnCl_2.4H_2O$, 0.00540 gm of $FeCl_3.6H_2O$, 0.00022 gm of $ZnSO_4.5H_2O$, 0.00022 gm $CuSO_4.5H_2O$ and 0.00012 gm of $Na_2MO_4.2H_2O$ in 1000 mL (qsp) de–ionized water, then autoclaved at 121 $^{o}$C temperature for 15 minutes on 15 psi pressure.

### 3.1.8 General nutrient broth medium (GNB)

For preparation of GN media, 20 gm glucose and 10 gm nutrient broth No. 2 in 1000 mL of de–ionized water. The medium was then autoclaved at 121 $^{o}$C temperature for 15 minutes on 15 psi pressure. It is an important media for the preparation of mycelia for transformation, spore productions and/or protoplasts formation [123].

### 3.1.9 Luria bertani medium (LB medium)

For preparation of Luria Bertani Broth, 10 gm Bacto–tryptone, 10 gm of sodium chloride (NaCl) and 5 gm yeast extract in 1000 mL of de–ionized water. After making 1 liter (qsp) solution the pH was adjusted to ~7.5 with 2M sodium hydroxide (NaOH) solution. It was then autoclaved at 121 $^{o}$C temperature for 15 minutes on 15 psi pressure.

### 3.1.10 Malt extract agar medium (MEA)

For preparation of MEA media, 50 gm of premixed components were taken according to the manufacturer's instructions in 1000 mL of de–ionized water. The medium was then autoclaved at 121 $^{o}$C temperature for 15 minutes on 15 psi pressure [124].

### 3.1.11 Nutrient agar medium (NAM)

Six (6 gm) of Bacteriological peptones, 2 gm of Yeast extract, 1 gm of Beef extract, 5 gm of NaCl and 15 gm Agar in 1000 mL (qsp) de–ionized water, then autoclaved at 121 $^{o}$C temperature for 15 minutes on 15 psi pressure. For Nutrient Broth (NB) there will be no agar in it [125].

### 3.1.12 Potato dextrose agar (PDA)

For preparation of Potato Dextrose Agar 4 gm of Potato starch, 20 gm of Dextrose and 14–15 gm of Agar in 1000 mL (qsp) de–ionized water, then autoclaved at 121 °C temperature for 15 minutes on 15 psi pressure. For Potato Dextrose Broth (PDB) there will be no agar in it.

### 3.1.13 Sabouraud dextrose agar (SDA)

Ten (10 gm) of mycological peptones, 40 gm of D–Glucose (Dextrose) and 14–15 gm of Agar in 1000 mL (qsp) de–ionized water, then autoclaved at 121 $^{o}$C temperature for 15 minutes on 15 psi pressure. For Sabouraud Dextrose Broth (SDB) there will be no agar in it.

## 3.2 Methodology Phase–I

This project was accomplished in the following three stages.

- A thorough literature survey was conducted to identify the fungal species for the production of biologically active secondary metabolites.
- Soil samples were collected, to isolate desired fungal species.
- Preliminary biological activities such as antibacterial and antifungal activity were conducted with the use of either currently recommended or newly developed methods.

### 3.2.1 Isolation of fungi

Fungi were isolated from the soil for the production of biologically important secondary metabolites. The soil samples were collected from the local hospitals of district Peshawar with care, in polyethylene bags in April 2010, and were shifted to Microbial Biotech; Laboratory, at the Centre of Biotechnology and Microbiology University of Peshawar, Pakistan.

The soil samples were dissolved in sterile water so as to avoid the microbial contamination in the soil samples. Because I needed the microbes (fungi) from the soil collected, therefore serial dilution method were applied for making ten (10) dilution of the each sample. These processes were carried out in the Laminar Flow Hood (LFH). Then potato dextrose agar (PDA) media were prepared and heated up to 60 $^{o}$C, for the homogenous mixing of all the ingredients, then transfer to Autoclave, along with petri plates (already wrapped in aluminium foil/paper) for 15 minutes at 121 $^{o}$C, this made the media and petri plates sterile.

After autoclaving the media was shifted to LFH, whereas the petri plates were placed in the oven at 184 $^{o}$C for complete dryness and then the petri plates were also

shifted to (LFH). Then the media were poured in the plates and allowed it to cool. Then with the help of sterile pasteur pipette three drops from each dilutions were poured on separate plates. Then the soil solution was spread with glass spreader and allowed for ten minutes. Then the plates were covered with para film and placed in incubator for seven days at 28 $^{o}$C. These experiments were repeated for five times and with different media till I got pure and isolated fungi [126-127].

#### 3.2.1.1 Experiment 1

These experiments were conducted to grow the fungal strains from soil samples for the production of biologically important secondary metabolites and were stored in the refrigerator at 4 $^{o}$C prior to the use in next experiments.

### 3.2.2 Antibacterial activity of metabolites in media of isolated fungi

The isolated and purified strains were grown in the petri plates for the preliminary antibacterial activity. Those strains were further preceded for the production and/or isolation of secondary metabolites that gave the antibacterial activity. In this method 40 mL of the nutrient broth (Sigma Aldrich, Germany) were taken in four test tubes. Test tubes were plugged with foam bung and transferred to autoclave along with nutrient agar, beaker, metallic cork borer, petri plates (wrapped in aluminium foil/paper) for 15 minutes at 121 $^{o}$C for making sterile.

After autoclave both the media (broth and agar) were shifted to (LFH), and petri plates were placed in oven at 184 $^{o}$C for complete dryness, then petri plates were also shifted to (LFH). Now 0.6 mL of each of the experimental organisms were transferred in to nutrient broth in different tubes at about 37 $^{o}$C and left for 10 to 20 minutes in the (LFH). The nutrient agar were poured in the plates and allowed to cool.

1.5 mL of each of the experimental organisms were taken from the broth and poured in the plates containing nutrient agar, spread with the help of sterile spreader to make the lawn of the experimental organisms and allowed for about 10 minutes. Then pustules were made with the help of cork borer from the seven day old culture of all the fungi, and were placed on the lawn of the experimental organisms in the petri plates.

The plates were covered with para film and placed in incubator for 24 hours at 37 $^{o}$C for recording the zone of inhibition. These experiments were repeated in triplicate so as to get the accurate zone of inhibition [27-28].

#### 3.2.2.1 Experiment 2

These experiments were conducted to determine the effect of the metabolites produced by fungi against the pathogenic bacteria i.e. *Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Salmonella typhi, Shigella flexneri* and *Staphylococcus aureus*, for further production of the metabolites to find some novel antibiotic drug.

### 3.2.3 Inoculums preparation and conservation

Isolated species were grown on the solid media and then the spores were preserved in glycerol, as inoculums for the inoculation to produced secondary metabolites. The suspensions of spores i.e. 106 spores/mL of Fungus were spread on a petri plate containing CYA medium and allowed for 8 days of incubation at 28 °C to grow. After the incubation period, 10 to 15 mL of 0.01% Tween–80 (Oliec acid) solution/or detergent solution was spread on each plate. The spores were scratched with the help of sterile blade and then poured in to spore solutions (glycerol 50% spores suspension (50:50 (v /v)) by sterile syringe in to 15 mL falcon tubes. The spore

solution was preserved in ultra freezing fridge at –23 or –80 $^{o}$C. From now and onward for all the experiments the spores counting were performed through Thoma Bright Line (TBL) counting chamber.

#### 3.2.3.1 Experiment 3

These experiments were conducted to keep the spores of biologically active fungi, preventing from germination and to keep them viable for long time for further inoculation to produced secondary metabolites.

### 3.2.4 Extraction of metabolites from liquid culture medium

Each fungus were grown in their respective broth medium for the production of secondary metabolites for further biological activities like antibacterial, antifungal, cytotoxic and phytotoxic. This work was done in Microbial Biotech: Laboratory, at the Centre of Biotechnology and Microbiology University of Peshawar, Pakistan and in the School of Chemistry, University of Bristol, UK.

The spore suspensions (106 spores/1 mL) were transferred to liquid medium Czapek Yeast–extract Broth (CYB). This process was conducted in the sterile condition. Then the culture was grown in shaking incubator at 28 $^{o}$C and 150 rpm for 12 days. After the incubation period, 200 to 500 µL of 40% concentrated HCl were added to acidify the culture medium for improve settling of the media. Then culture was grinded with blender and then filtered with Whatman filter paper no. 1 or cheese cloth or filter paper.

Then equal volume of ethyl acetate/chloroform (some time) were added to the medium and mixed well by shaking about 20 to 30 minutes. Then the mixture were transferred to separating funnel and allowed for 10 minutes to settle down. Two separate phases were formed, the ethyl acetate/chloroform (lower phase) were

recovered in the round bottom flask, then the organic layers was washed with 2M brine solution. After washing the organic portion containing metabolites was purified with anhydrous sodium sulphate ($Na_2SO_4$). Then the metabolites were concentrated by rotary evaporator at 45 °C with a slight rotation of 150 rpm. The crude extract (mixture of few compounds) was recovered from rotary evaporator by methanol in were dried in the fume hood under liquid nitrogen and were subjected to biological activities.

#### 3.2.4.1 Experiment 4

These experiments were conducted to isolate secondary metabolites from different broth medium for further biological activities like antibacterial, antifungal, cytotoxic and phytotoxic to evaluate the potential of the secondary metabolites. The potent extract will be subjected for the isolation of secondary metabolites.

### 3.2.5 Optimization of media and the use of epigenetic modifiers

In order to recognize the best media for the maximum production of secondary metabolites and the incubation period for the production, five different media these includes, Czapek–dox Broth (CB), Czapek Yeast–extract Broth (CYB), Yeast Extract Sucrose (YES), Potato Dextrose Broth (PDB) and Czapek–dox (supplemented with glucose & starch) Broth (CGSB) and five different concentrations of the two epigenetic modifiers (suberoyl anilide hydroxamic acid; SAHA and 5–azacytidine; 5–AZA) were used.

Then 300 mL of each media were prepared. 30 clear conical flasks (100 mL) were taken in which a set of five flasks served for each medium. Then 50 mL of the

respective medium was added to each of the flask and were capped with cotton. These flasks were then autoclaved at 121 $^{o}$C for 15 minutes. After autoclaving, the flasks were brought to the laminar flow hood and were allowed to cool down to 25 $^{o}$C. Then each of the flasks containing the broth medium was inoculated with a spore suspension 106 spores/mL with the help of a micropipette and was incubated at 25 °C with continuous shaking of 150 rpm in shaking incubator.

After periods of three days of incubation, one flask of each media were taken out form the incubator, their pH was determined with pH meter, and then filtered through a pre–weighed Whatman filter paper no. 1. The mycelia in the filter paper were allowed to dry. The filter papers containing the dry mycelia were weighed again and the weight of dry mycelia were determined by subtracting the weight of the clear filter paper from the weight of the filter paper containing the dry mycelia.

The filtrate was placed in a separating funnel and concentrated HCl was added in a concentration of 1 mL HCl/100 mL of the filtrate, to degrade the media components. Mix by inverting the bulb of the funnel time to time, and then chloroform was gently added in a ratio of 1:1 (v/v). Mix again several times by inversion, the gas was removed by opening the cap. The mixture was left to settle down for several minutes (let the cap of the funnel open). The chloroform phase (lower phase) was recovered in a flask and evaporated through a Rotary aspirator at 50 °C with a slight rotation of 150 rpm. The extract was collected in a pre–weighed glass vials. The vials were again weighed to determine the weight of the secondary metabolite extract. In this way one flask of each media was taken out from the incubator after 6, 9, 12, 15 and 18 days incubation respectively, their pHs, weights of mycelia and weights of secondary metabolite extract were determined [128]. After the

media optimization different concentrations (1, 5 10 15 and 20 mM/100 mL) of media were used to obtained the highest production of metabolites.

#### 3.2.5.1 Experiment 5 & 6

These experiments were conducting to produce greater quantity of the crude secondary metabolites, for their respective biological activities and isolation of pure secondary metabolites.

### 3.2.5 Antibacterial activities

The ethyl acetate, *n*–Hexane and aqueous fractions of *A. carbonarius* (NRRL 369), *A. oryzae* (tenS.PKS/dmbC), *C. carrionii* (F–2) and *C. resinae* (NRRL 6437) were screened against six human pathogenic bacterial strains i.e. *Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Salmonella typhi, Shigella flexneri* and *Staphylococcus aureus*.

The agar well diffusion method was used for the screening of antibacterial activities [27, 129-131]. (10 mL x 6) of nutrient broth (different additive) were taken and the experimental organisms (pathogenic bacteria) were ingrained at 37 $^{o}$C for 24 hours for the production of fresh culture.

Then (20 mL x 6) of the nutrient agar were taken in sterile petri plates and allowed to cool; 0.2 mL of each experimental organisms were taken from broth culture and poured on the agar media, therefore made a homogenous lawn. Specific numbers of well were made. Stock solutions were prepared in sterile di–methyl sulfoxide (DMSO) with dose concentration 1, 10, 20, 50, 100, 250 and 500 µg/mL, now equal volume of each concentration was poured in each well. Carbenicillin was used as standard drug with concentration of 100 µg/mL.

The plates were left at room temperature for 2–3 hours for the diffusion of the samples and then transferred to incubator for 24 hours incubation at 37 $^{o}$C. The experiments were conducted in duplicate and the zone of inhibitions was determined by the following formula.

$$\text{Inhibition \%} = \left( \frac{\text{Zone of sample}}{\text{Zone of control}} \right) \text{x } 100$$

#### 3.2.5.1 Experiment 7

These experiments were conducted to determine the effect of using the extract and fraction of different fungi, against the pathogenic bacteria. Carbenicillin was used as standard drug for the comparative results of the test samples for further isolation and standardization to find a new standard antibacterial drug.

### 3.2.6 Antifungal Activities

The ethyl acetate extract and *n*–Hexane fraction of *A. carbonarius* (NRRL–369), *A. oryzae* (tenS.PKS/dmbC), *C. carrionii* (F–2) and *C. resinae* (NRRL–369) were screened against six human, animal and plants pathogenic fungi i.e. *Aspergillus flavus, Candida albicans, Candida glabrata, Fusarium solani, Microsporum canis* and *Trichophyton longifusus*.

The agar tube diffusion method was applied for the screening of Antifungal Activities [27-28, 130-131]. (25 mL x 6) of potato dextrose agar (PDA–different additive) were taken and the experimental organisms (pathogenic fungi) were inoculated at 28 $^{o}$C for 7 days for the production of fresh culture.

Then (10 mL x 6) of the potato dextrose agar were taken in sterile test tubes and the test samples were added with dose concentrations of 10, 20, 50, 100, 250, 500 and 1000 µg/mL from stock solution prepared in sterile di–methyl sulfoxide (DMSO) allowed in slanted position to cool; then a small piece of about 4 mm in diameter was detached from the old culture (7 days old) of fungi and implanted. Miconazole were used as standard drug with concentration of 100 µg/mL. The test tubes were transferred to incubator for 7 days and 28 $^{o}$C. The experiments were conducted in duplicate and the line of inhibitions was determined by the following formula.

$$\text{Inhibition \%} = 100 - \left( \frac{\text{Growth of sample}}{\text{Growth of controle}} \right) \text{x } 100$$

### 3.2.6.1 Experiment 8

These experiments were conducted to determine the effect of using the extract and fraction of different fungi, against the pathogenic fungi. Miconazole and Amphotericin–B were used as standard drug for the comparative results of the test samples for further isolation and standardization to find a new standard antifungal drug.

### 3.2.7 Brine–shrimp cytotoxicity

The ethyl acetate extract and *n*–Hexane fraction of *A. carbonarius* (NRRL–369), *A. oryzae* (tenS.PKS/dmbC), *C. carrionii* (F–2) and *C. resinae* (NRRL– 6437) were screened against Artemia salina (*brine–shrimp* eggs) to measure the mortality/lethality [132]. When placed in artificial seawater, the eggs hatched within 48 hours, providing large number of larvae. These tiny shrimp larvae were used as a tool to determine the cytotoxic activities of the samples considered for the present

study. This is a rapid inexpensive, in–house, general bioassay, which is used as a rapid and simple preliminary monitor for bioactive compounds from the natural product.

Artificial hatching media or sea water was made by dissolving 3.8 gm of sea salt in 1000 mL of de–ionized water and then filtered. One milligram (1 mg) of *brine shrimp* eggs were transferred to the hatching media (sea water) in to a small tank; and were covered with aluminium foil. It was then left for 24 hours at 25 $^{o}$C so numerous larvae were hatched. Stock solution was prepared in ethanol (30 mg/1.5 mL). The test samples were transferred to sea water with dose concentration of 1000, 100 and 10 µL/mL. Then ten (10) shrimps were transferred to each vial and were left for 24 hours, the surviving shrimps were recorded and value of $LD_{50}$ was calculated.

$$\text{Mortality \%} = 100 - \left( \frac{\text{Shrimps in sample}}{\text{Total shrimps}} \right) \text{x } 100$$

After the calculation of percentage, the median lethal dose ($LD_{50}$) was calculated with the help of software (Probit Analysis) with 95% confidence intervals and also by conventional method.

**3.2.7.1 Experiment 9**

These experiments were conducted to demonstrate the effect of using the extract of different fungi, against Artemia salina (*brine shrimp* eggs). To determined the cytotoxicity of the samples and particularly to find the $LD_{50}$, to establish a background for the discovery of anticancer drugs.

### 3.2.8 Phytotoxic activities

The ethyl acetate extract and *n*–Hexane fraction of *A. carbonarius* (NRRL–369), *A. oryzae* (tenS.PKS/dmbC), *C. carrionii* (F–2) and *C. resinae* (NRRL– 369) were screened against *Lemna acquinoctialis* to determine the phytotoxic activity by *Lemna* bioassay [133-135]. See section 3.1.1.7.

The artificial medium was made by mixing various inorganic ingredients in de–ionize water (1000 mL) and the pH was adjusted (5.5–6.5) by two molar solution of potassium hydroxide (2M of KOH). The E–medium was sterilized using autoclave at 121 $^{o}$C for 15 minutes. Stock solution was prepared in ethanol (30 mg/1.5 mL). The test samples were transferred to E–medium with dose concentration of 1000, 100 and 10 µL/mL. The solution was allowed for 24 hours to evaporate the excessive solvent under aseptic condition.

After 24 hours 20 mL of the medium with slightly basic pH was added to all of the sterilized flasks and ten healthy plants of *Lemna acquinoctialis*, having three fronds each were transferred to a growth cabinet/chamber for seven days. The temperature was maintained at 30 $^{o}$C and light intensity was 9000 lux, where humidity were maintained around 60 by keeping sterilized de–ionized water in open beaker in the cabinet/chamber. The flasks containing *Lemna* were plug with cotton and kept in the growth cabinet/chamber for seven days. On eighth day the fronds was measured. Parquet was used as positive control [27].

$$\text{Growth inhibition \%} = 100 - \left( \frac{\text{No of fronds in sample}}{\text{No of fronds in control}} \right) \text{x } 100$$

#### 3.2.8.1 Experiment 10

These experiments were conducted to demonstrate the effect of using the extract and different fungi, against *L. acquinoctialis*. To determined the toxicity toward other herbs.

## 3.3 Methodology Phase–II

Phase–II methodology is consisting to cover the following part of the current research project.

- Standardization of the biologically active secondary metabolites and/or compound(s) was done successfully.
- Estimation of the Gene (s) responsible for the secondary metabolites [$MH^+$ 211, 213] was also done successfully by feeding 1–labelled acetate.

### 3.3.1 Isolation of metabolites by column chromatography techniques

First of all, the slurry of the sample were made in silica gel, and then allowed to dry till to make the powder of the samples in silica; then I mix the silica in 100% *n*–Hexane and poured in the column. When the silica gel made a clear bed in the column then with the help of funnel the sample was poured on the silica bed. Then 100% *n*–Hexane was used/passed as solvent of the mobile phase. While passing the solvent three times isolated no compound showed that there is no non polar compound in the sample mixture.

Then the polarity were increased by adding 15 mL of the ethyl acetate to 285 mL of the *n*–Hexane [5/95 V/V (Ethyl acetate; *n*–Hexane)]. However this mobile phase was also unsuccessful and produced nothing. By the same way the polarity was increased to 10/90 V/V (Ethyl acetate; *n*–Hexane) and this phase isolated only one

compounds. The polarity were gradually increased to 11, 12 13% and so on, because to increase the polarity with smaller fraction gave more perfect chance for the isolation of the single compound [136].

#### 3.3.1.1 Experiment 11

These experiments were conducting to isolate a pure compound from the mixture of metabolites, so as to know their biological activities against the pathogenic bacteria, which will then be standardized as novel antibiotic.

## 3.4 LC–MS/HPLC analysis

Liquid chromatography mass spectrometry (LCMS) is an instrument (Waters 2795HT HPLC system) used for the analysis of fungal secondary metabolites. The instrument is equipped with Waters 2998 photodiode array detector joined to a Micro–Mass ZQ spectrometer for the detection of UV between 200–400 nm. Whereas; High Performance Liquid Chromatography (HPLC) is an instrument with Waters 2545 pump system used for the purification of fungal secondary metabolites. The instrument is equipped with Waters UV 2990 photodiode array detector joined to 175–Quatro–Micro Mass spectrometer for the detection of UV between 200–400 nm [137]. There are two type of system for the analysis of metabolites, to detect the desirable metabolites is done by the analytical run whereas, to isolate is done by the preparative run.

### 3.4.1 Procedure–I (Analytical Run)

The crude extracts (10 mg/mL) were dissolved in HPLC grade methanol (MeOH). Then the solution was centrifuged for two (2) minutes to remove the un–dissolved/suspended particles and then filtered by chess cloth. Then 20 µL of the clear solution was injected to LC–MS for analytical run of 15 minutes Phenomenex Luna reversed phase column with specification (C–18, 5 µ, 100 Å and 250 × 4.6 mm) or Phenomenex Kinetex column with specification (C–18, 2.6 µ, 100 Å and 100 × 4.6 mm). While in either case the column were protected by a guard column, with specification (Luna C–5 300 Å). This column is working as security guard before the analytical column. The flow rate was 1 mL/minute for analytical run. The secondary metabolites were detected by UV between 200–400 nm and simultaneously by electro–spray (ES) mass spectrometry using a Waters ZQ spectrometer between $MH^{+/-}$ (100–400 m/z units) [137].

Solvents for both the analytical and preparative systems**:**

**(A)** Ultra–pure water, $H_2O$ (HPLC grade) with 0.050% formic acid,

**(B)** Methanol, MeOH (HPLC grade) with 0.045% formic acid,

**(C)** Acetonitrile, $CH_3CN$ (HPLC grade) with 0.045% formic acid),

**Method 1:-**

Luna/MeOH: 0 minute, 25% B; 5 minute, 25% B; 51 minute, 95% B; 53 minute, 95% B; 55 minute, 25% B; 59 minute, 25% B; 60 minute, 25% B. (It was used for analysis of extracts containing $MH^+$ 143 and 245).

**Method 2:-**

Kinetex/CH3CN: 0 minute, 10% C; 10 minute, 90% C; 12 minute, 90% C; 13 minute, 10% C; 15 minute, 10% C. (It was used for analysis of extracts containing $MH^+$ 211 and 213).

### 3.4.2 Procedure–II (Preparative Run)

After the confirmation of the desired metabolites the remaining extracts (20–50 mg/mL) were dissolved in HPLC grade methanol (MeOH). Then the solution was centrifuged for 2 minute so as to remove the un–dissolved/suspended particles and then filtered by chess cloth. Then 200–500 µL (depending upon the purity of chromatogram) of the clear solution was injected to HPLC for preparative run of 30 minute. Phenomenex Luna reversed phase column with specification (C–18, 5 µ, 100 Å and 250 × 10 mm). Like in LC–MS the preparative column were also protected by a guard column, with specification (Luna C–5 300 Å). This column is working as security guard before the preparative column. The flow rate was 4 mL/minute for preparative run. The secondary metabolites were detected by UV between 200–400 nm and simultaneously by electro–spray (ES) mass spectrometry using a Waters QM spectrometer between $MH^{+/-}$ (100–400 m/z units). The quantities of the desire metabolites were analysed by Evaporative Light Scattering Detector (ELSD) connected to Waters QM spectrometer. All the desired/selected metabolites were collected in glass tubes with an automatic fraction collector. The eluent was then evaporated completely to get the metabolite ready for further NMR experiments [137].

**Method 1:-**

Luna/MeOH: 0 minute, 25% B; 5 minute, 25% B; 51 minute, 95% B; 53 minute, 95% B; 55 minute, 25% B; 59 minute, 25% B; 60 minute, 25% B. (It was used for analysis of extracts containing $MH^+$ 143 and 245).

**Method 2:-**

Kinetex/CH3CN: 0 minute, 05% C; 02 minute, 05% C; 20 minute, 60% C; 22 minute, 95% C; 24 minute, 95% C; 26 minute, 05% C and 30 minute, 05% C. (It was used for analysis of extracts containing $MH^+$ 211 and 213)

#### 3.4.1.1 Experiment 12 & 13

These experiments were conducted for estimation and purification of the desire metabolites for further structure elucidation through different spectroscopic techniques.

### 3.4.2 Structure elucidation

The purified compounds were mainly elucidated using 1D and 2D NMR techniques along with MS methods, while some additional parameters such as IR properties and melting point were also determined. Literature searches were done using Dictionary of Natural Product (DNP) and Chemical Abstracts (Scifinder). Three structures were designated as new.

#### 3.4.2.1 Nuclear magnetic resonance (NMR) spectroscopy

Both the (1D and 2D) NMR spectra of $^{1}H$, $^{13}C$ were recorded from a Varian NMR 500 MHz.

#### 3.4.2.2 Mass spectrometry

Electro–spray Ionization (ESI) mass spectra were measured from an API–150 EX mass spectrometer with a turbo ion spray source.

#### 3.4.2.3 IR spectra

Infrared (IR) spectra were measured on a Perkin Elmer System 2000 FT–IR. The wave number is indicated in $cm^{-1.}$

### 3.4.3 Feeding $^{13}C$ labelled precursor, LCMS/HPLC and NMR analysis

Feeding with isotopic–labelled acetate is an important study because the fungal polyketide synthases use acetate as starting unit for the production of polyketides. Therefore it was necessary to feed *A. oryzae* with C–13 acetate to understand the biosynthetic pathways of its biologically active metabolites [62]. We have applied the same procedures as it was applied before in the section 3.1.4, 3.2.2 and 3.3.

#### 3.4.3.1 Experiment 14 & 15

These experiments were conducted to feed the isotopic labelled acetate as precursor for the biosynthesis of polyketides and to determine the pattern of their incorporation to estimate the biosynthetic pathways of the two new isolated biologically potent metabolites by using high NMR technology.

# CHAPTER 4

# RESULTS OF BIOASSAY SCREENINGS

# 4 RESULTS OF BIOASSAY SCREENINGS

## 4.1 Optimization of media and biological activities

Various concentrations of ethyl acetate extract and *n*–Hexane fraction of *Aspergillus and Cladosporium species* were screened against, antibacterial, antifungal, *Brine–shrimp* cytotoxicity and *Lemna* phytotoxic activities.

### 4.1.1 Optimization of growth media for *Aspergillus carbonarius*

Optimization of media is an important factor for the growth and maximum production of metabolites therefore; five different media were used. The results have shown in figure 4.1. *A. carbonarius* produced 9 mg in czapek–dox broth (CB) on 2$^{nd}$ day, with gradual increase while maximum amounts (52, 64 and 62 mg) were produced on 7$^{th}$, 8$^{th}$ and 9$^{th}$ days respectively. While, (26, 27 and 29 mg) were produced in czapek yeast–extract broth (CYB), yeast extract sucrose (YES) and potato dextrose broth (PDB) on 8$^{th}$ day respectively, therefore these media were observed ordinary medium for the production of metabolites. However, czapek–dox (supplemented with glucose & starch) Broth (CGSB) were observed comparatively a better media for *A. carbonarius*, for the maximum production of metabolites. Nothing were produced till 3$^{rd}$ day except the leak production (6 mg), then a very rapid increase were observed in the production metabolites (25, 40 and 52 mg) on 4$^{th}$, 5$^{th}$ and 6$^{th}$ days respectively, whereas, maximum amount (72, 85 and 75 mg) were produced on 7$^{th}$, 8$^{th}$ and 9$^{th}$ days respectively. The highest amount of secondary metabolites with most media type was produced on 8$^{th}$ day and the production was highest (85 mg) for CGSB media type as compared to CB (64 mg), PDB (29 mg), YES (27 mg) and CYB (26 mg), therefore, CGSB media type was recorded as the optimum media for *A. carbonarius*.

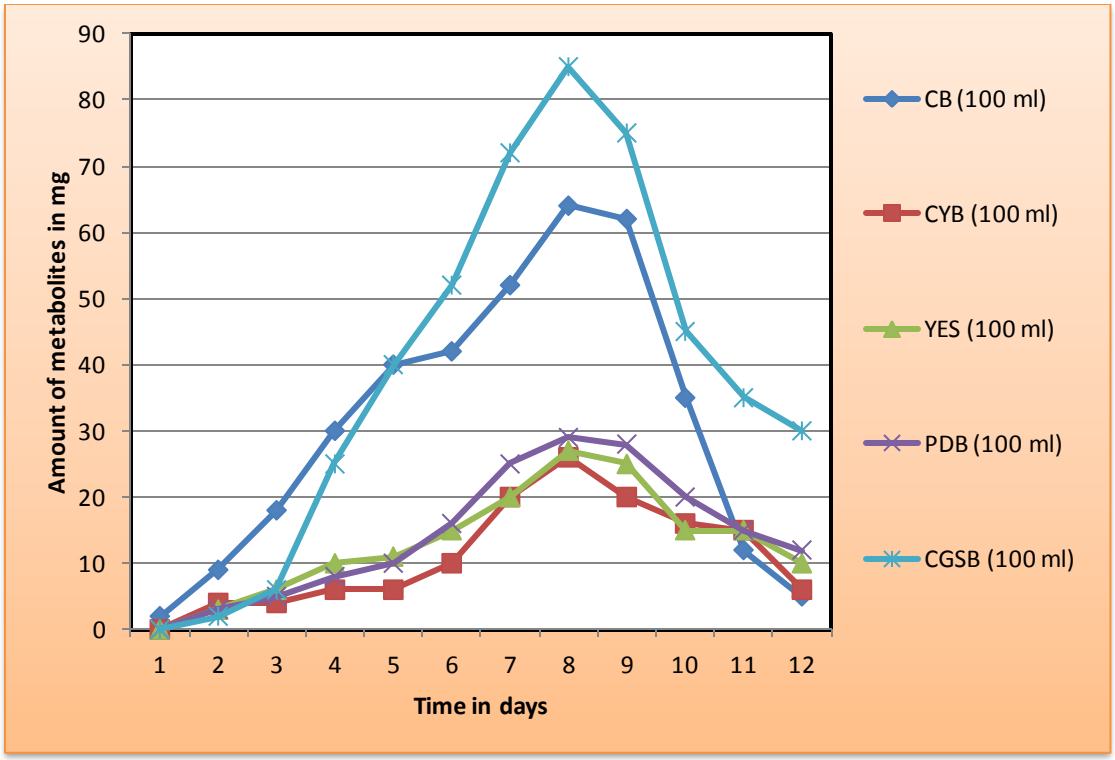



**Figure 4.1 Optimization of media for production of secondary metabolites, CB= Czapek–dox Broth, CYB= Czapek Yeast–extract Broth, YES= Yeast Extract Sucrose, PDB= Potato Dextrose Broth and CGSB= Czapek–dox (supplemented with glucose & starch) Broth.**

### 4.1.2 Use of epigenetic modifiers for *Aspergillus carbonarius*

Two epigenetic modifiers suberoyl anilide hydroxamic acid (SAHA) and 5–azacytidine (5–AZA) were used in Czapek–dox (Supplemented with Glucose & Starch) Broth (CSGB) media and the results have shown in figure 4.2. Different concentrations (1, 5, 10, 15 and 20 µM/100 mL) of both the modifiers were used for *A. carbonarius*.

A dose of 10 µM of SAHA resulted in increased secondary metabolites production by 17 mg, gradual increasing the amount from 25 to 42 mg on 4$^{th}$ day. Also a gradual increase in the metabolites productions (15, 25 and 23 mg) were observed by gradual increasing the amount from 40 to 55, 52 to 75 and 72 to 95 mg on 5$^{th}$, 6$^{th}$ and 7$^{th}$ day respectively, whereas the highest amount (105 mg) was

observed on 8$^{th}$ day with an increase of 20 mg by gradual increasing the amount from 85 to 105 mg and then a gradual decrease.

Therefore, it was found that a dose 10 µM/100 mL of SAHA was observed an effective modifier for the increase of secondary metabolites production. Never the less, a dose of 15 µM/100 mL of 5–AZA resulted in increased secondary metabolites production by 30 mg, gradual increasing the production from 25 to 55 mg on 4$^{th}$ day.

Then fast increase in the metabolites productions (28, 58 and 28 mg) were observed by gradual increasing the amount from 40 to 68, 52 to 110 and 72 to 100 mg on 5$^{th}$, 6$^{th}$ and 7$^{th}$ days respectively. Whereas, the highest amount (115 mg) was observed on 8$^{th}$ day with an increase of 30 mg by gradual increasing the amount from 85 to 115 mg and then a gradual decrease.

It was found that both epigenetic modifiers produced the highest amount of secondary metabolites on day 8 with 5–AZA at 15 µM/100 mL concentration producing marginally higher amount of secondary metabolites (115 mg) than SHAH at 10 µM/100 mL concentration (105 mg). Therefore, the 5–AZA at 15 µM/100 mL concentration was considered as the most effective modifier for *A. carbonarius*.

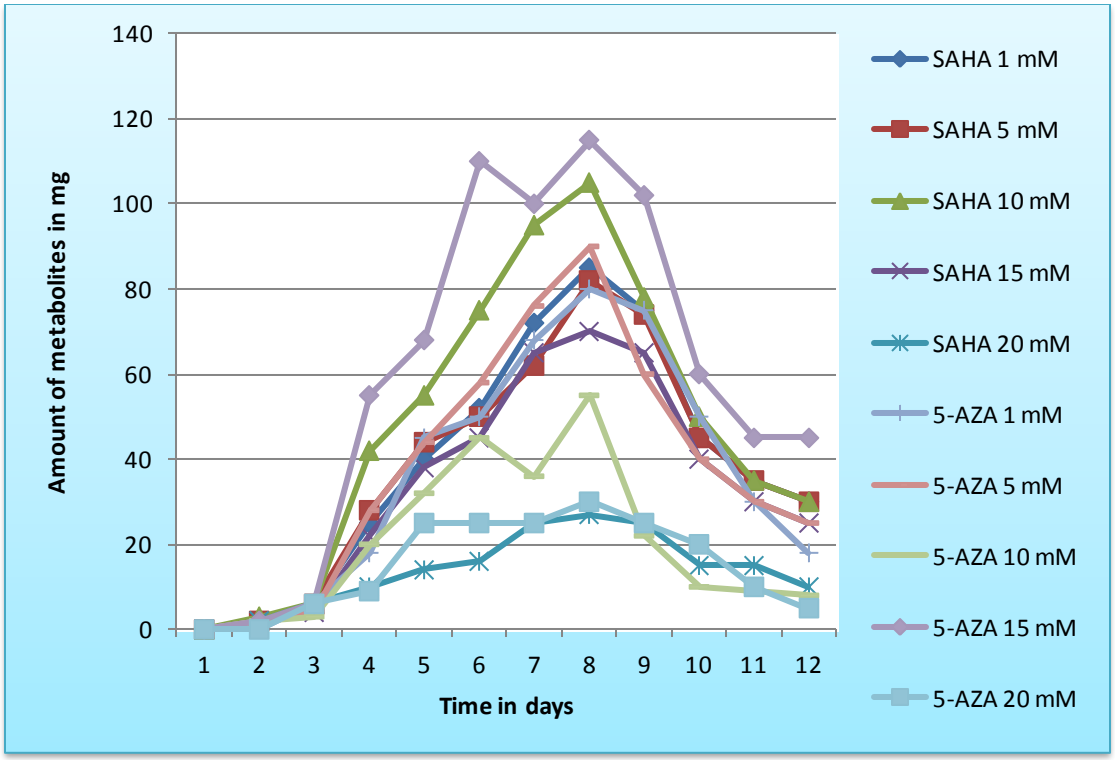



**Figure 4.2 Epigenetic modifier (suberoyl anilide hydroxamic acid (SAHA) and 5–azacytidine (5–AZA)) were used in (CGSB) media for the expression of the silent genes for their metabolites production.**

### 4.1.3 Antibacterial activities of *Aspergillus carbonarius*

Antibacterial activities of the crude metabolites of *Aspergillus carbonarius* (NRRL–369) were tested against the pathogenic bacteria (*Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Salmonella typhi, Shigella flexneri* and *Staphylococcus aureus*), the results have shown in figure 4.3 & 4.4. Different dose inhibited the growth with different zone however, a dose from 1 and 10 µg/mL showed no zone of inhibition and all the pathogenic species survived. Then a dose of 50 µg/mL inhibited the growth of *B. Subtilis* (7.5%), *S. flexneri* (23.5%) and *S. aureus* (13%) and 100 µg/mL inhibited the growth of *S. flexneri* (26%) and *S. aureus* (25%). While a dose of 250 µg/mL inhibited all the experimental species with different zone however, higher inhibition were recorded against *B. subtilis* and *S. aureus* (36 and 31%) respectively and low inhibition against *E. coli*, *K. pneumoniae* and *S. flexneri*

(25, 25 and 27%) respectively whereas, very much low inhibitions against S. *typhi* (15%). However, a higher does 500 µg/mL inhibited the growth of *B. subtilis* and *S. flexneri* (64.5 and 59.5%) respectively, *K. pneumoniae* (51%) and *E. coli*, *S. typhi* and *S. aureus* (35, 38.5 and 36%) *respectively*.

The first four dose concentrations of *n*–Hexane fraction i.e. from 1 to 50 µg/mL have shown no inhibition against any of the pathogenic bacteria. However; 100 and 250 µg/mL inhibited the growth of *E. coli* (10 and 32%) respectively, and 500 µg/mL inhibited the growth of *E. coli* (57.5%) and *S. aureus* (22.5%).

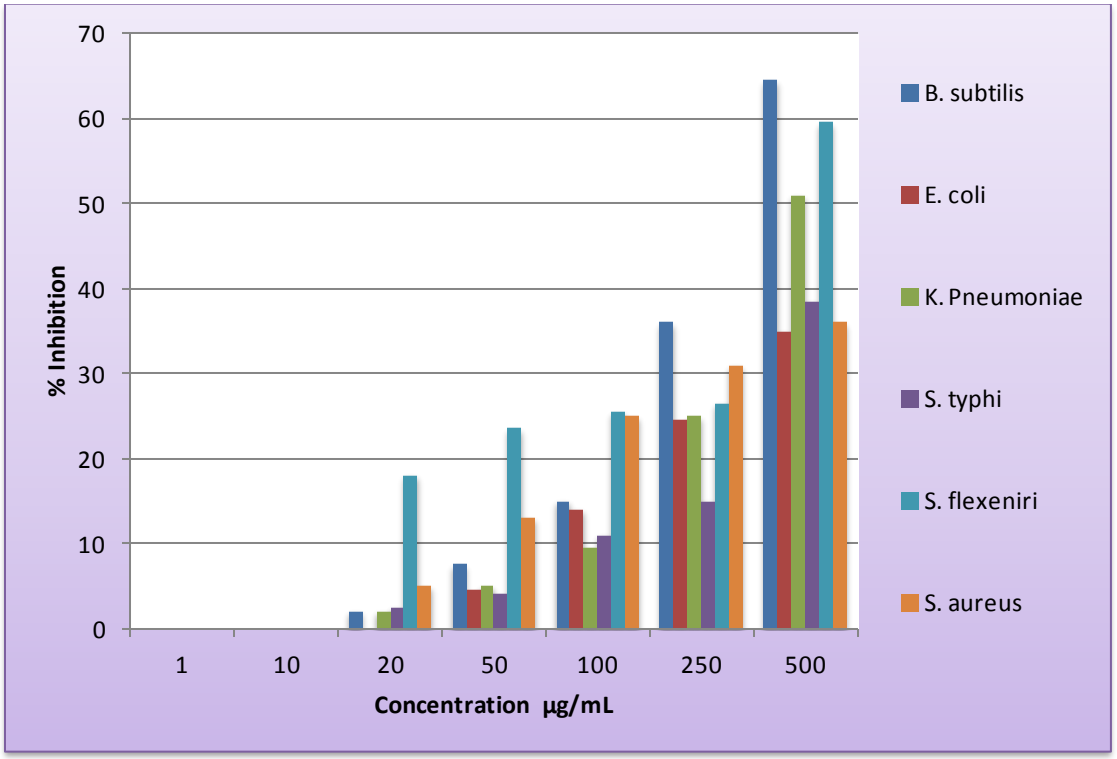



**Figure 4.3 Antibacterial activities of ethyl acetate extract of *A. carbonarius* (NRRL–639).**

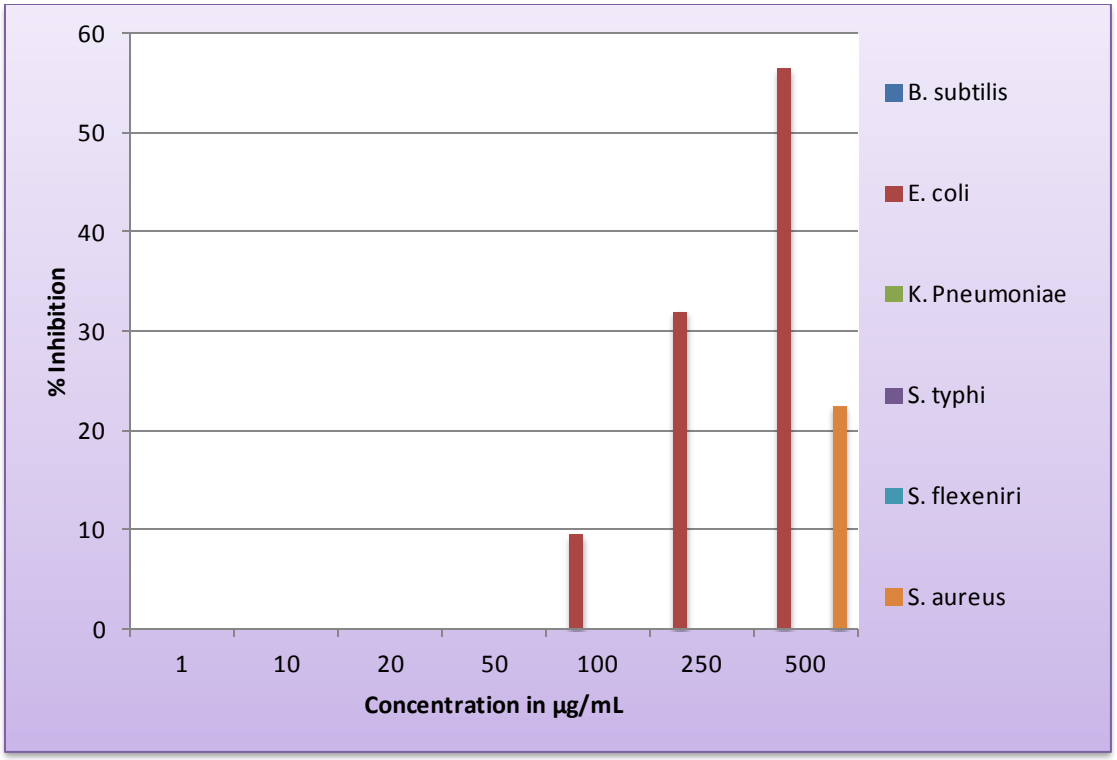



**Figure 4.4 Antibacterial activities of *n*–Hexane fraction of *A. carbonarius* (NRRL–639).**

### 4.1.4 Antifungal activities of *Aspergillus carbonarius*

Antifungal activities of the crude metabolites of *Aspergillus carbonarius* (NRRL–369) were tested against the pathogenic fungi (*Aspergillus flavus, Candida albicans, Candida glabrata, Fusarium solani, Microsporum canis* and *Trichophyton longifusus*), the results have shown in figure 4.5 & 4.6. Different dose concentrations inhibited the growth with different zone but a dose of 10, 20 & 50 µg/mL showed no linear inhibition and all the pathogenic species survived. Then a dose 100 µg/mL inhibited the growth of almost all the fungi except *M. canis*. Then a dose 250 µg/mL inhibited the linear growth of *A. flavus, C. albicans, C. glabrata,* and *T. longifusus* by (10, 15.5, 14.5 and 16%) respectively, *F. Solani* (6%), and no inhibitions against *M. canis*. While a dose of 500 µg/mL inhibited the linear growth of *C. Albicans* (35%), *C. glabrata* and *T. longifusus* (28.5 and 24.5%) and *A. Flavus, F. solani* and *M. canis*

by (15, 13.5, 5.5%) respectively. However, a higher dose 1000 µg/mL inhibited the growth of *C. Albicans* (72%), *C. glabrata* and *T. Longifusus* (58.5% 57%), *A. Flavus, F. solani* and *M. canis* (28, 30 and 10%).

The first five dose concentrations of *n*–Hexane fraction i.e. from 10 to 250 µg/mL showed no inhibition against any of the pathogenic fungi, however, 500 µg/mL inhibited the linear growth of *M. canis* (13%) and 1000 µg/mL inhibited the linear growth of *C. albicans* and *M. Canis* (14 and 35.5%).

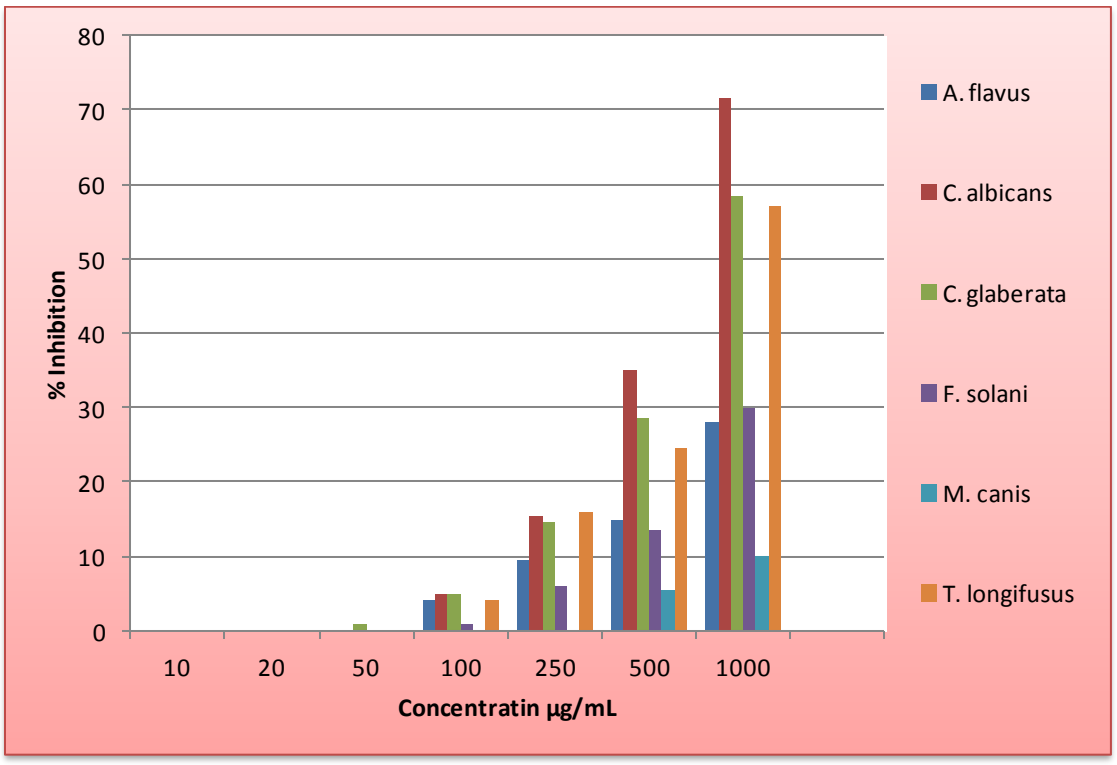



**Figure 4.5 Antifungal activities of ethyl acetate extract of *A. carbonarius* (NRRL–639).**

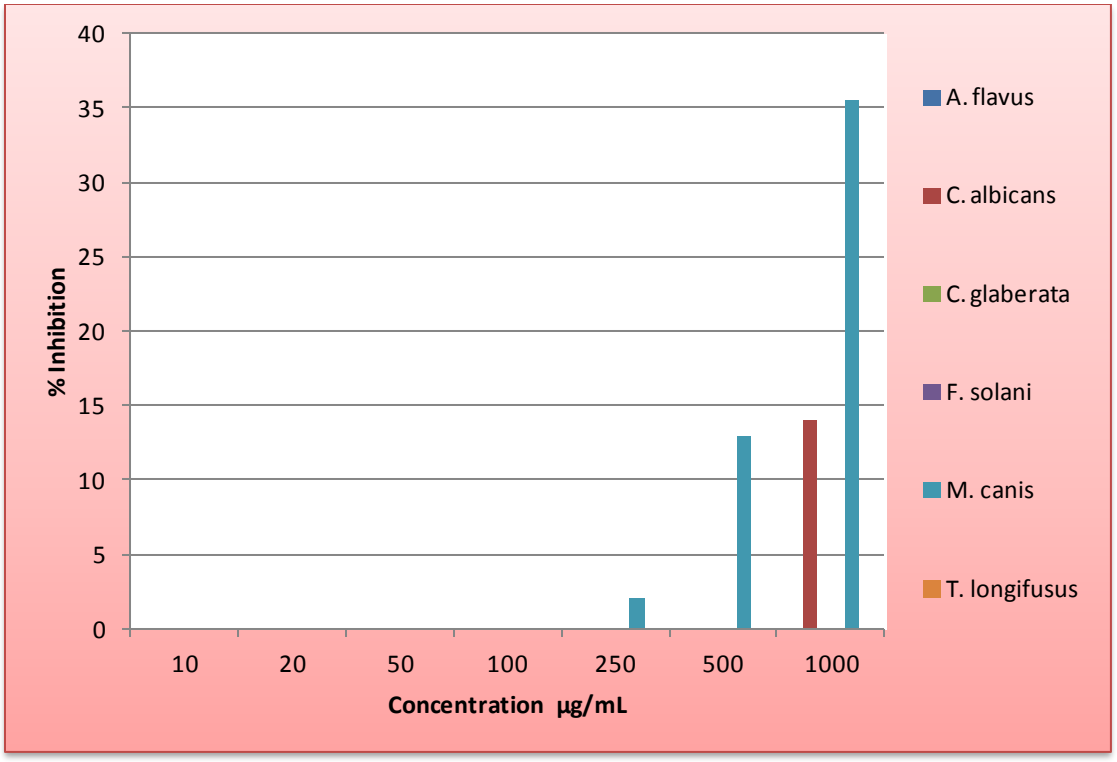



**Figure 4.6 Antifungal activities of *n*–Hexane fraction of *A. carbonarius* (NRRL–639).**

### 4.1.5 Cytotoxic activities of *Aspergillus carbonarius*

Cytotoxic activities of the crude metabolites of *Aspergillus carbonarius* (NRRL–369) were tested against (b*rine shrimps*), the results have shown in figure 4.7. A total of three dose concentrations 10, 100 and 1000 µg/mL was used to measure the toxicity of the metabolites. A dose of 10, 100 and 1000 µg/mL showed 50, 77 and 93% mortality against b*rine shrimps* respectively, whereas the same concentrations of *n*–Hexane fraction showed 37, 73 and 90% mortality against b*rine shrimps* respectively. Both the ethyl acetate and *n*–Hexane fraction displayed very low $LD_{50}$ value i.e. 61.18 and 136.29 respectively.

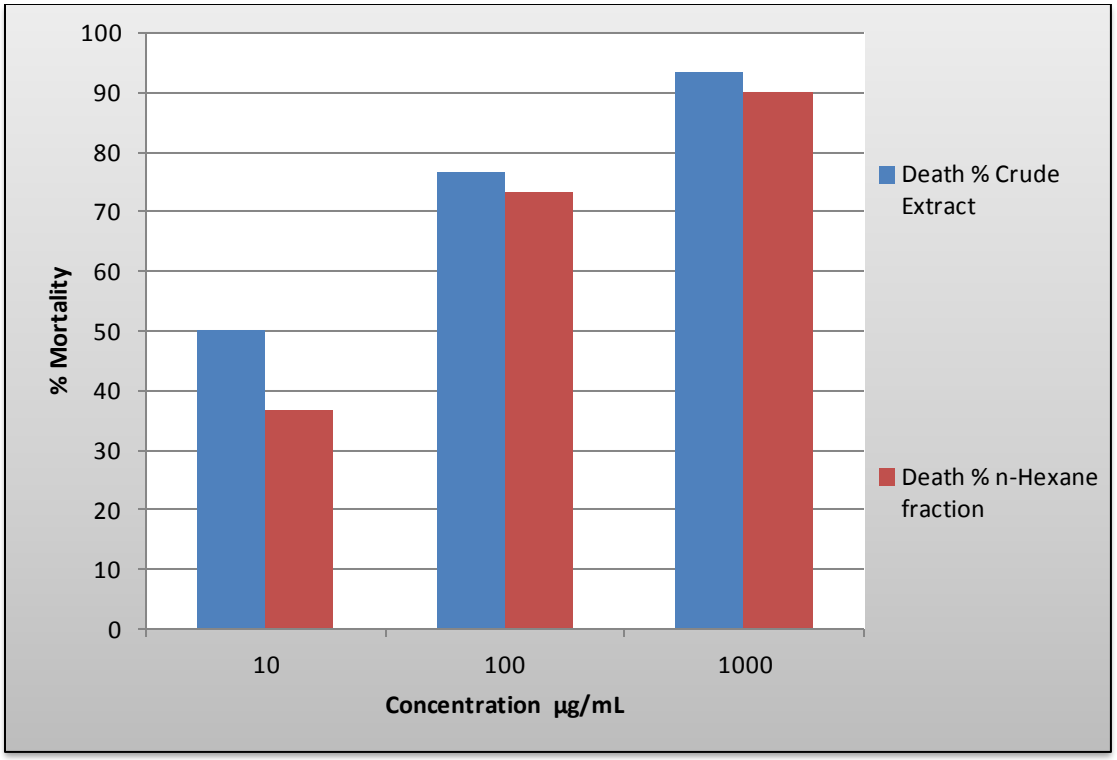



**Figure 4.7 Cytotoxic activities of ethyl acetate extract and *n*–Hexane fraction of *A. carbonarius* (NRRL–639).**

### 4.1.6 Phytotoxic activities of *Aspergillus carbonarius*

Phytotoxic activities of the crude metabolites of *Aspergillus carbonarius* (NRRL–369) were tested against (*Lemna*), the results have shown in figure 4.8. A total of three dose concentrations 10, 100 and 1000 µg/mL was used to measure the phytotoxicity of the metabolites. A dose of 10, 100 and 1000 µg/mL showed 40, 63 and 90% mortality against *Lemna* respectively, whereas the same concentrations of *n*–Hexane fraction showed 30, 80 and 93% mortality against *Lemna* respectively. Both the ethyl acetate and *n*–Hexane displayed very low $LD_{50}$ value i.e. 159.25 and 43.37 respectively.

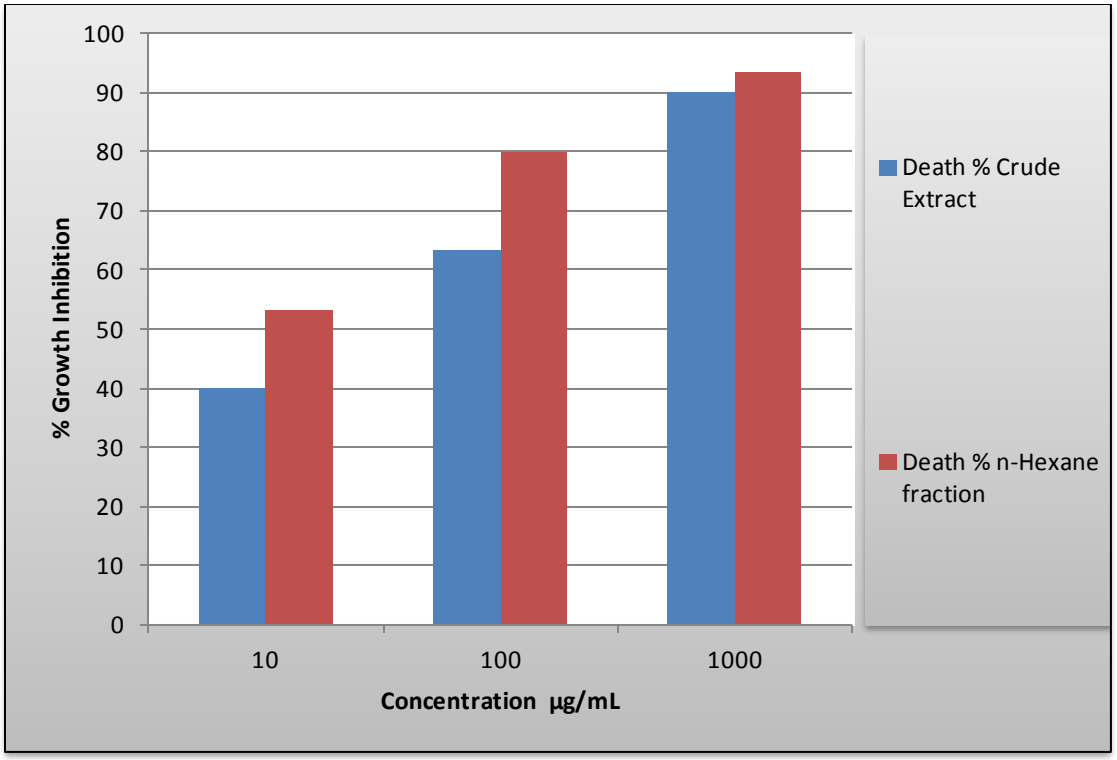



**Figure 4.8 Phytotoxic activities of ethyl acetate extract and *n*–Hexane fraction of *A. carbonarius* (NRRL–639).**

### 4.2.1 Optimization of growth media for *Aspergillus oryzae*

Optimization of media is an important factor for the growth and maximum production of metabolites therefore; five different media were used. The results have shown in figure 4.9. *A. oryzae* produced 10 mg in czapek–dox broth (CB) on 3$^{rd}$ day, with gradual increase while maximum amounts (20, 28 and 20 mg) were produced on 7$^{th}$, 8$^{th}$ and 9$^{th}$ days respectively. While, (52, 60 and 38 mg) were produced in czapek yeast–extract broth (CYB), yeast extract sucrose (YES) and potato dextrose broth (PDB) on 8$^{th}$ day respectively, therefore these media were observed ordinary medium for the production of metabolites. However, Czapek–dox (supplemented with glucose & starch) Broth (CGSB) were observed comparatively a better media for *A. oryzae*, for the maximum production of metabolites. Nothing were produced till 3$^{rd}$ day except the leak production (2 mg), then a very rapid increase were observed in the production metabolites (45, 65 and 72 mg) on 4$^{th}$, 5$^{th}$ and 6$^{th}$ days respectively, whereas, maximum amount (80, 100 and 92 mg) were produced on 7$^{th}$, 8$^{th}$ and 9$^{th}$ days respectively, therefore, (CGSB) were recorded as the optimum media for *A. oryzae*. The highest amount of secondary metabolites with most media type was produced on 8$^{th}$ day and the production was highest (100 mg) for CGSB media type as compared to CB (28 mg), CYB (52 mg), PDB (38 mg), YES (60 mg) and therefore, CGSB media type was recorded as the optimum media for *A. oryzae*.

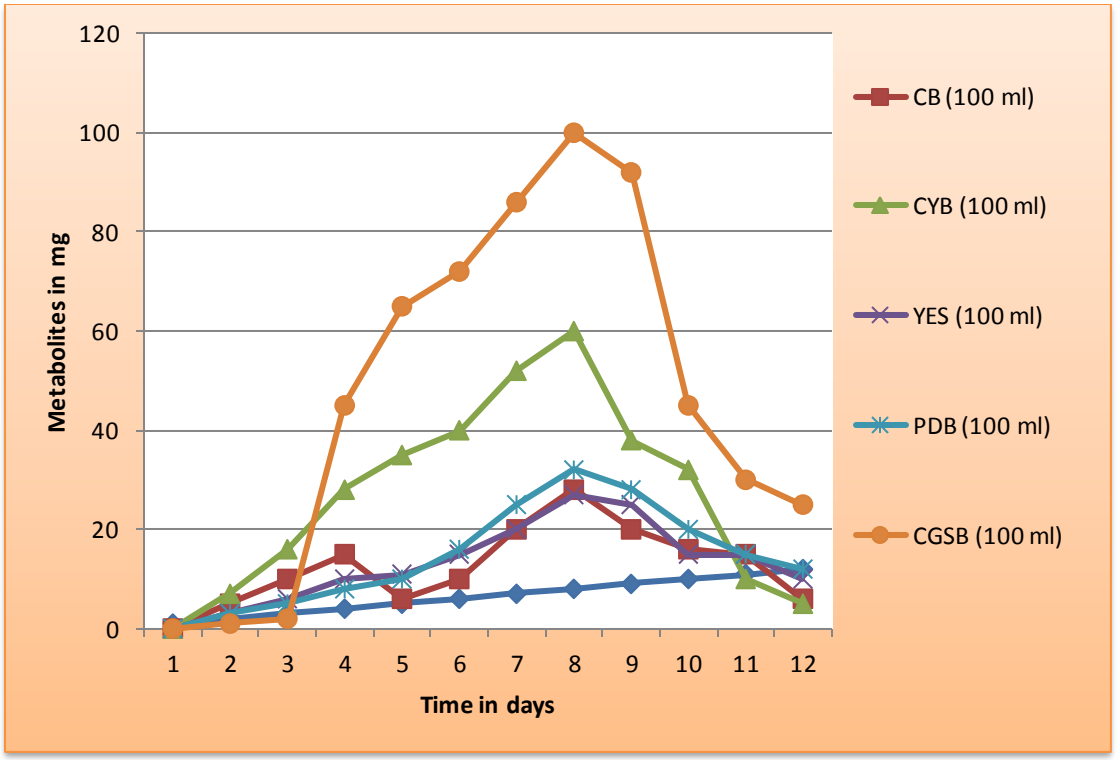



**Figure 4.9 Optimization of media for production of secondary metabolites, CB= Czapek–dox Broth, CYB= Czapek Yeast–extract Broth, YES= Yeast Extract Sucrose, PDB= Potato Dextrose Broth and CGSB= Czapek–dox (supplemented with glucose & starch) Broth.**

### 4.2.2 Use of epigenetic modifiers for *Aspergillus oryzae*

Two epigenetic modifiers suberoyl anilide hydroxamic acid (SAHA) and 5–azacytidine (5–AZA) were used in Czapek–dox (Supplemented with Glucose & Starch) Broth (CSGB) media and the results have shown in figure 4.10. Different concentrations (1, 5, 10, 15 and 20 µM/100 mL) of both the modifiers were used for *A. oryzae*.

A dose of 10 µM of SAHA resulted in increased secondary metabolites production by 13 mg, gradual increasing the amount from 45 to 58 mg on 4$^{th}$ day. Also a gradual increase in the metabolites productions (20, 23 and 44 mg) were observed by gradual increasing the amount from 65 to 85, 72 to 95 and 86 to 130 mg on 5$^{th}$, 6$^{th}$ and 7$^{th}$ day respectively, whereas the highest amount (140 mg) was

observed on 8$^{th}$ day with an increase of 40 mg by gradual increasing the amount from 100 to 140 mg and then a gradual decrease.

Therefore, it was found that a dose 10 µM/100 mL of SAHA was observed an effective modifier for the increase of secondary metabolites production. Never the less, a dose of 15 µM/100 mL of 5–AZA resulted in increased secondary metabolites production by 23 mg, gradual increasing the production from 45 to 68 mg on 4$^{th}$ day.

Then fast increase in the metabolites productions (47, 46 and 54 mg) were observed by gradual increasing the amount from 65 to 112, 72 to 118 and 86 to 140 mg on 5$^{th}$, 6$^{th}$ and 7$^{th}$ days respectively. Whereas, the highest amount (155 mg) was observed on 8$^{th}$ day with an increase of 55 mg by gradual increasing the amount from 100 to 155 mg and then a gradual decrease.

It was found that both epigenetic modifiers produced the highest amount of secondary metabolites on day 8 with 5–AZA at 15 µM/100 mL concentration producing marginally higher amount of secondary metabolites (155 mg) than SHAH at 10 µM/100 mL concentration (140 mg). Therefore, the 5–AZA at 15 µM/100 mL concentration was considered as the most effective modifier for *A. oryzae*.

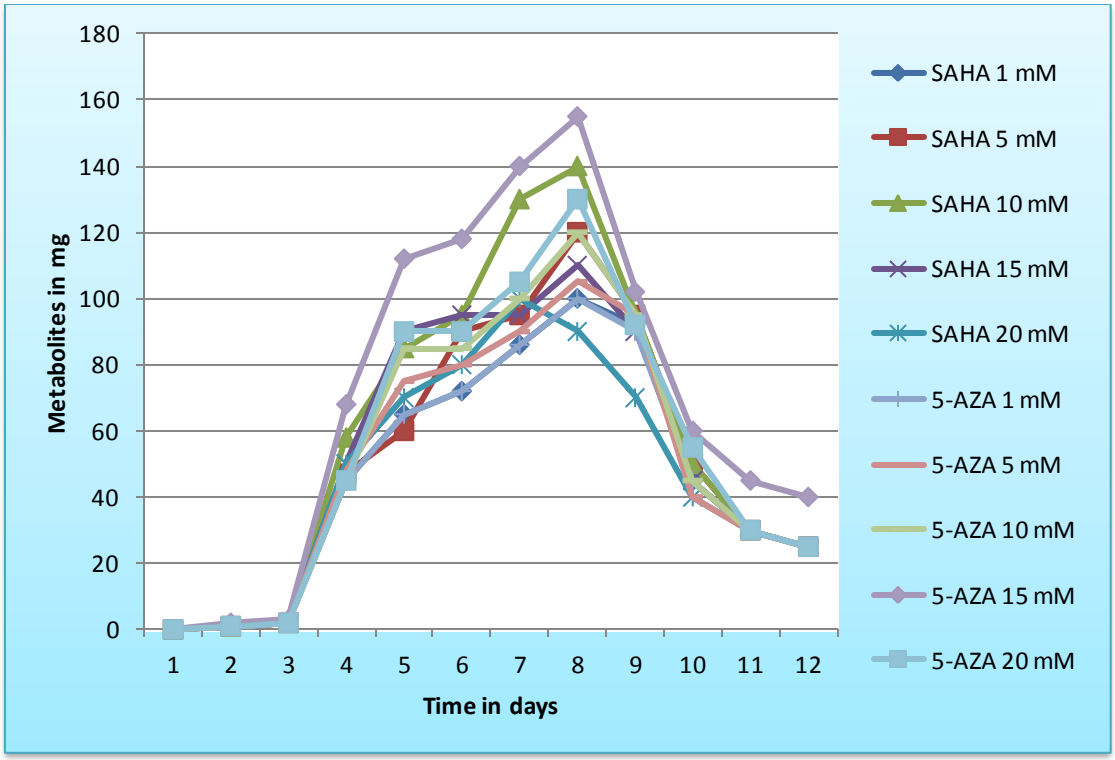



**Figure 4.10 Epigenetic modifier (suberoyl anilide hydroxamic acid (SAHA) and 5–azacytidine (5–AZA)) were used in (CGSB) media for the expression of the silent genes for their metabolites production.**

### 4.2.3 Antibacterial activities of *Aspergillus oryzae*

Antibacterial activities of the crude metabolites of *Aspergillus oryzae* (tenS.PKS/dmbC) were tested against the pathogenic bacteria (*Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Salmonella typhi, Shigella flexneri* and *Staphylococcus aureus*), the results have shown in figure 4.11 & 4.12. Different dose concentrations inhibited the growth with different zone however, a dose from 1 and 10 µg/mL showed no zone of inhibition and all the pathogenic species survived. Then a dose of 50 µg/mL inhibited the growth of *B. subtilis* and *E. coli* and *S. aureus* (13, 10 and 9%) and 100 µg/mL inhibited the growth of *B. Subtilis*, *E. coli* and *S. aureus* (23.5, 23 and 23%) and *S. typhi* (16.5%). While a dose of 250 µg/mL inhibited the growth of all the experimental species *B. subtilis, E. coli, K. pneumoniae, S. typhi, S. flexneri* and *S. aureus* (46, 43.5, 26, 36, 13 and 44.5%) respectively. However, a

higher does 500 µg/mL inhibited the growth of all the experimental species *B. subtilis, E. coli, K. pneumoniae, S. typhi, S. flexneri* and *S. aureus* by (94, 84, 50, 74, 31 and 91%) respectively.

The first four dose concentrations of *n*–Hexane fraction i.e. from 1 to 50 µg/mL have shown no inhibition against any of the pathogenic bacteria. However, 100 and 250 µg/mL inhibited the growth of *S. aureus* (22 and 36%) respectively and 500 µg/mL inhibited the growth of *S. flexneri* and *S. aureus* (32 and 71%) respectively.

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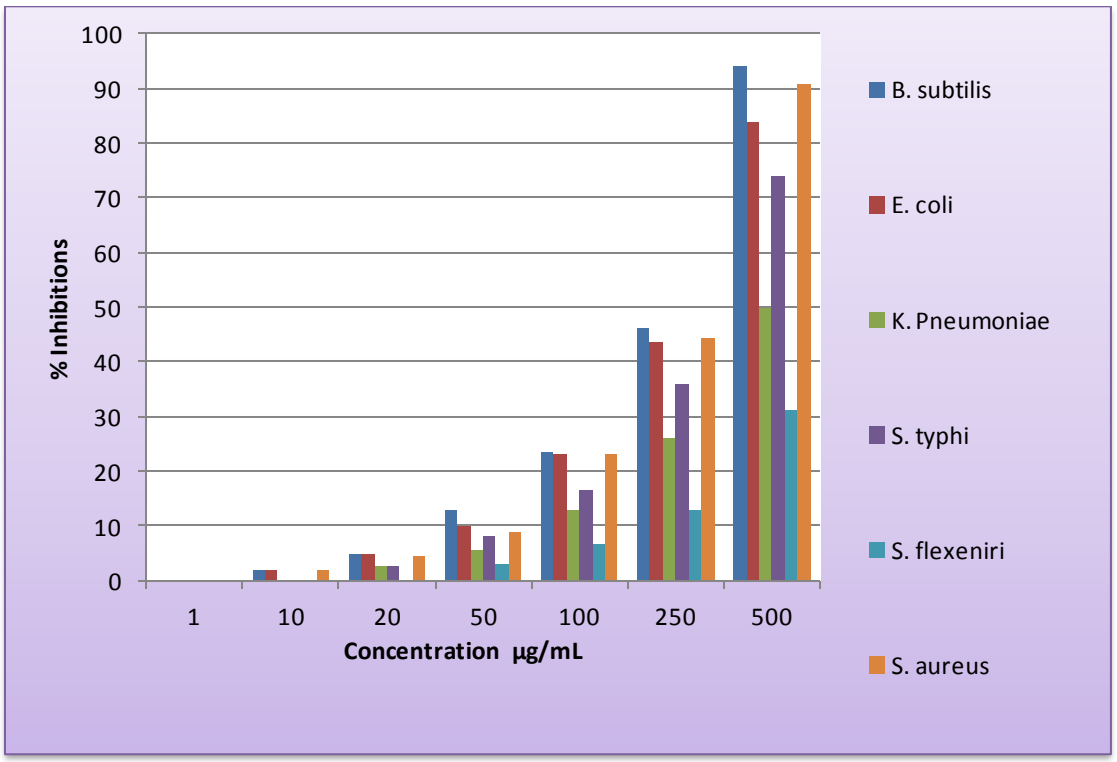



**Figure 4.11 Antibacterial activities of ethyl acetate extract of A. oryzae (tens.PKS/dmbC).**

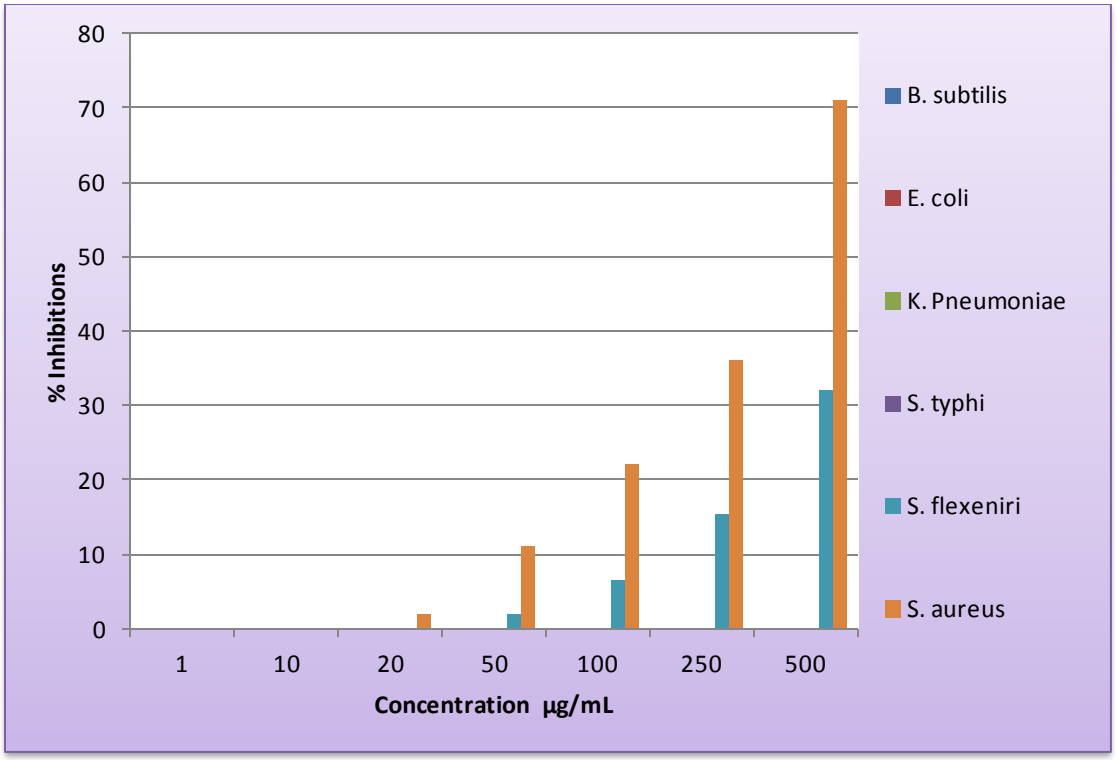



**Figure 4.12 Antibacterial activities of n–Hexane fraction of A. oryzae (tens.PKS/dmbC).**

### 4.2.4 Antifungal activities *Aspergillus oryzae*

Antifungal activities of the crude metabolites of *Aspergillus oryzae* (tenS.PKS/dmbC) were tested against the pathogenic fungi (*Aspergillus flavus, Candida albicans, Candida glabrata, Fusarium solani, Microsporum canis* and *Trichophyton longifusus*), the results have shown in figure 4.13 & 4.14. Different dose concentrations inhibited the growth with different zone but a dose of 10, 20 & 50 µg/mL showed no linear inhibition and all the pathogenic species survived. Then a dose of 100 µg/mL inhibited the growth almost all the fungi especially *M. Canis* by 10% . Then a dose 250 µg/mL inhibited the linear growth of *A. flavus, C. albicans, C. glabrata,* and *M. canis* respectively (19, 13, 18, and 21%), *F. solani* and *T. Longifusus* by 9 and 6% respectively. While a dose of 500 µg/mL inhibited the linear growth of *A. Flavus* and *M. canis* (40 and 41%) respectively, *C. albicans* and *C.*

*glabrata* (27, 34%) respectively, while, *F. solani* and *T. longifusus* by 19 and 11% respectively. However, a higher dose 1000 µg/mL inhibited the growth of *M. Canis* (84%), *A. flavus* and *C. glabrata* (76 and 71%) respectively, while C. *albicans, F. solani* and *T. longifusus* (52, 40 and 21%) respectively.

The first five dose concentrations of *n*–Hexane fraction i.e. from 10 to 250 µg/mL showed no inhibition against any of the pathogenic fungi; however, 500 and 1000 µg/mL inhibited the linear growth of *T. longifusus* by 25 and 50% respectively.

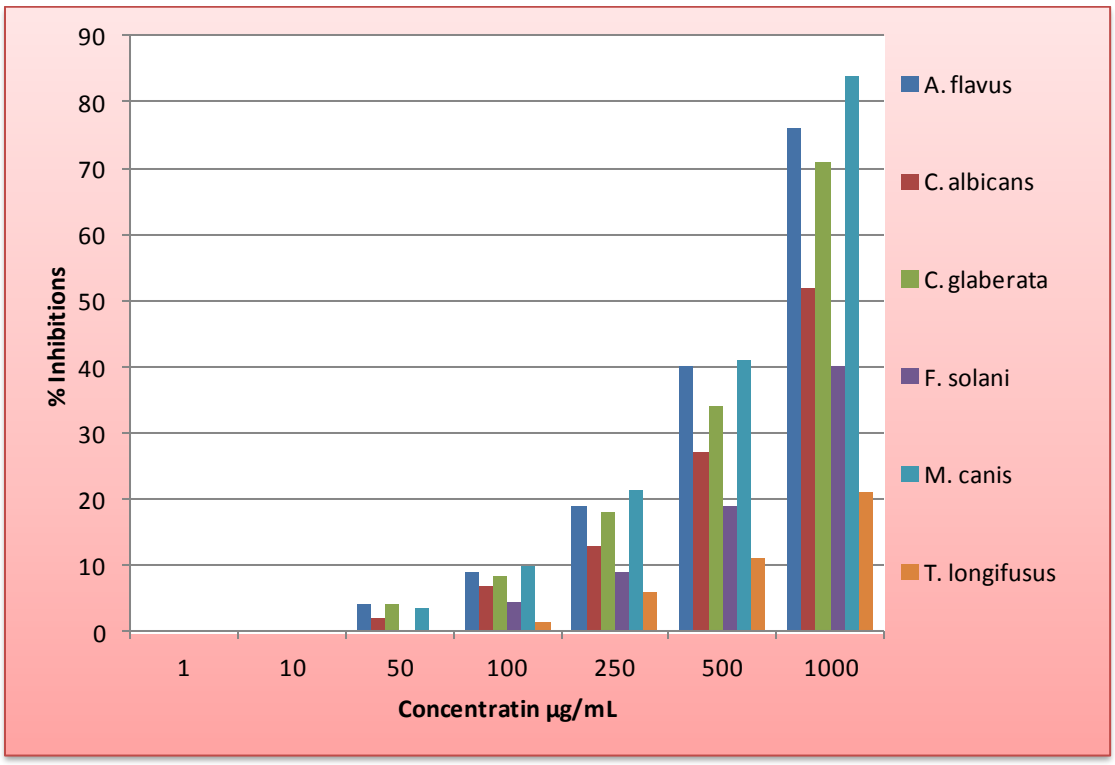



**Figure 4.13 Antifungal activities of ethyl acetate extract of A. oryzae (tenS.PKS/dmbC).**

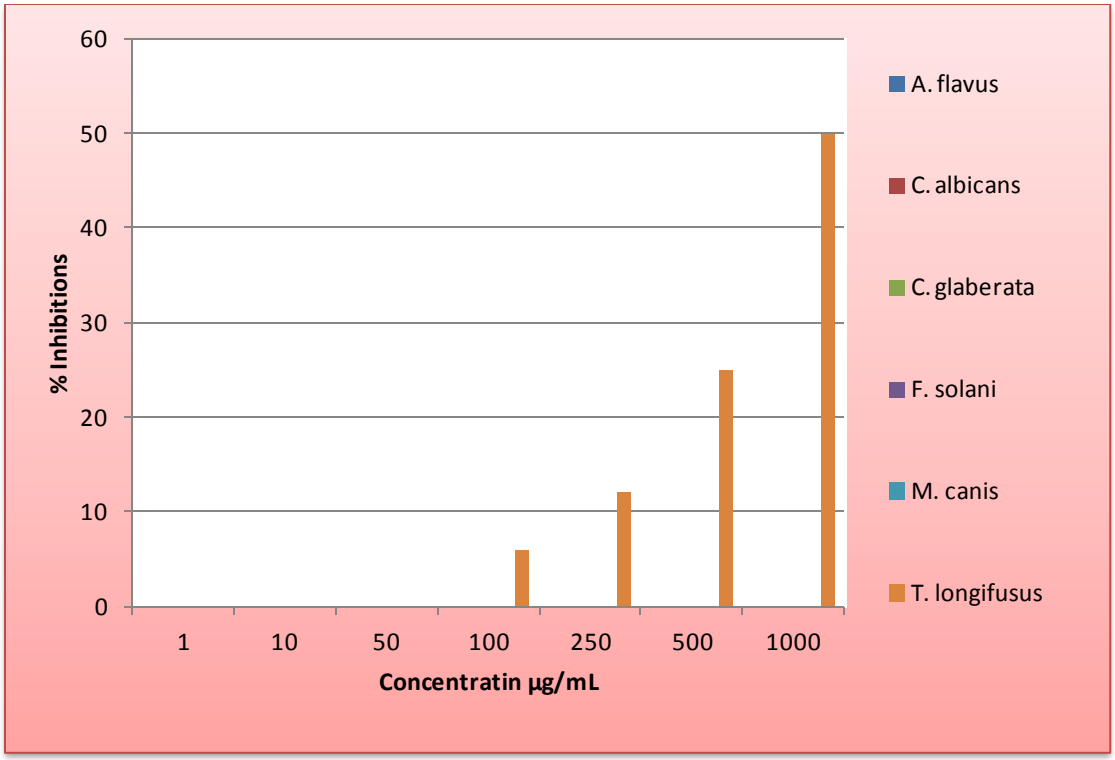



**Figure 4.14 Antifungal activities of n–Hexane fraction of A. oryzae (tenS.PKS/dmbC).**

### 4.2.5 Cytotoxic activities of *Aspergillus oryzae*

Cytotoxic activities of the crude metabolites of *Aspergillus oryzae* (tenS.PKS/dmbC) were tested against (b*rine shrimps*), the results have shown in figure 4.15. A total of three dose concentrations 10, 100 and 1000 µg/mL was used to measure the toxicity of the metabolites. Dose concentrations (10, 100 and 1000 µg/ mL) showed 3, 13 and 52% mortality respectively, whereas the same concentrations of *n*–Hexane fraction showed 3, 18 and 57% mortality respectively. Both the ethyl acetate and *n*–Hexane fraction displayed very low $LD_{50}$ value i.e 1027.04 and 819.48, respectively.

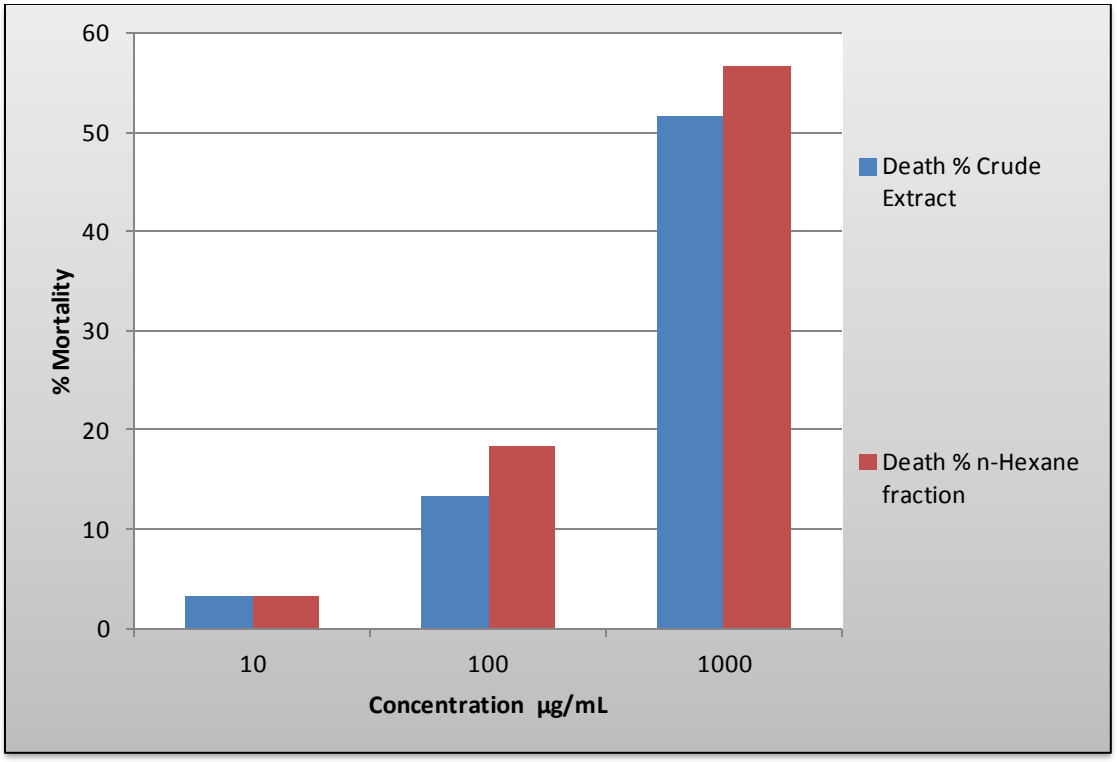



**Figure 4.15 Cytotoxic activities of ethyl acetate extract & n–Hexane fraction of A. oryzae (tenS.PKS/dmbC).**

### 4.2.6 Phytotoxic activities of *Aspergillus oryzae*

Phytotoxic activities of the crude metabolites of *Aspergillus oryzae* (tenS.PKS/dmbC) were tested against (*Lemna*), the results have shown in figure 4.16. A total of three dose concentrations 10, 100 and 1000 µg/mL was used to measure the phytotoxicity of the metabolites. Dose concentrations (10, 100 and 1000 µg/ mL) showed 3, 17 and 67% mortality respectively, whereas the same concentrations of *n*–Hexane fraction showed 3, 17 and 50% mortality respectively. Both the ethyl acetate and n–Hexane displayed very low $LD_{50}$ value i.e. 159.25 and 43.37 respectively.

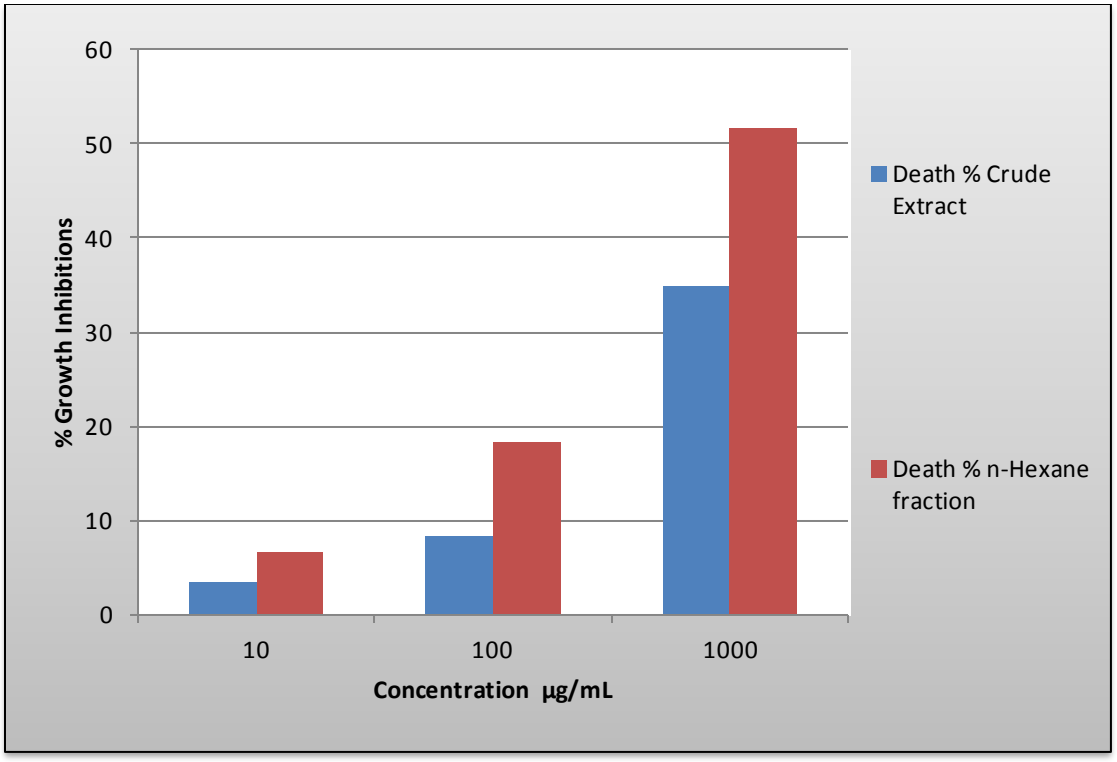



**Figure 4.16 Phytotoxic activities of ethyl acetate extract and n–Hexane fraction of A. oryzae (tenS.PKS/dmbC).**

### 4.3.1 Optimization of growth media for *Cladosporium carrionii*

Optimization of media is an important factor for the growth and maximum production of metabolites therefore; five different media were used. The results have shown in figure 4.17. *C. carrionii* produced (6 mg) in Czapek–dox Broth (CB) on 3$^{rd}$ day, with gradual increase while maximum amount (20, 25 and 20 mg) were produced on 6$^{th}$, 7$^{th}$, and 8$^{th}$ days respectively. While, (10, 24 and 30 mg) were produced in Yeast Extract Sucrose (YES), Potato Dextrose Broth (PDB) and Czapek–dox (supplemented with glucose & starch) Broth (CGSB) on 8$^{th}$ day respectively, therefore these media were observed ordinary medium for the production of metabolites. However, Czapek Yeast–extract Broth (CYB) was observed comparatively a better media for *C. carrionii*, for the maximum production of metabolites. *C. carrionii* produced 5 mg on 3$^{rd}$ day, then a gradual increase were observed in the production metabolites (18, 40 and 50 mg) on 4$^{th}$, 5$^{th}$ and 6$^{th}$ days respectively, whereas, maximum amount (70 mg) were produced on both 7$^{th}$ and 8$^{th}$ days, therefore, (CYB) were recorded as the optimum media for *C. carrionii*.

The highest amount of secondary metabolites with most media type was produced on 7$^{th}$ and 8$^{th}$ day and the production was highest (70 mg) for CYB media type as compared to CB (25 mg), CGSB (30 mg), PDB (24 mg), YES (10 mg) and therefore, CYB media type was recorded as the optimum media for *C. carrionii*.

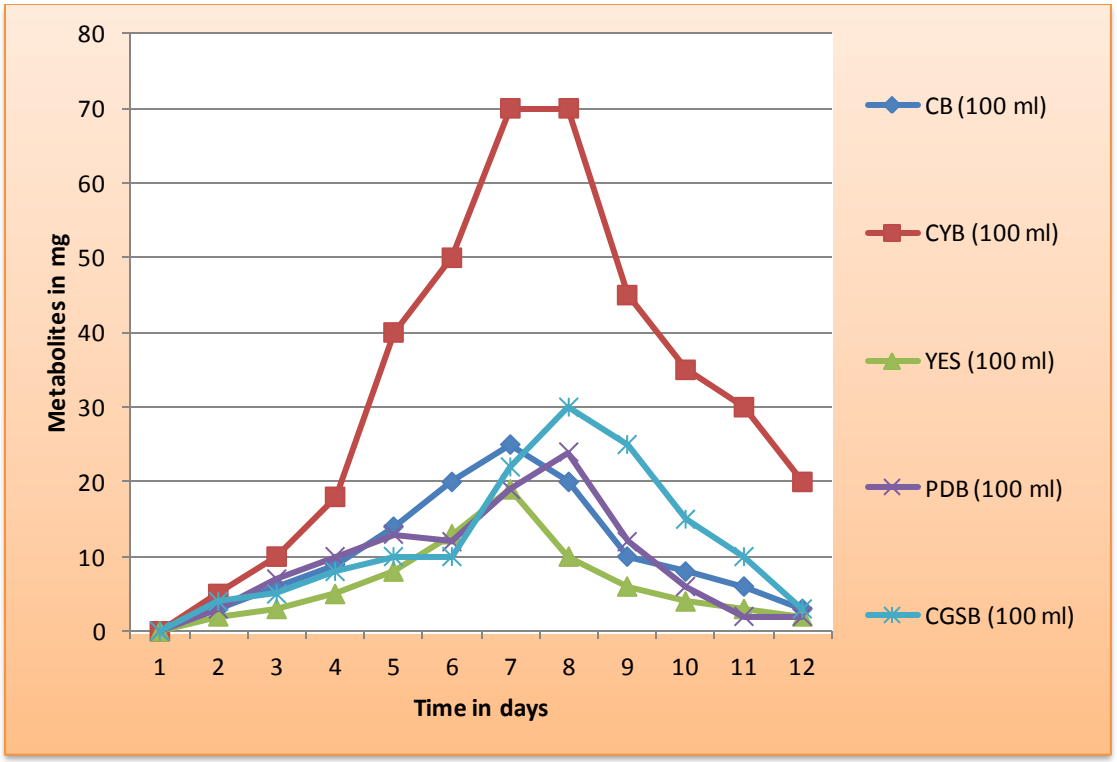



**Figure 4.17 Optimization of media for production of secondary metabolites, CB= Czapek–dox Broth, CYB= Czapek Yeast–extract Broth, YES= Yeast Extract Sucrose, PDB= Potato Dextrose Broth and CGSB= Czapek–dox (supplemented with glucose & starch) Broth.**

### 4.3.2 Antibacterial activities of *Cladosporium carrionii*

Antibacterial activities of the crude metabolites of *Cladosporium carrionii* were tested against the pathogenic bacteria (*Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Salmonella typhi, Shigella flexneri* and *Staphylococcus aureus*), the results have shown in figure 4.18 & 4.19. Different dose concentrations inhibited the growth with different zone however, a dose from 1 and 10 µg/mL showed no zone of inhibition and all the pathogenic species survived. A dose of 20 µg/mL inhibited the growth of only *B. subtilis* by 2.0% . Similarly a dose 50 µg/mL inhibited the growth of *E. coli* (5%) and *B. subtilis* and *S. typhi* (6%) each. Then a dose 100 µg/mL inhibited the growth of *K. pneumoniae* (4%), *S. flexneri* (6%), *S. typhi* (11%), *E. coli* (12%) and *B. subtilis* (17%) respectively. Similarly a dose 250

µg/mL inhibited the growth almost all the species *B. subtilis* (34%), *E. coli* (25%) and *S. typhi* (24%). However, a higher does 500 µg/mL inhibited the growth of *B. subtilis* (66%), *S. typhi* (59%) respectively, while good activity was shown against *E. coli* (51%). However, low activity was against *S. aureus* (30%), *K. pneumoniae* (27%) and *S. flexneri* (22%) respectively.

The first three dose concentrations of *n*–Hexane fraction i.e. from 1 to 20 µg/mL have shown no inhibition activity against any of the pathogenic bacteria while a dose of 50 and 100 µg/mL slightly inhibited the growth of *S. flexneri* by 8% and 13% respectively. However, a dose 250 µg/mL inhibited the growth of *S. flexneri* (25%) and *S. typhi* (21%), whereas, a dose 500 µg/mL inhibited the growth of *S. flexneri* (52%), *S. typhi* (40%) and *E. coli* (22%) and no inhibition were recorded against *S. aureus* and *K. pneumoniae*.

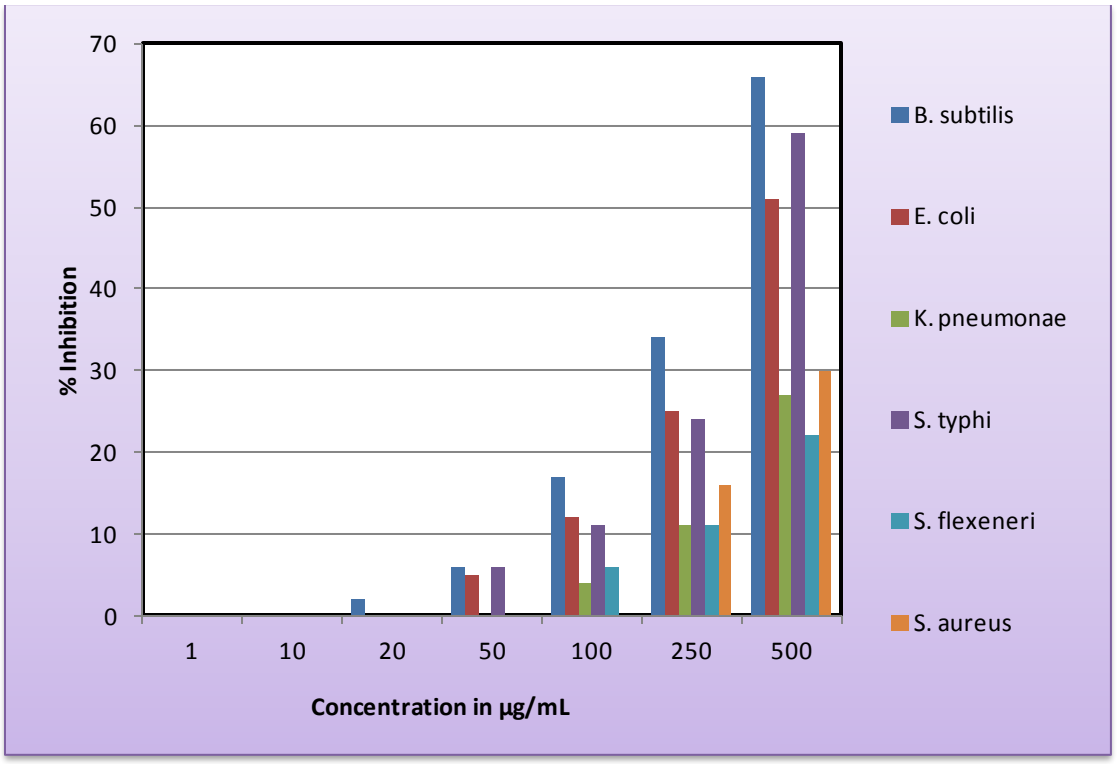



**Figure 4.18 Antibacterial activities of ethyl acetate extract of C. carrionii.**

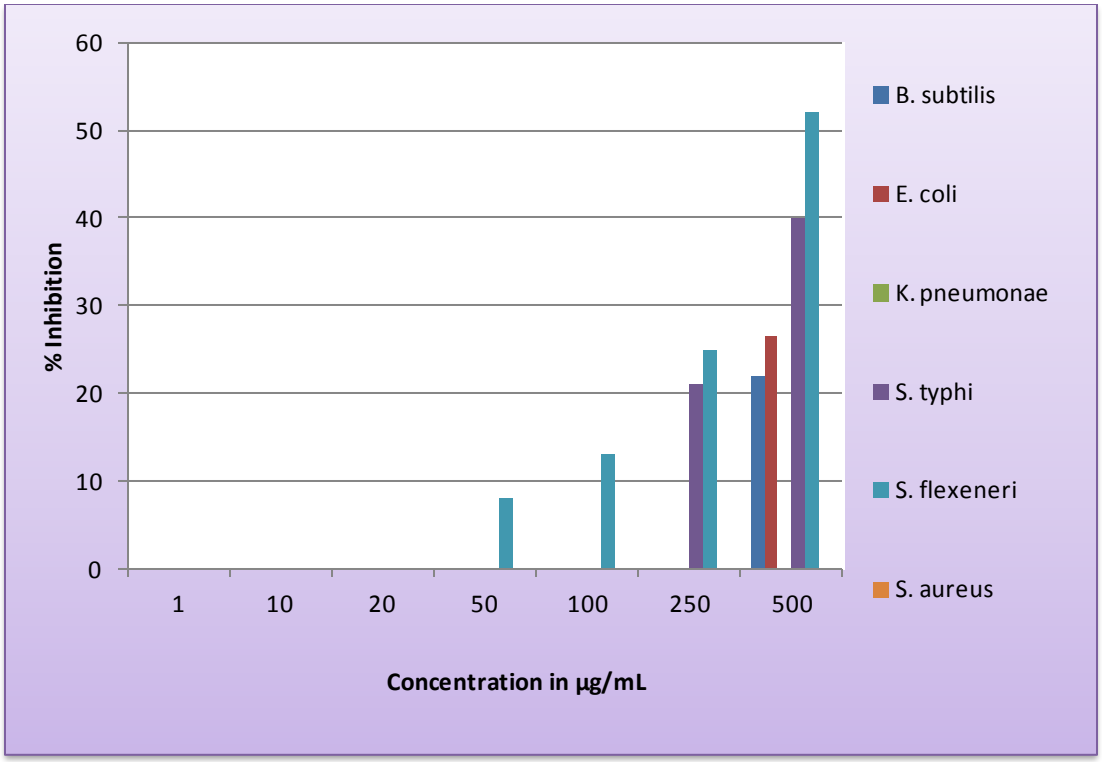



**Figure 4.19 Antibacterial activities of n–Hexane fraction of C. carrionii**

### 4.3.3 Antifungal activities *Cladosporium carrionii*

Antifungal activities of the crude metabolites of *Cladosporium carrionii* were tested against the pathogenic fungi (*Aspergillus flavus, Candida albicans, Candida glabrata, Fusarium solani, Microsporum canis* and *Trichophyton longifusus*), the results have shown in figure 4.20 & 4.21. Different dose concentrations inhibited the growth with different zone but a dose of 10, 20 & 50 µg/mL showed no linear inhibition and all the pathogenic species survived. Then a dose 100 µg/mL inhibited the growth only *C. glabrata* (5%). Then a dose 250 µg/mL inhibited the linear growth of *A. flavus, C. albicans* and *C. glabrata* (10, 12 and 16%) respectively, no linear inhibition observed against *M. canis, F. solani* and *T. longifusus*. While a dose 500 µg/mL inhibited the linear growth of *C. albicans* (30%), *C. glabrata* (26%), *A. flavus* (25.5%), while *M. Canis, T. longifusus* and *F. solani* by 14, 13 and 7% respectively. However, a higher dose 1000 µg/mL inhibited the growth of *C. albicans* (60%), *C.*

*glabrata* and *A. flavus* (54 and 47%) also *T. longifusus* and *M. canis* (28, 36%) respectively and *F. solani* by 12%.

The first five dose concentrations of *n*–Hexane fraction i.e. from 10 to 250 µg/mL showed no inhibition activity against any of the pathogenic fungi except *F. solani* (7%). However, 500 µg/mL inhibited the linear growth of *F. solani* (13%) and *C. glabrata* (16.5%) and 1000 µg/mL inhibited the linear growth of *C. glabrata* (52%), low activities against *C. albicans* (36.5%) and *F. solani* (25%) and *A. flavus* (19%) respectively, while no linear inhibition observed against *M. canis* and *T. longifusus*.

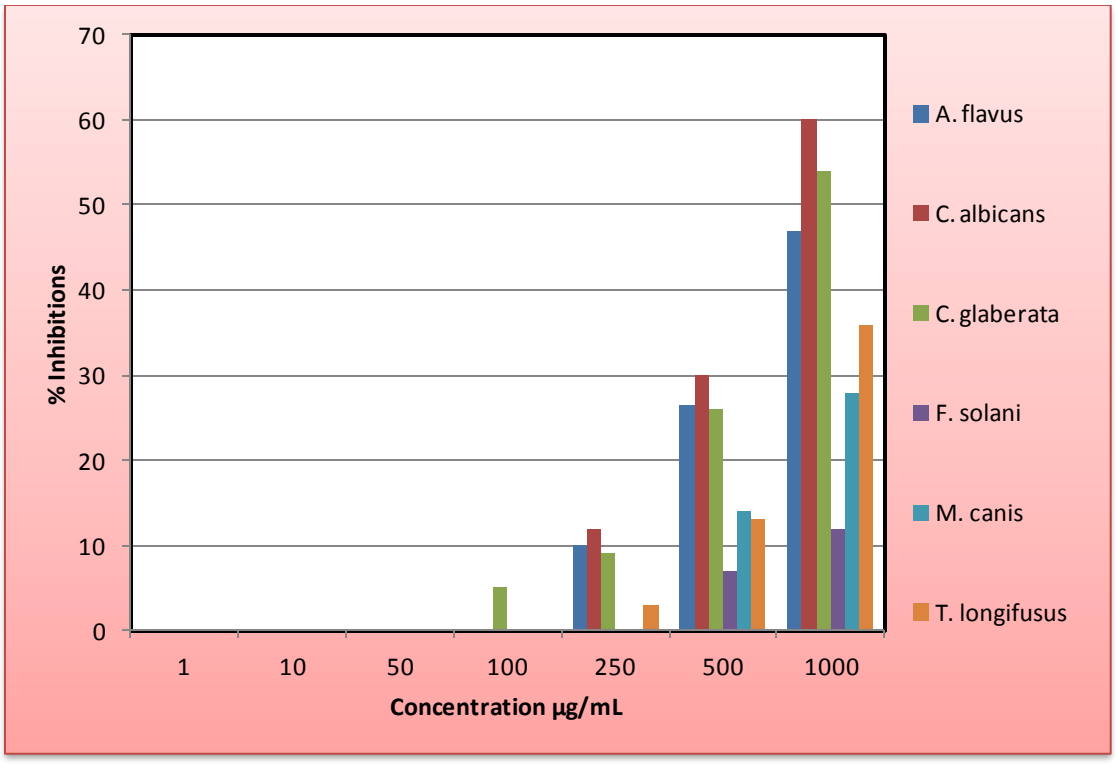



**Figure 4.20 Antifungal activities of ethyl acetate extract of C. carrionii.**

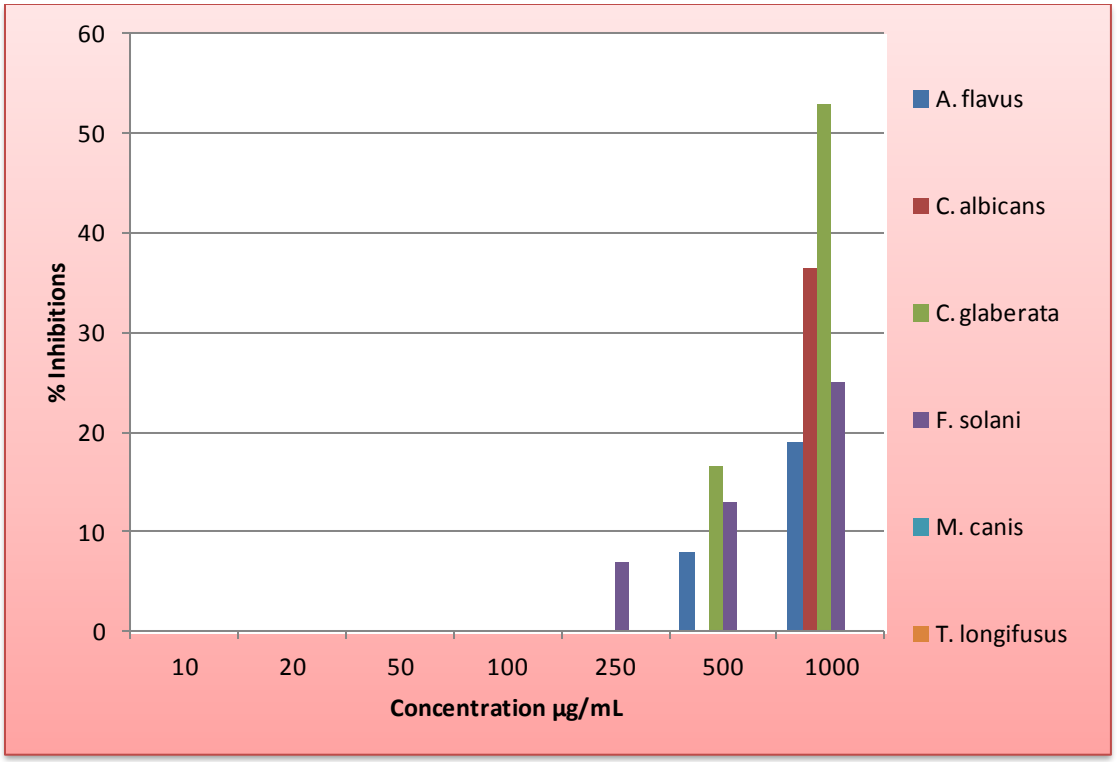



**Figure 4.21 Antifungal activities of n–Hexane fraction of C. carrionii.**

### 4.3.4 Cytotoxic activities of *Cladosporium carrionii*

Cytotoxic activities of the crude metabolites of *Cladosporium carrionii* were tested against (b*rine shrimps*), the results have shown in figure 4.22. A total of three dose concentrations 10, 100 and 1000 µg/mL was used to measure the toxicity of the metabolites. Dose concentrations of 10, 100 and 1000 µg/mL showed 13, 63 and 87% mortality respectively, whereas, a dose of 10, 100 and 1000 µg/mL of *n*–Hexane fraction showed 15, 68 and 90% mortality respectively. Both the ethyl acetate and *n*–Hexane displayed very low $LD_{50}$ value i.e. 304.59 and 258.37 respectively.

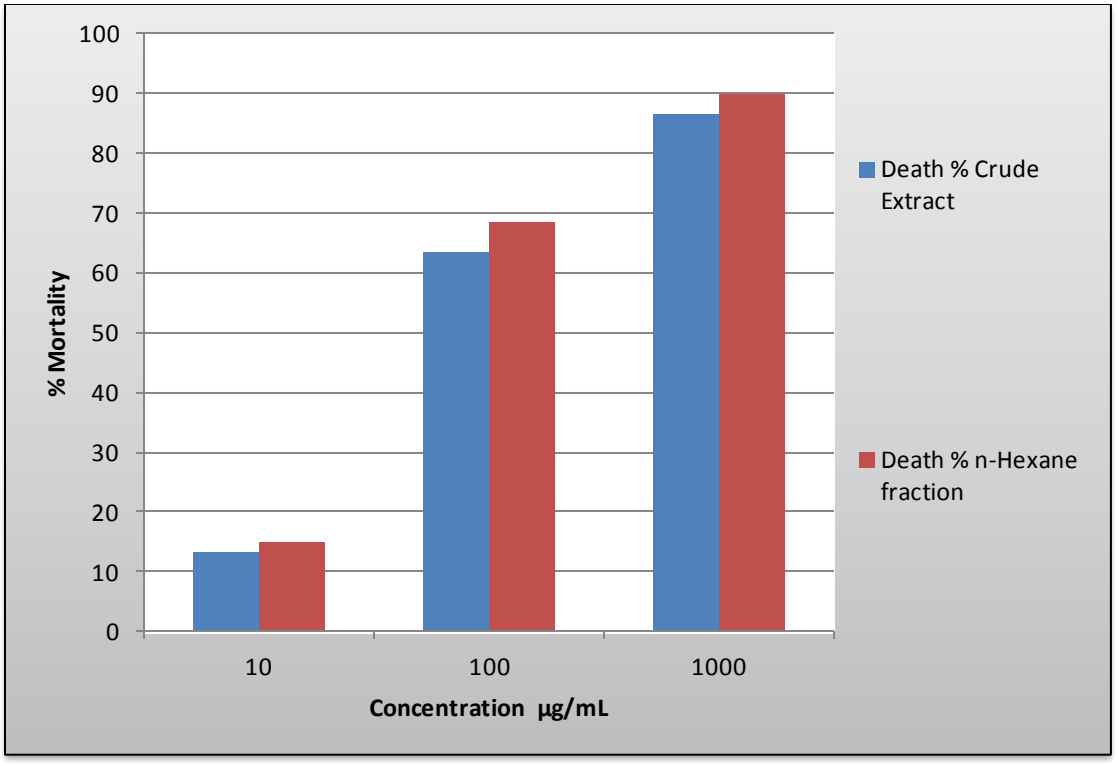



**Figure 4.22 Cytotoxic activities of ethyl acetate extract & n–Hexane fraction of *C. carrionii*.**

### 4.3.5 Phytotoxic activities of *Cladosporium carrionii*

Phytotoxic activities of the crude metabolites of *Cladosporium carrionii* were tested against (*Lemna*), the results have shown in figure 4.23. A total of three dose concentrations 10, 100 and 1000 µg/mL was used to measure the phytotoxicity of the metabolites. Dose concentration of 10, 100 and 1000 µg/mL showed 23, 57 and 80% mortality respectively, whereas, a dose of 10, 100 and 1000 µg/mL of *n*–Hexane fraction showed 37, 63 and 87% mortality respectively. Both the ethyl acetate and *n*–Hexane displayed very low $LD_{50}$ value i.e. 390.71 and 229.97 respectively.

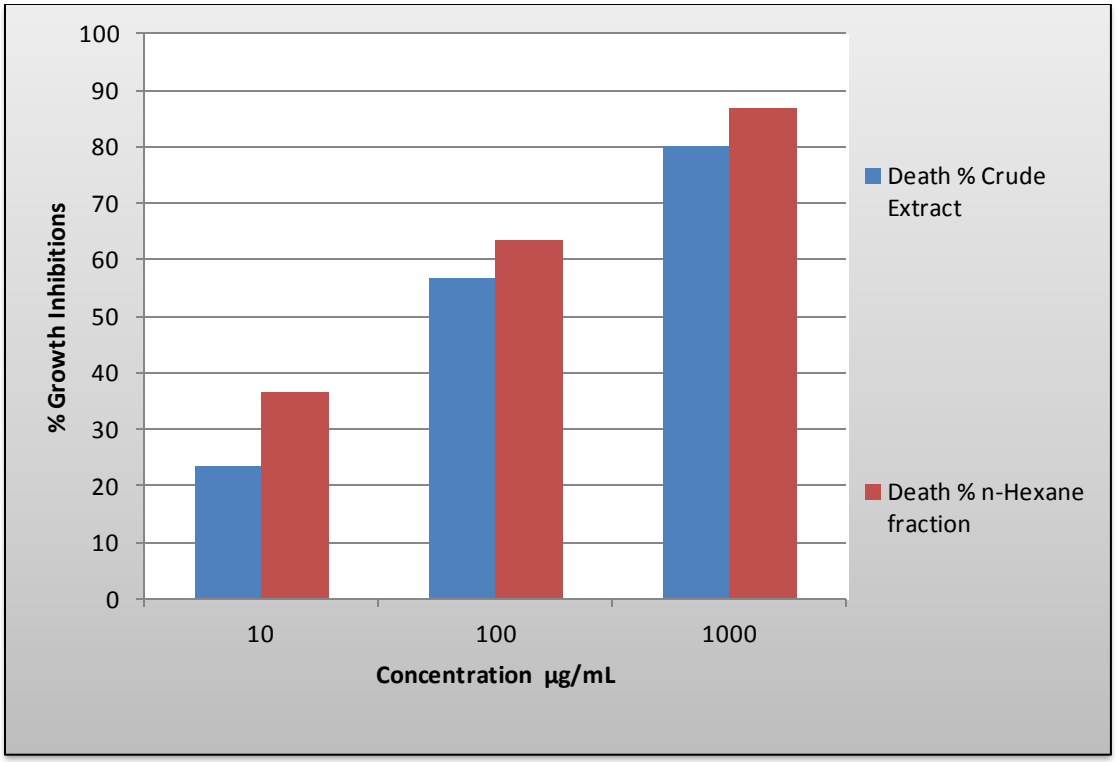



**Figure 4.23 Phytotoxic activities of ethyl acetate extract & n–Hexane fraction of *C. carrionii*.**

### 4.4.1 Optimization of growth media for *Cladosporium resinae*

Optimization of media is an important factor for the growth and maximum production of metabolites therefore; five different media were used. The results have shown in figure 4.24. *C. resinae* produced (10 mg) in Czapek–dox Broth (CB) on 3$^{rd}$ day, with gradual increase while maximum amount (30, 35 and 45 mg) were produced on 6$^{th}$, 7$^{th}$, and 8$^{th}$ days respectively. While, (30, 25 and 26 mg) were produced in Yeast Extract Sucrose (YES), Potato Dextrose Broth (PDB) and Czapek–dox (supplemented with glucose & starch) Broth (CGSB) on 8$^{th}$ day respectively, therefore these media were observed ordinary medium for the production of metabolites. However, Czapek Yeast–extract Broth (CYB) was observed comparatively a better media for *C. carrionii*, for the maximum production of metabolites. *C. resinae* produced 6 mg on 3$^{rd}$ day, then an irregular increase were observed in the production metabolites (35, 40, 45 and 40 mg) on 4$^{th}$, 5$^{th}$, 6$^{th}$ and 7$^{th}$ days respectively, whereas, maximum amount (60 and 65 mg) were produced on both 8$^{th}$ and 9$^{th}$ days, therefore, (CYB) were recorded as the optimum media for *C. resinae*.

The highest amount of secondary metabolites with most media type was produced on 9$^{th}$ day and the production was highest (65 mg) for CYB media type as compared to CB (45 mg), CGSB (26 mg), PDB (25 mg), YES (30 mg) and therefore, CYB media type was recorded as the optimum media for *C. resinae*.

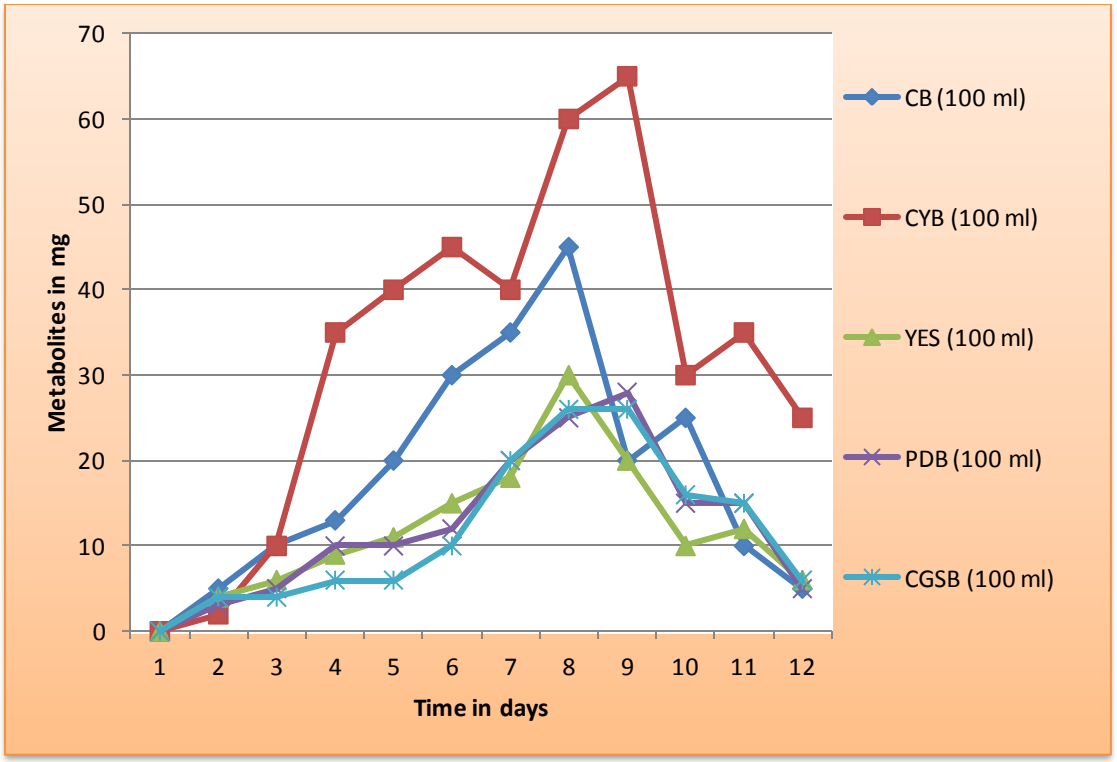



**Figure 4.24 Optimization of media for production of secondary metabolites, CB= Czapek–dox Broth, CYB= Czapek Yeast–extract Broth, YES= Yeast Extract Sucrose, PDB= Potato Dextrose Broth and CGSB= Czapek–dox (supplemented with glucose & starch) Broth.**

### 4.4.2 Use of epigenetic modifiers for *Cladosporium resinae*

Two epigenetic modifiers suberoyl anilide hydroxamic acid (SAHA) and 5–azacytidine (5–AZA) were used in Czapek–dox (Supplemented with Glucose & Starch) Broth (CSGB) media and the results have shown in figure 4.25. Different concentrations (1, 5, 10, 15 and 20 µM/100 mL) of both the modifiers were used for *C. resinae*.

A dose of 15 µM of SAHA resulted in increased secondary metabolites production by 10 mg, gradual increasing the amount from 35 to 45 mg on 4$^{th}$ day. Also a gradual increase in the metabolites productions (15, 30 and 50 mg) were observed by gradual increasing the amount from 40 to 55, 45 to 75 and 40 to 90 mg on 5$^{th}$, 6$^{th}$ and 7$^{th}$ day respectively, whereas the highest amount (110 mg) was

observed on 8$^{th}$ day with an increase of 50 mg by gradual increasing the amount from 60 to 110 mg and then a gradual decrease.

Therefore, it was found that a dose 10 µM/100 mL of SAHA was observed an effective modifier for the increase of secondary metabolites production. Never the less, a dose of 15 µM/100 mL of 5–AZA resulted in increased secondary metabolites production by 15 mg, gradual increasing the production from 35 to 50 mg on 4$^{th}$ day.

Then fast increase in the metabolites productions (28, 35 and 45 mg) were observed by gradual increasing the amount from 40 to 68, 45 to 80 and 40 to 85 mg on 5$^{th}$, 6$^{th}$ and 7$^{th}$ days respectively. Whereas, the highest amount (100 mg) was observed on 9$^{th}$ day with an increase of 35 mg by gradual increasing the amount from 65 to 100 mg and then a gradual decrease.

It was found that both epigenetic modifiers produced the highest amount of secondary metabolites on day 8 with SAHA at 15 µM/100 mL concentration producing marginally higher amount of secondary metabolites (110 mg) than 5–AZA at 10 µM/100 mL concentration (100 mg). Therefore, the SAHA at 15 µM/100 mL concentration was considered as the most effective modifier for *C. resinae*.

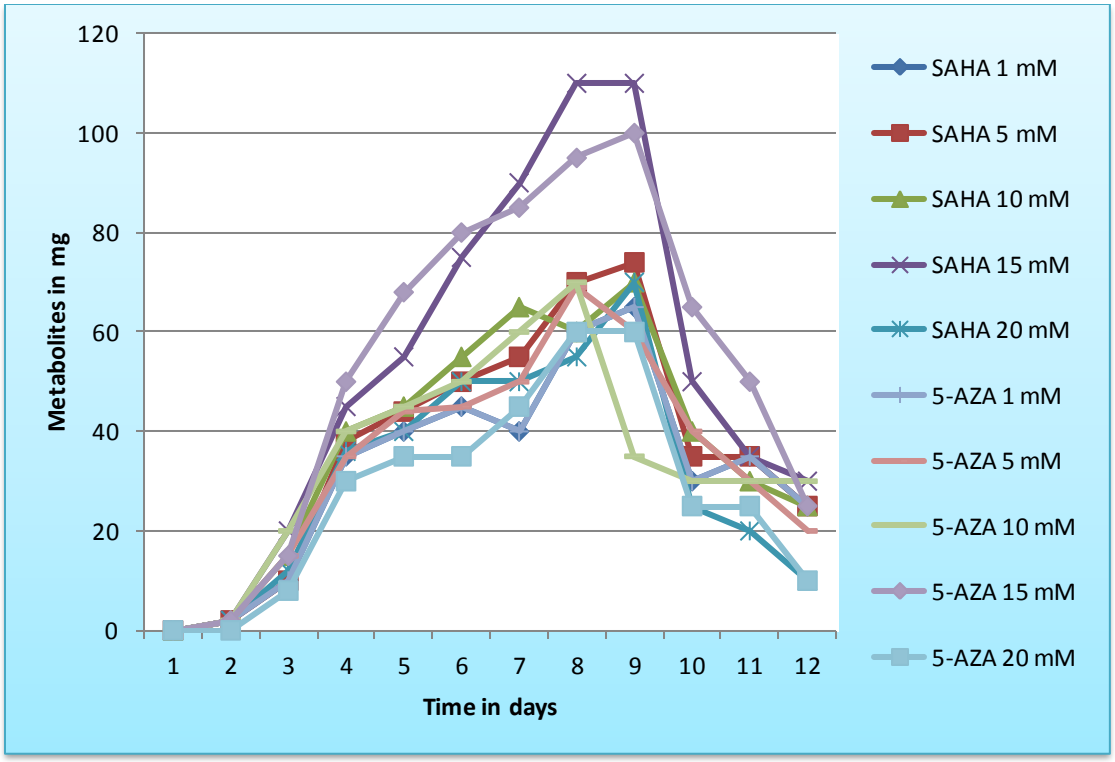



**Figure 4.25 Epigenetic modifier (suberoyl anilide hydroxamic acid (SAHA) and 5–azacytidine (5–AZA)) were used in (CYB) media for the expression of the silent genes for their metabolites production.**

### 4.4.3 Antibacterial activities of *Cladosporium resinae*

Antibacterial activities of the crude metabolites of *Cladosporium resinae* (NRRL–6437) were tested against the pathogenic bacteria (*Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Salmonella typhi, Shigella flexneri* and *Staphylococcus aureus*), the results have shown in figure 4.26 & 4.27. Different dose concentrations inhibited the growth with different zone however, a dose from 1 and 10 µg/mL showed no zone of inhibition and all the pathogenic species survived. Then a dose of 50 µg/mL inhibited the growth of *E. coli* and *S. typhi* (5.5 and 6.5%) respectively and *S. aureus* by 13% and 100 µg/mL inhibited the growth of all the species *B. subtilis, E. coli, K. pneumoniae, S. typhi, S. flexneri* and *S. aureus* (8, 18, 7, 15.5, 10 and 27%) respectively. While a dose of 250 µg/mL inhibited *B. Subtilis* (20.5%), *E. coli* (37%), *K. Pneumoniae* (25%), *S. typhi* (33.5%), *S. flexneri* (26.5%)

and *S. aureus* (47%). However, a higher does 500 µg/mL inhibited the growth of *S. aureus* (81%), *E. coli* and *S. typhi* (73.5 & 79%) respectively, whereas *K. Pneumoniae*, *S. flexneri* and *B. subtilis* (51, 59.5 & 44.5%) respectively.

The first four dose concentrations of *n*–Hexane fraction i.e. from 1 to 50 µg/mL showed no zone of inhibition activity against any pathogenic bacteria. However, 100 and 250 µg/mL inhibited the growth of *B. Subtilis* (11 and 32%) respectively and 500 µg/mL inhibited the growth *B. subtilis* (61%), *S. flexneri* (51%), *S. aureus* (42.5%), and *E. coli* (37%).

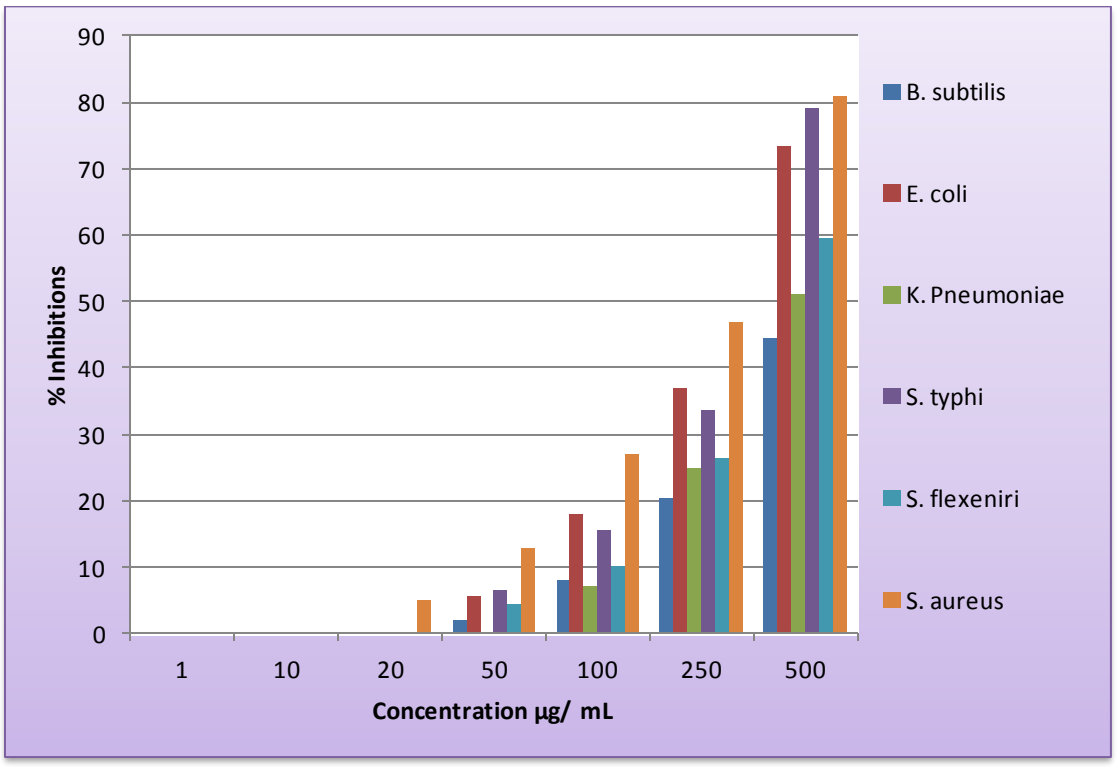



**Figure 4.26 Antibacterial activities of ethyl acetate extract of *C. resinae* (NRRL–6437)**

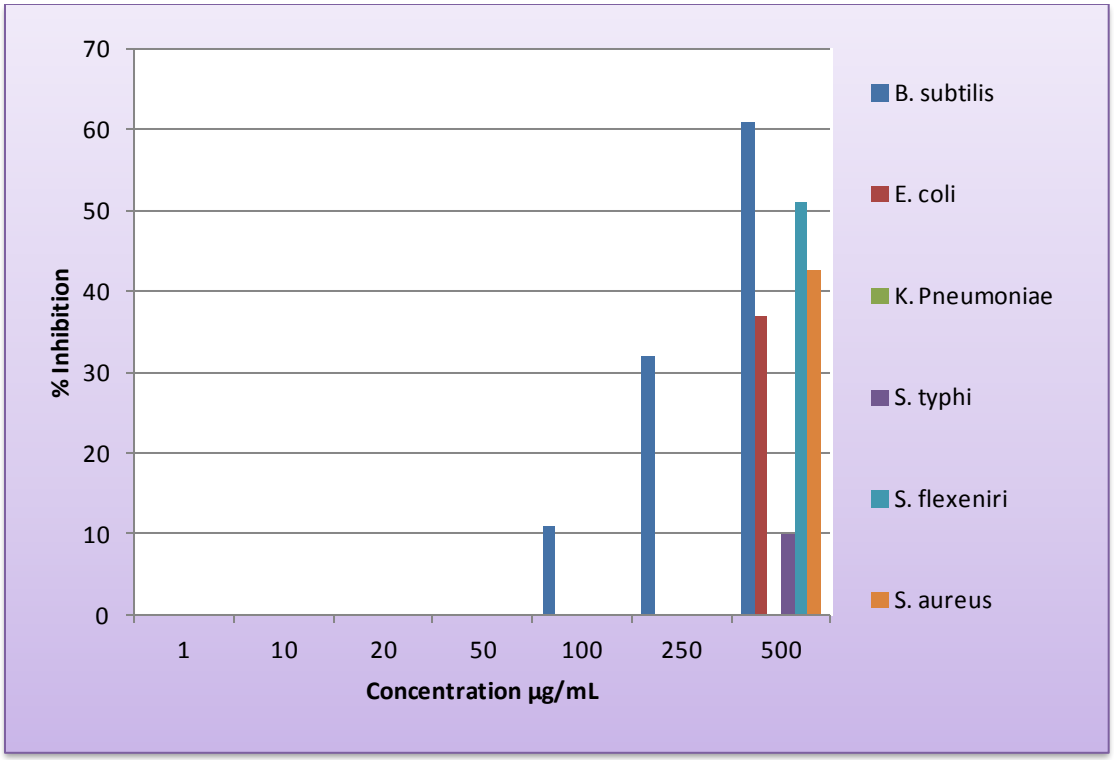



**Figure 4.27 Antibacterial activities of *n*–Hexane fraction of *C. resinae* (NRRL–6437).**

### 4.4.4 Antifungal activities of *Cladosporium resinae*

Antifungal activities of the crude metabolites of *Cladosporium resinae* (NRRL–6437) were tested against the pathogenic fungi (*Aspergillus flavus*, *Candida albicans*, *Candida glabrata*, *Fusarium solani*, *Microsporum canis* and *Trichophyton longifusus*), the results have shown in figure 4.28 & 4.29. Different dose concentrations inhibited the growth with different zone but a dose of 10, 20, 50 & 100 µg/mL showed no linear inhibition and all the pathogenic species survived. A dose 250 µg/mL inhibited the linear growth of F. solani by 6%. Then a dose 500 µg/mL inhibited the growth of A. flavus and F. solani (8 and 11%) respectively, similarly, 1000 µg/mL inhibited the growth of A. flavus and F. solani (15 and 23%) respectively.

The first four dose concentrations of *n*–Hexane fraction i.e. from 10 to 100 µg/mL showed no linear growth inhibition against any of the pathogenic fungi, except

*C. glabrata* (5%). A dose of 250 µg/mL inhibited the linear growth of *A. flavus, C. albicans, C. glabrata and T. longifusus* (9, 9, 11, 2 and 6%) respectively. Then a dose of 500 µg/mL inhibited the linear growth of *A. flavus* (16%), *C. albicans* (20%), *C. glabrata* (25%), *M. canis* (13%) and *T. Longifusus* (13%) while 1000 µg/mL of n–Hexane also showed inhibitions against the same pathogens, *A. flavus* (33%), *C. albicans* (45%), *C. glabrata* (52%), *M. canis* (35.5%) and *T. Longifusus* (26%).

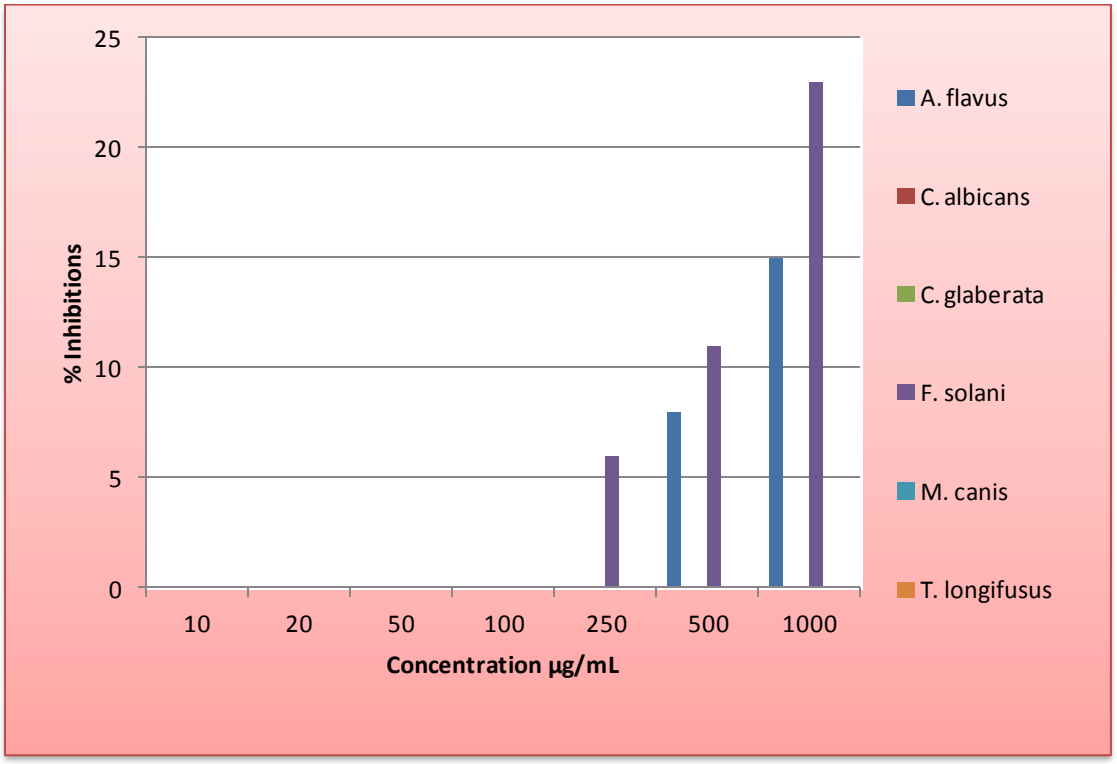



**Figure 4.28 Antifungal activities of ethyl acetate extract of *C. resinae* (NRRL–6437)**

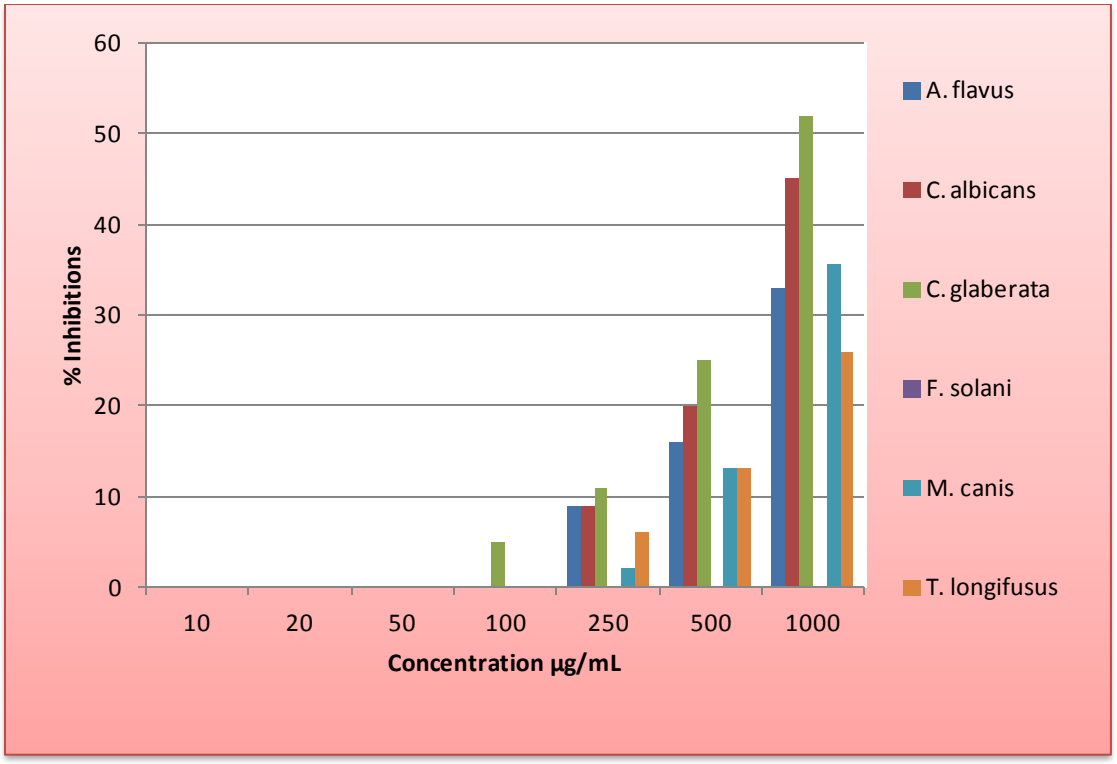



**Figure 4.29 Antifungal activities of *n*–Hexane fraction of *C. resinae* (NRRL–6437)**

### 4.4.5 Cytotoxic activities of *Cladosporium resinae*

Cytotoxic activities of the crude metabolites of *Cladosporium resinae* (NRRL–6437) were tested against (b*rine shrimps*), the results have shown in figure 4.30. A total of three dose concentrations 10, 100 and 1000 µg/mL was used to measure the toxicity of the metabolites. Dose concentrations (10, 100 and 1000 µg/mL) showed 33, 73 and 93% mortality respectively, whereas the same concentrations of *n*–Hexane fraction showed 25, 68 and 90% mortality respectively. Both the ethyl acetate and *n*–Hexane displayed very low $LD_{50}$ value i.e. 38.93 and 82.54 respectively.

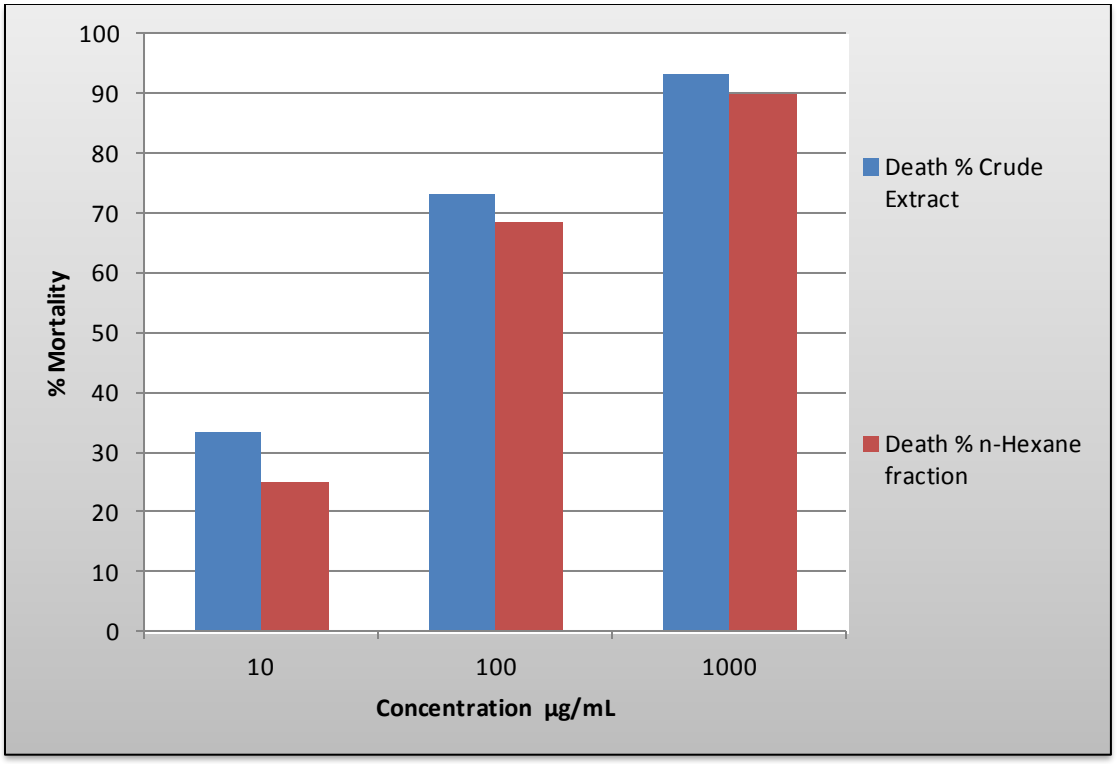



**Figure 4.30 Cytotoxic activities of ethyl acetate extract & *n*–Hexane fraction of *C. resinae* (NRRL–6437).**

### 4.4.6 Phytotoxic activities of *Cladosporium resinae*

Phytotoxic activities of the crude metabolites of *Cladosporium resinae* (NRRL–6437) were tested against (*Lemna*), the results have shown in figure 4.31. A total of three dose concentrations 10, 100 and 1000 µg/mL was used to measure the phytotoxicity of the metabolites. Dose concentrations (10, 100 and 1000 µg/ mL) showed 27, 50 and 80% mortality respectively, whereas the same concentrations of *n*–Hexane fraction showed 40, 60 and 87% mortality respectively. Both the ethyl acetate and *n*–Hexane displayed very low $LD_{50}$ value i.e. 119.33 and 35.53 respectively.

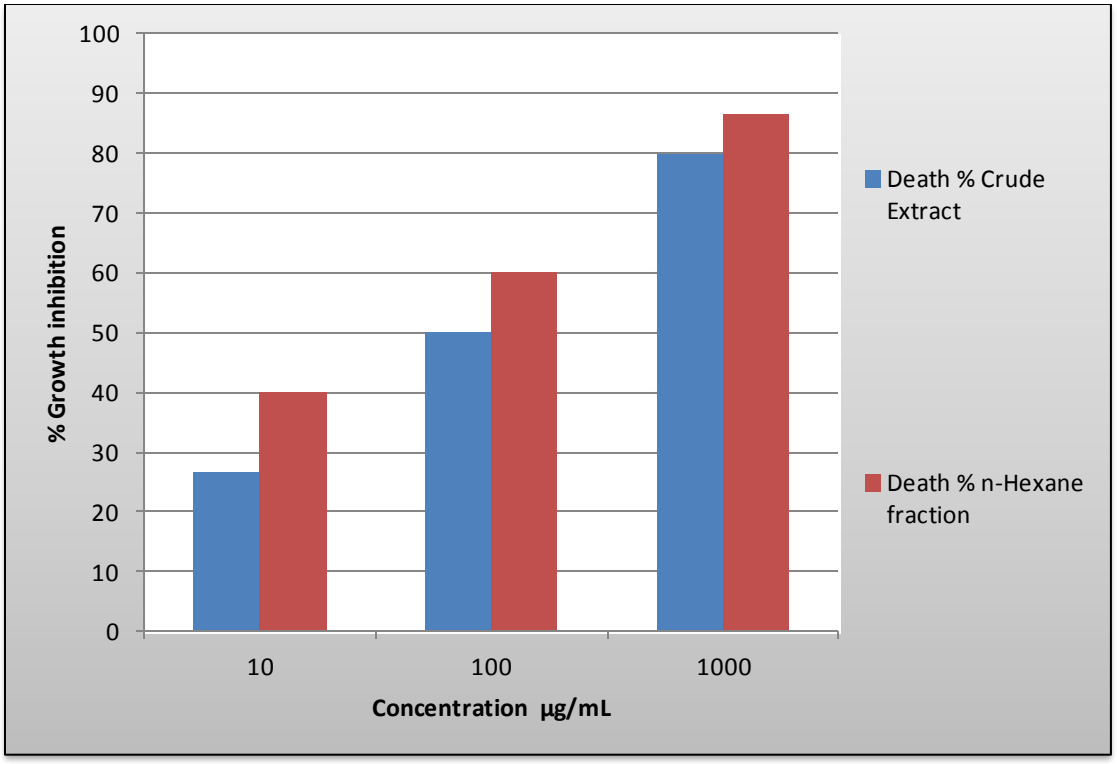



**Figure 4.31 Phytotoxic activities of ethyl acetate extract & *n*–Hexane fraction of *C. resinae* (NRRL–6437).**

The test samples showed different inhibitions. *A. oryzae* (tenS.PKS/dmbC) and *C. carrionii* displayed greater inhibitions against the experimental organisms as compared to *A. carbonarius* and *C. resinae*. Therefore, based on the biologically activities of the crude extracts from the two fungal species *A. oryzae* (tenS.PKS/dmbC) and *C. carrionii* were further selected for the isolation of secondary metabolites, the results have been summarized in table 4.1.

**Table 4.1 Different biological activities of the four fungi**

| Fungi | Sample | Antibacterial Activities | | | Antifungal Activities | | | Cytotoxic Activities | | | Phytotoxic Activities | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | L | M | H | L | M | H | L | M | H | L | M | H |
| *Aspergillus carbonarius* (NRL-369) | Ethyl acetate | | ✓ | | ✓ | | | | | ✓ | | ✓ | |
| | n-Hexane | ✓ | | | ✓ | | | | ✓ | | | ✓ | |
| *Aspergillus oryzae* (tenS.PKS/dmbC) | Ethyl acetate | | | ✓ | | | ✓ | ✓ | | | ✓ | | |
| | n-Hexane | ✓ | | | ✓ | | | ✓ | | | ✓ | | |
| *Cladosporium carrionii* (F-2) | Ethyl acetate | | | ✓ | | ✓ | | | ✓ | | ✓ | | |
| | n-Hexane | | ✓ | | ✓ | | | | ✓ | | | ✓ | |
| *Cladosporium resinae* (NRL-6437) | Ethyl acetate | ✓ | | | | | | | | ✓ | | ✓ | |
| | n-Hexane | | ✓ | | ✓ | | | | ✓ | | | | ✓ |

**Note: L=Low, M= Medium and H= High.**

# CHAPTER 5

# DISCRIPTION OF METABOLITES

# 5 DISCRIPTION OF METABOLITES

After the detail biological work conducted in laboratory and explained in previous chapter, summarized in table 4.1, have been used as tool for the direction of exploitation of fungi for biologically active secondary metabolites. Therefore *A. oryzae* (*ten*S.PKS/*dmb*C) and *C. carrionii* (F–2) were selected for the isolation of secondary metabolites (compounds). Based on the "bioassay guided isolation" and our hypothesis that the (tenS.PKS/dmbC) might produce the straight chain polyketides with mass of $M[H]^+$ 211, $M[Na]^+$ 233 in $ES^+$ and 209 in $ES^-$ as shown in figure 5.1.

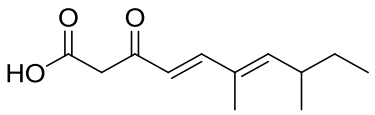



**Figure 5.1 Proposed metabolites (40) from the heterologous expressed *A. oryzae.***

However, the heterologous expression of (*tenS* and *dmbC*) in *A. oryzae*, resulted incorrectly programmed compounds. Therefore, different peaks were isolated and forwarded for antibacterial testing. Mean while two peaks in the extract of *A. oryzae* were remained the most interesting by showing high inhibition against *B. subtilis* and *E. coli*. The peak **A** was eluted at 22.7 retention time (rt) and peak **B** was eluted just after the peak **A** at 23.3 retention time (rt) as shown in figure 5.2. The peak **A** showed mass of $M[H]^+$ 213 and $M[Na]^+$ 235 in $ES^+$ while 211 in $ES^-$ as shown in figure 5.3, while the peak **B** showed mass of $M[H]^+$ 211, $M[Na]^+$ 233 in $ES^+$ and 209 in $ES^-$ as shown in figure 5.6. Both these metabolites did not show strong UV absorption, however the UV spectra showed, UV λ max (MeOH) = 210 nm for peak **A** and λ max (MeOH) = 230 nm for peak **B**.

The peak **B** created more interest because the mass of that peak was exactly the same as we were expecting from that transformant. However these compounds were produced in less quantity. Therefore, we purified 5.5 mg of $M[H]^+$ 213 and 5 mg of $M[H]^+$ 211 from a total of 350 mg of crude extract by preparing 50 mg/mL concentration solution of the crude extract in (HPLC) grade methanol for the purification of the desire metabolites. About 28 runs, each of 30 minutes in methanol/water system (40–80%) program were carried out. The HPLC chromatogram of *A. oryzae* has shown in figure 5.2. whereas, the extract of *C. carrionii* were straight away transfered to HPLC for isoaltion based on their prelimenary biassy seceenings. The chromatogram of the extract of *C. carrionii* has shown in figure 5.9. Same like *A. oryzae* the two peaks in the extract of *C. carrionii* were shown interest by inhibiting the *B. subtilis* and *E. coli*. These peaks were called as **C** and **D**. The **C** was eluted at 4.02 retention time (rt) and peak **D** was eluted after the peak **C** at 5.89 retention time (rt) as shown in figure 5.9. The peak **C** showed mass of $M[H]^+$ 143 in $ES^+$ while 141 in $ES^-$ as shown in figure 5.10, while the peak **D** showed mass of $M[H]^+$ 245, $M[Na]^+$ 267 in $ES^+$ and 243 in $ES^-$ as shown in figure 5.12. Both these metabolites showed UV absorption, which showed, UV λ max (MeOH) = 221 and 266 nm for peak **C** and λ max (MeOH) = 258 and 264 nm for peak **D**.

## 5.1 Chromatogram of *Aspergillus oryzae*

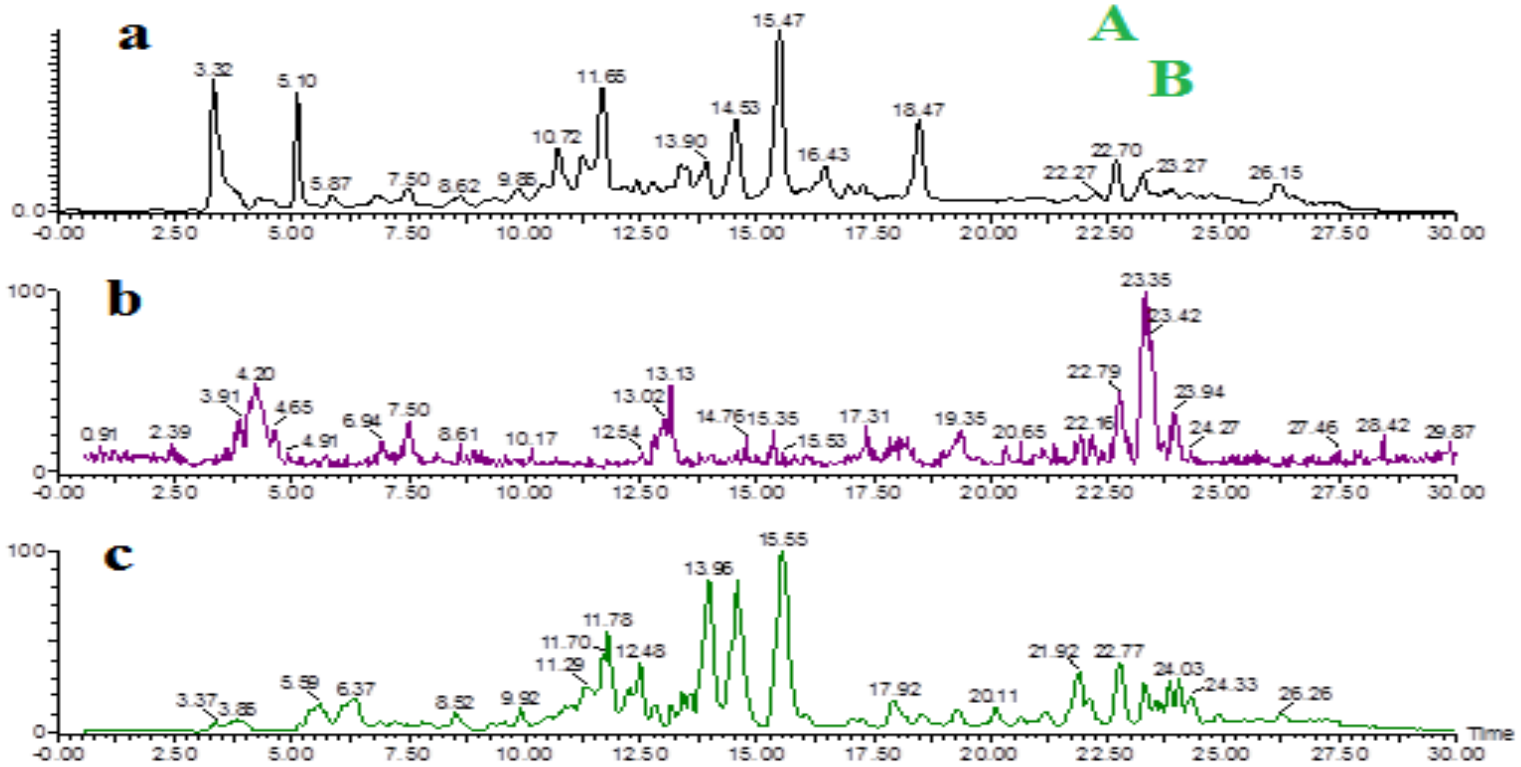



**Figure 5.2 LCMS chromatogram of *A. oryzae* (tenS.PKS/dmbC) showing UV profile of the extract (trace a), $ES^-$ profile of the extract (trace b) and $ES^+$ profile of the extract (trace c). While A and B are the targeted compounds eluted at 22.77 and 23.35 rt respectively.**

### 5.1.1 Chromatogram of new compound (A–41)

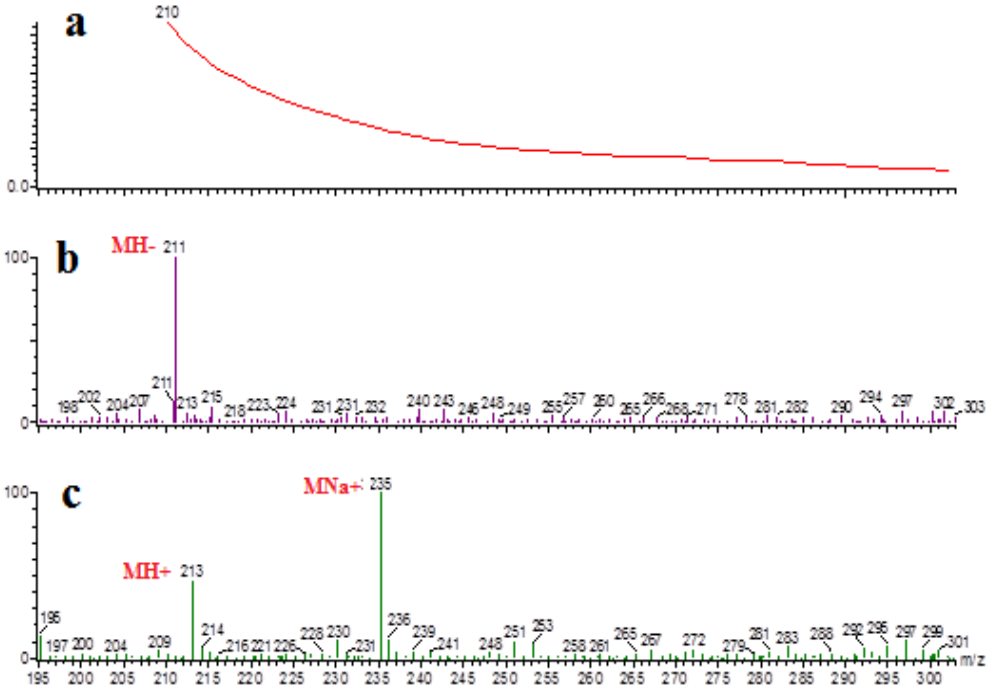



**Figure 5.3 LCMS chromatogram of *A. oryzae* (tenS.PKS/dmbC) showing UV profile of the extract (trace a), $ES^-$ profile of the extract (trace b) and $ES^+$ profile of the extract (trace c) of compound (A–41).**

#### 5.1.1.1 New compound (A–41)

The molecular formula of the new compound was established by HR–ESI–MS and $^{13}C$ NMR (Broad Band and DEPT). HR–ESI–MS showed pseudo molecular ion peaks [$M^+$] at *m/z* 213.1129 [M]$H^+$ and 235.0949 [M]$Na^+$ corresponding to the molecular formula $C_{11}H_{16}O_4$ [Calculated as $C_{11}H_{16}O_4$ + H = 213.1129]. The IR spectrum indicated the presence of an amide carbonyl (1660 $cm^{-1}$) and benzene ring (1625 and 1391$cm^{-1}$). The absorption maxima in the UV spectrum (258 and 264 nm) also exhibited the presence of a benzene ring.

Then 5.5 mg of pure compound (**41**) was dissolved in 0.70 mL of deutrated methanol ($CD_3OD$). A number of 1D and 2D NMR experiments were carried out on 500 MHz NMR Spectrometer for the structure elucidation.

$^1H$–NMR ($CD_3OD$, 500 MHz) showed 11 signals for protons. There was a methyl triplet at the right end of the spectrum at δ 0.95 giving integration of 3 protons and then two signals gave an integration of 2 protons each at δ 1.40 and 1.48 corresponds to $CH_2$. All the rest of proton signals gave integration of one proton each.

The 1D $^{13}C$ NMR showed 11 signals corresponds to 11 carbons skeleton of the compound showing 1 quaternary carbon at δ 138.59 while 2 carbonyl carbons at δ 168.31 and 179.11 respectively. The 2D HSQC presented 8 protonated carbons as shown in table 5.1. The 2D HMBC and $^1H$–$^1H$ COSY disclosed many of the correlations and the elucidated structure from these data as shown in figure 5.4.

**Table 5.1** $^{1}H$ and $^{13}C$–NMR and Chemical Shifts of Compound (**41**) (ppm, $CD_3OD$, 500)

| C. No | Multiplicity (DEPT) | $^{13}C$–NMR | $^{1}H$ ($J$ = Hz) |
|---|---|---|---|
| **1** | $CH_3$ | 14.27 | 0.95 t ($J$ = 5.0, 10 Hz) |
| **2** | $CH_2$ | 23.54 | 1.40 m |
| **3** | $CH_2$ | 28.80 | 1.48 m |
| **4** | $CH_2$ | 36.22 | 1.68, 1.79 m |
| **5** | CH | 80.99 | 4.50 m |
| **6** | $CH_2$ | 36.14 | 2.07, 2.52 m |
| **7** | CH | 46.74 | 3.76 d ($J$ = 5.0 Hz) |
| **8** | –C– | 138.59 | ---------- |
| **9** | C=O | 168.31 | ---------- |
| **10** | $CH_2$ | 130.06 | 5.87, 6.36 s |
| **11** | C=O | 179.11 | ---------- |

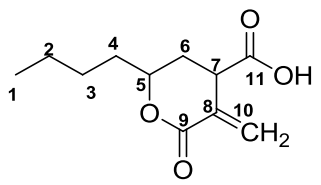



**Figure 5.4 structure of the new compound (41)**

In $^{1}H–^{1}H$ COSY, the protons of C–2 showed correlation with the proton of C–3, also the proton of C–4 with the proton of C–5 and that of C–5 with C–6. While in HMBC, the methyl proton 3H–1 shows two bonds correlation to C–2 and the proton H–2 shows two bonds correlation to C–3. The two protons H–6a and H–6b showed two bond correlations to C–5 and C–7and 3 bond correlations to quaternary C–8, carboxylic group at C–11 and methylene C–4 on the alkane chain. Proton H–7 showed tow bonds correlation to quaternary C–8, carboxylic group at C–11 and three bonds correlation to carboxylic group at C–9. The two alkene protons H–10a and H–10b shows two bonds correlations with C–8, three bonds correlation to C–7 and carbonyl carbon at C–9. The 2D ($^{1}H–^{1}H$ COSY and HMBC) supported that this compound has a six membered ring attached to a four carbon chain of alkane.


**Figure 5.5 COSY correlations have shown by the red arms, while the HMBC correlations have shown by the blue arrows.**

### 5.1.2 Chromatogram of new compound (B–42)

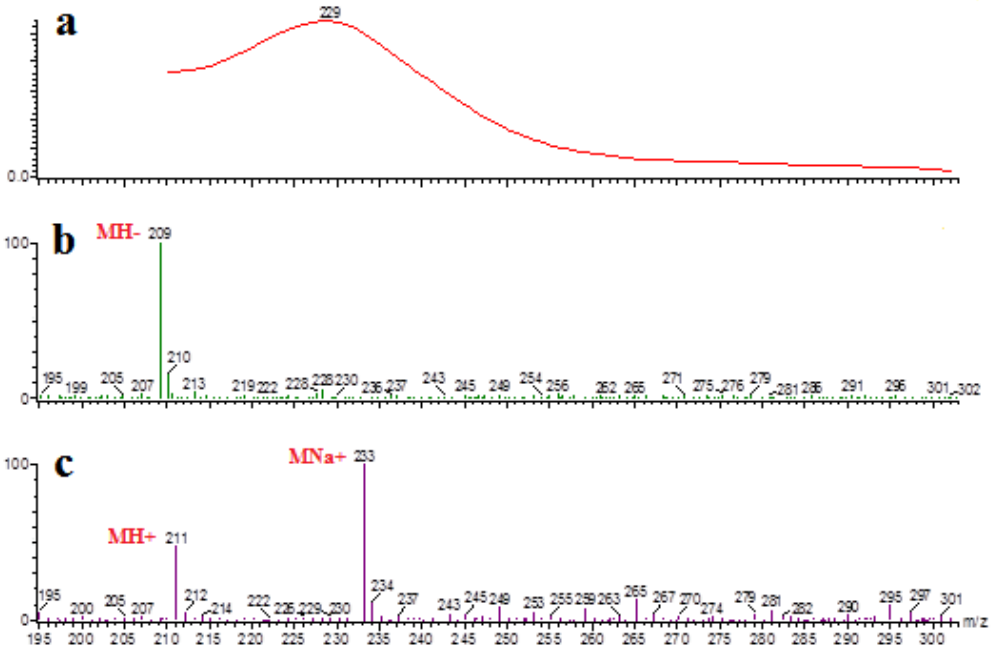



**Figure 5.6 LCMS chromatogram of A. oryzae (tenS.PKS/dmbC) showing UV profile of the extract (trace a), $ES^-$ profile of the extract (trace b) and $ES^+$ profile of the extract (trace c) of compound (B–42).**

**5.1.2.1 New compound (B–42)**

The molecular formula of the new compound was established by HR–ESI–MS and $^{13}C$ NMR (Broad Band and DEPT). HR–ESI–MS showed pseudo molecular ion peak [$M^+$] at *m/z* 233.0801 [M]$Na^+$ corresponding to the molecular formula $C_{11}H_{14}O_4$ [Calculated as $C_{11}H_{14}O_4$ + Na = 233.0801]. The IR spectrum indicated the presence of an amide carbonyl (1660 $cm^{-1}$), and benzene ring (1625 and 1391$cm^{-1}$). The absorption maxima in the UV spectrum (258 and 264 nm) also exhibited the presence of a benzene ring.

The 5 mg of pure compound (**42**) was dissolved in 0.70 mL of deuterated methanol ($CD_3OD$). A number of 1D and 2D NMR experiments were carried out on 500 MHz NMR Spectrometer for structure elucidation.

$^1H$–NMR ($CD_3OD$, 500 MHz) showed the 09 signals for protons. There was a methyl triplet at the right end of the spectrum at δ 0.96 giving the integration of 3 protons and then two signals gave an integration of 2 protons each at δ 1.40 and 1.41 corresponds to $CH_2$. All the rest of proton signals gave integration of one proton each.

The 1D $^{13}C$ NMR showed 11 signals correspond to 11 carbons skeleton of the compound, showing 2 quaternary carbons at δ 126.57 and 131.59 while 2 carbonyl carbons at δ 168.50 and 173.73 respectively. The 2D HSQC presented 7 protonated carbons as shown in table 5.2. The 2D HMBC and $^1H$–$^1H$ COSY disclosed many of the correlations and the elucidated structure as shown in figure 5.7.

**Table 5.2** $^{1}H$ and $^{13}C$–NMR and Chemical Shifts of Compound (**42**) (ppm, $CD_3OD$, 500 MHz)

| **C. No** | **Multiplicity (DEPT)** | **$^{13}C$–NMR** | **$^{1}H$ ($J$ = Hz)** |
|---|---|---|---|
| **1** | $CH_3$ | 14.20 | 0.96 t ($J$ = 5.0, 10 Hz) |
| **2** | $CH_2$ | 23.90 | 1.40 m |
| **3** | $CH_2$ | 28.23 | 1.41 m |
| **4** | $CH_2$ | 33.94 | 1.67, 1.83 m |
| **5** | CH | 82.37 | 5.08 m |
| **6** | CH | 155.40 | 8.01s |
| **7** | –C– | 126.57 | ---------- |
| **8** | –C– | 131.59 | ---------- |
| **9** | C=O | 168.50 | ---------- |
| **10** | $CH_2$ | 130.41 | 6.56, 6.83 s |
| **11** | C=O | 173.73 | ---------- |

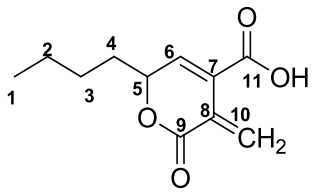



**Figure 5.7 Structure of the new compound (42).**

In $^{1}H–^{1}H$ COSY, the protons of C–1 showed correlation with the proton of C–2, also the proton of C–4 with the proton of C–5 and that of C–5 with C–6. While in HMBC, the methyl proton 3H–1 shows two bonds correlation to C–2 and three bonds correlation to C–3. The remaining protons of alkane series showed coupling to each other. Proton C–5 showed two bonds correlation to C–4 and C–6 while three bonds correlation to C–7. The alkene protons H–6 showed two bond correlations to C–7 and 3 bond correlations to quaternary C–8, carboxylic group at C–11. The two alkene protons H–10a and H–10b shows three bonds correlations with C–7 and Carbonyl carbon at C–9. The 2D ($^{1}H–^{1}H$ COSY and HMBC) supported that this compound has a six membered ring attached to a four carbon chain of alkane in figure 5.8.

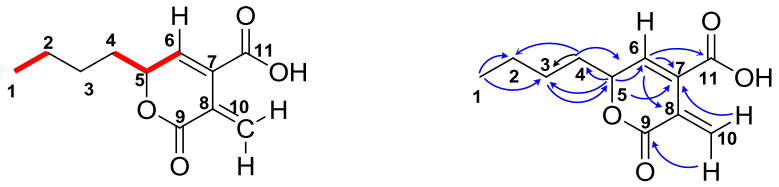



**Figure 5.8 COSY correlations have shown by the red arms, while the HMBC correlations have shown by the blue arrows.**

## 5.2 Chromatogram of *Cladosporium carrionii*

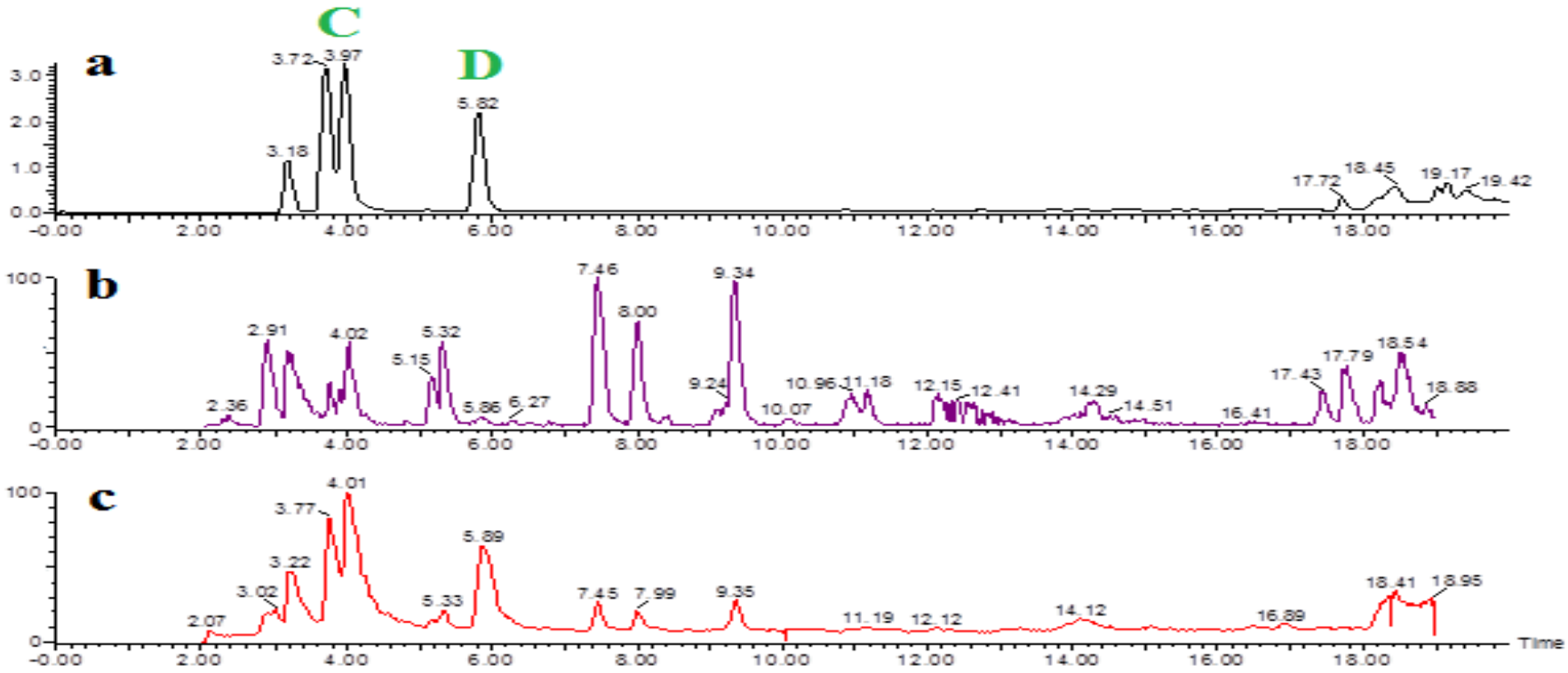



**Figure 5.9 LCMS chromatogram of *C. carrionii*, showing UV profile of the extract (trace a), $ES^-$ profile of the extract (trace b) and $ES^+$ profile of the extract (trace c). While C and D are the targeted compounds eluted at 4.02 and 5.89 rt respectively.**

### 5.2.1 Chromatogram of known compound (C–43)

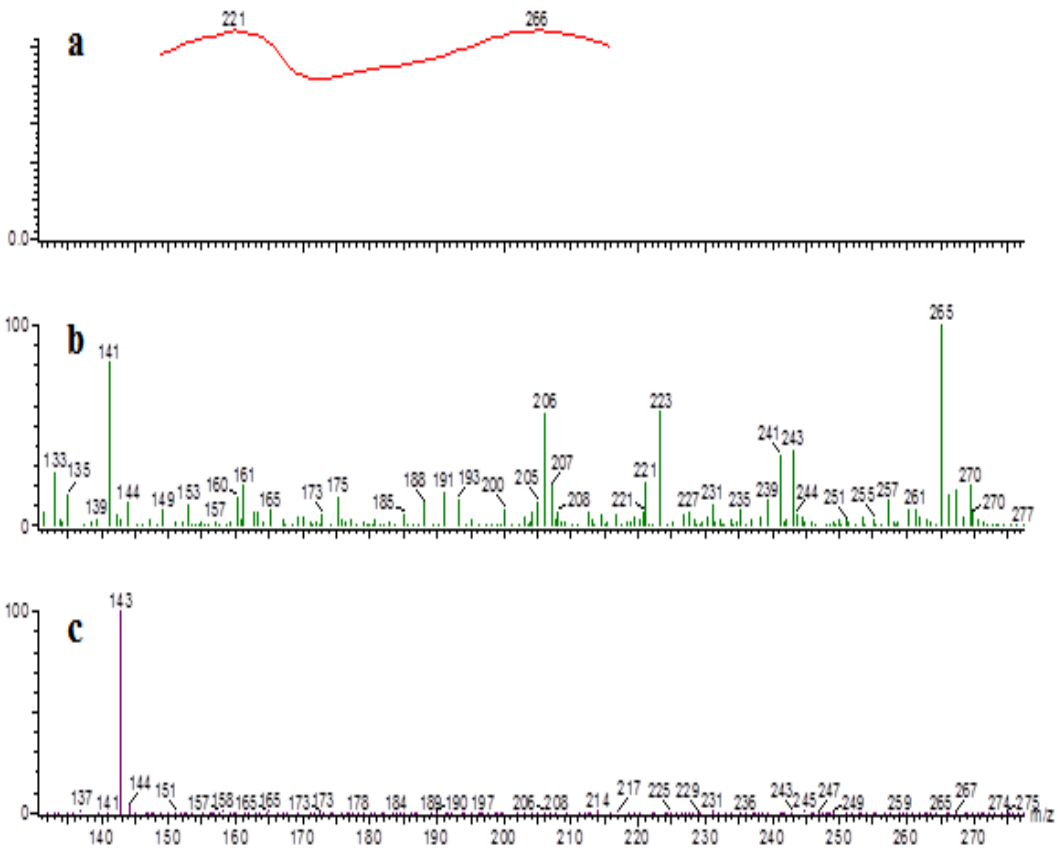



**Figure 5.10 LCMS chromatogram of *C. carrionii*, showing UV profile of the extract (trace a), $ES^-$ profile of the extract (trace b) and $ES^+$ profile of the extract (trace c) of compound (C–43).**

**5.2.1.1 Known compound (C–43)**

The molecular formula of the known compound was established by HR–$EI^+$ and $^{13}C$ NMR (Broad Band and DEPT). HR–ESI–MS showed pseudo molecular ion peak [$M^+$] at *m/z* 143.1 $[M+H]^+$ corresponding to the molecular formula $C_6H_6O_4$ [Calculated for $C_6H_6O_4$ + H = 143.1]. The IR spectrum indicated the presence of an amide carbonyl (1660 $cm^{-1}$), and benzene ring (1625 and 1391$cm^{-1}$). The absorption maxima in the UV spectrum (221, and 266 nm) also exhibited the presence of a benzene ring.

Then 10 mg of pure compound (**43**) was dissolved in 0.70 mL of deutrated (DMSO). A number of 1D and 2D NMR experiments were carried out on 500 MHz NMR Spectrometer for structure elucidation.

$^1H$–NMR (DMSO, 500 MHz) showed the 03 signals for protons. There was a duplet at the right side of the spectrum at δ 4.28 giving the integration of 2 protons correspond to $CH_2$ and two signals gave an integration of 1 protons each at δ 6.33 and 8.22 corresponds to CH.

The 1D $^{13}C$ NMR showed 06 signals correspond to 06 carbons skeleton of the compound, showing 2 quaternary carbons at δ 168.11and 145.70 respectively while 1 carbonyl carbons at δ 173.96. The 2D HSQC presented 3 protonated carbons as shown in table 5.3. The 2D ($^1H$–$^1H$ COSY and HMBC) disclosed many of the correlations and the elucidated structure as shown in figure 5.11.

**Table 5.3** $^{1}H$ and $^{13}C$–NMR and Chemical Shifts of Compound (**43**) (ppm, DMSO, 500 MHz)

| **C. No** | **Multiplicity (DEPT)** | **$^{13}C$–NMR** | **$^{1}H$ (*J* = Hz)** |
|---|---|---|---|
| **1** | $CH_2$ | 59.48 | 4.28 d ( *J* = 5.0 Hz) |
| **2** | –C– | 168.11 | ---------- |
| **3** | CH | 109.86 | 6.33 s |
| **4** | C=O | 173.93 | ---------- |
| **5** | –C– | 145.70 | ---------- |
| **6** | CH | 139.27 | 8.22 s |

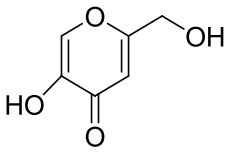



**Figure 5.11 Structure of known compound (43).**

### 5.2.2 Chromatogram of new compound (D–44)

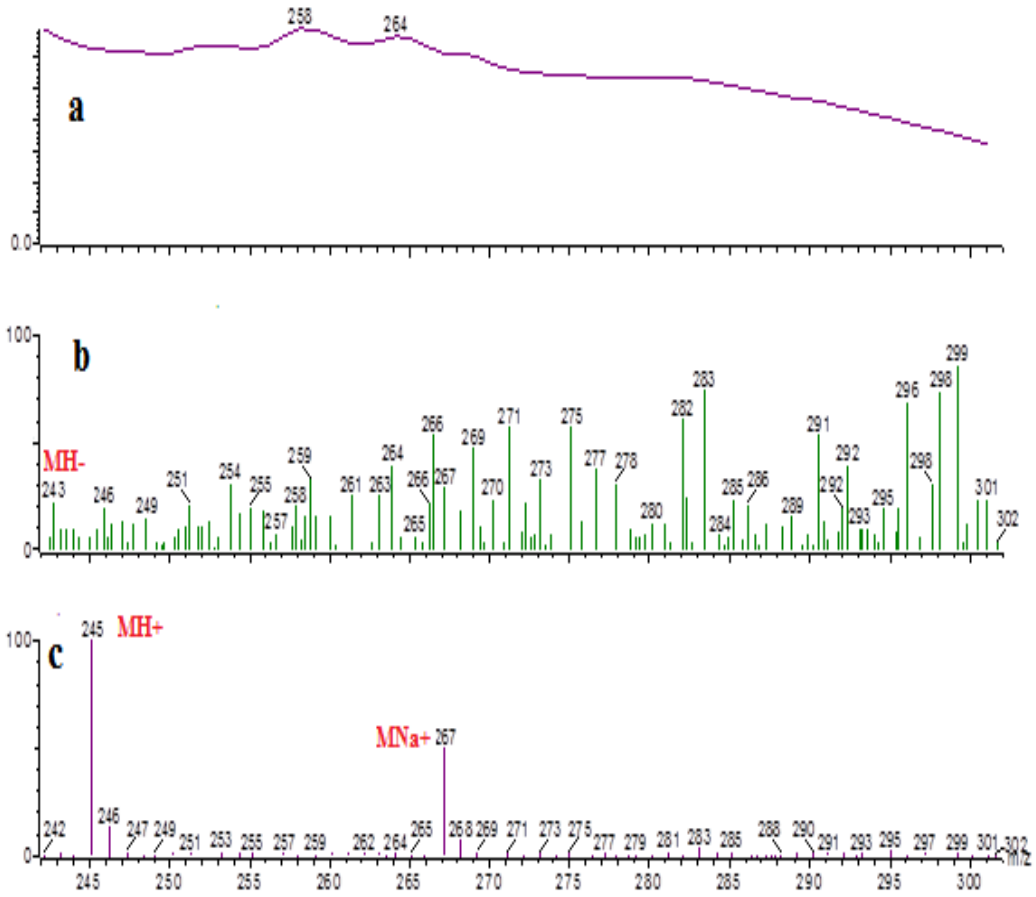



**Figure 5.12 LCMS chromatogram of *C. carrionii*, showing UV profile of the extract (trace a), $ES^-$ profile of the extract (trace b) and $ES^+$ profile of the extract (trace c) of compound (D–44).**

**5.2.2.1 New compound (D–44)**

The molecular formula of the new compound was established by HR–ESI–MS and $^{13}C$ NMR (Broad Band and DEPT). HR–ESI–MS showed pseudo molecular ion peaks [$M^+$] at *m/z* 245.1292 [M]$H^+$ and 267.1111 [M]$Na^+$ corresponding to the molecular formula $C_{14}H_{16}N_2O_2$ [Calculated as $C_{14}H_{16}N_2O_2$ + Na = 267.1103]. The IR spectrum indicated the presence of an amide carbonyl (1660 $cm^{-1}$), and benzene ring (1625 and 1391$cm^{-1}$). The absorption maxima in the UV spectrum (258, and 264 nm) also exhibited the presence of a benzene ring.

The 9.5 mg of pure compound (**44**) was dissolved in 0.70 mL of deuterated chloroform ($CD_3Cl$). A number of 1D and 2D NMR experiments were carried out on 500 MHz NMR Spectrometer for structure elucidation.

$^1H$–NMR ($CD_3Cl$, 500 MHz) showed the 12 signals for protons. There were two signals gave an integration of 2 protons each at δ 3.17 and 3.56 correspond to $CH_2$. All the rest of proton signals gave integration of one proton each.

The 1D $^{13}C$ NMR showed 14 signals correspond to 14 carbons skeleton of the compound, showing 1 quaternary carbons at δ 137.33 while 2 carbonyl carbons at δ 170.91 and 166.90 respectively. The 2D HSQC presented 11 protonated carbons as shown in table 5.4. The 2D ($^1H$–$^1H$ COSY and HMBC) disclosed many of the correlations and the elucidated structure as shown in figure 5.13.

**Table 5.4** $^{1}H$ and $^{13}C$–NMR and Chemical Shifts of Compound (**44**) (ppm, $CD_3OD$, 500 MHz)

| **C. No** | **Multiplicity (DEPT)** | **$^{13}C$–NMR** | **$^{1}H$ (*J* = Hz)** |
|---|---|---|---|
| **1** | $CH_2$ | 116.56 | 6.77d ( *J* = 5.0 Hz),<br>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** | $CH_2$ | 38.82 | 3.56 d ( *J* = 5.0 Hz) |
| **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 |

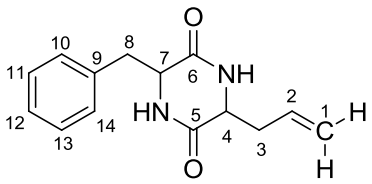



**Figure 5.13 Structure of new compound (44).**

In $^{1}H$–$^{1}H$ COSY, the alkene proton of H–2 showed correlation with the proton of C–3, also the proton H–3 showed correlation with the proton of C–4. The proton H–7 showed correlation the protons of C–8. While in HMBC, the methylen proton H–1a and H–1b shows three bonds correlation to C–3 and four bonds correlation to C–4. Again the methylene proton H–2 shows three bonds correlation to 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 showed three bond correlations with carbonyl carbon at C–5. The aromatic proton H–10 showed three bonds correlation with C–8, and the protons H–8 showed two bonds correlation to C–7, three bonds correlation with carbonyl carbon at C–6 and weak four bonds correlation with carbonyl carbon at C–5. The 2D ($^{1}H$–$^{1}H$ COSY and HMBC) alond with UV and IR supported that this compound has a benzene ring attached to six member ring of herto groups and a unique three carbon chain of alkene.

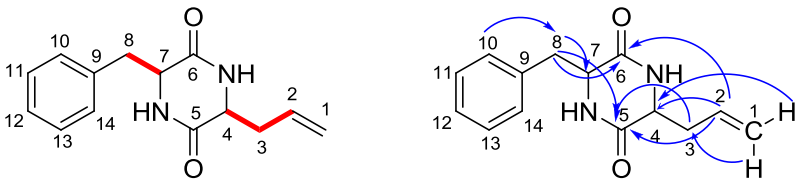



**Figure 5.14 COSY correlation has shown by the red arms, while the HMBC correlations have shown by the blue arrows.**

### 5.2.3 Known compound (45)

The isoprenylindole–3–carboxylic acid was produced for the first time by *C. carrionii* was isolated by column chromatography and readily identified by comparing its $H^{+}NMR/^{13}CNMR$ data with those in the literature [138], HR–ESI–MS showed pseudo molecular ion peak [$M^{+}$] at m/z 230.0801 [139]+ corresponding to the molecular formula $C_{14}H_{15}NO_2$. The 5 mg of pure compound was dissolved in 0.70 mL of deutrated chloroform ($CDCl_3$). A number of 1D and 2D NMR experiments were carried out on 500 MHz NMR machine for the structure elucidation. The analogous NMR data of the compound has explained as;

$^{1}H$–NMR ($CD_3Cl$, 500 MHz) showed the signals of 9 protons. There were two signals gave an integration of 3 protons each at δ 1.77 and 1.78 correspond to two $CH_3$. One signal gave an integration of 2 protons at 3.48 corresponded to $CH_2$. All the rest of proton signals gave integration of one proton each.

The 1D $^{13}C$ NMR showed 14 signals correspond to 14 carbons skeleton of the compound, showing 5 quaternary carbons at δ 124.1, 137.5, 129.0, 137.5 and 34.4 while 1 carbonyl carbons at δ 171.5 in figure 5.15.

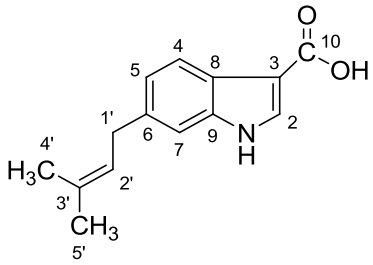
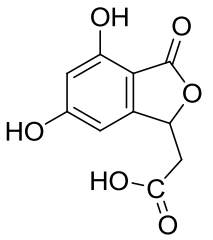


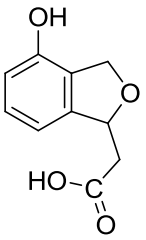

**Figure 5. 15 Compound isolated from the defatted ethyl acetate fraction of *C. carrionii* [138].**

### 5.2.4 Known compounds (46 and 47)

2-(4,6-dihydroxy-3-oxo-1,3-dihydroisobenzofuran-1-yl) acetic acid (**46**) and 2-(4-hydroxy-1,3-dihydroisobenzofuran-1-yl) acetic acid (**47**) also isolated using column chromatography and elucidated by NMR.




Compound (**46**)




Compound (**47**)

**Figure 5.1 1 Compounds isolated from the defatted ethyl acetate fraction of C. carrionii [140-141].**

# CHAPTER 6

# ESTIMATION OF POLYKETIDE BIOSYNTHESIS

# 6 ESTIMATION OF POLYKETIDE BIOSYNTHESIS

Natural products chemistry (NPC) is a combinatorial branch of biology and chemistry which deals with the isolation, identification and structure elucidation studies of the natural metabolites either from bacteria, fungi or plants and then the biosynthesis of the interested metabolites produced by the putative organisms.

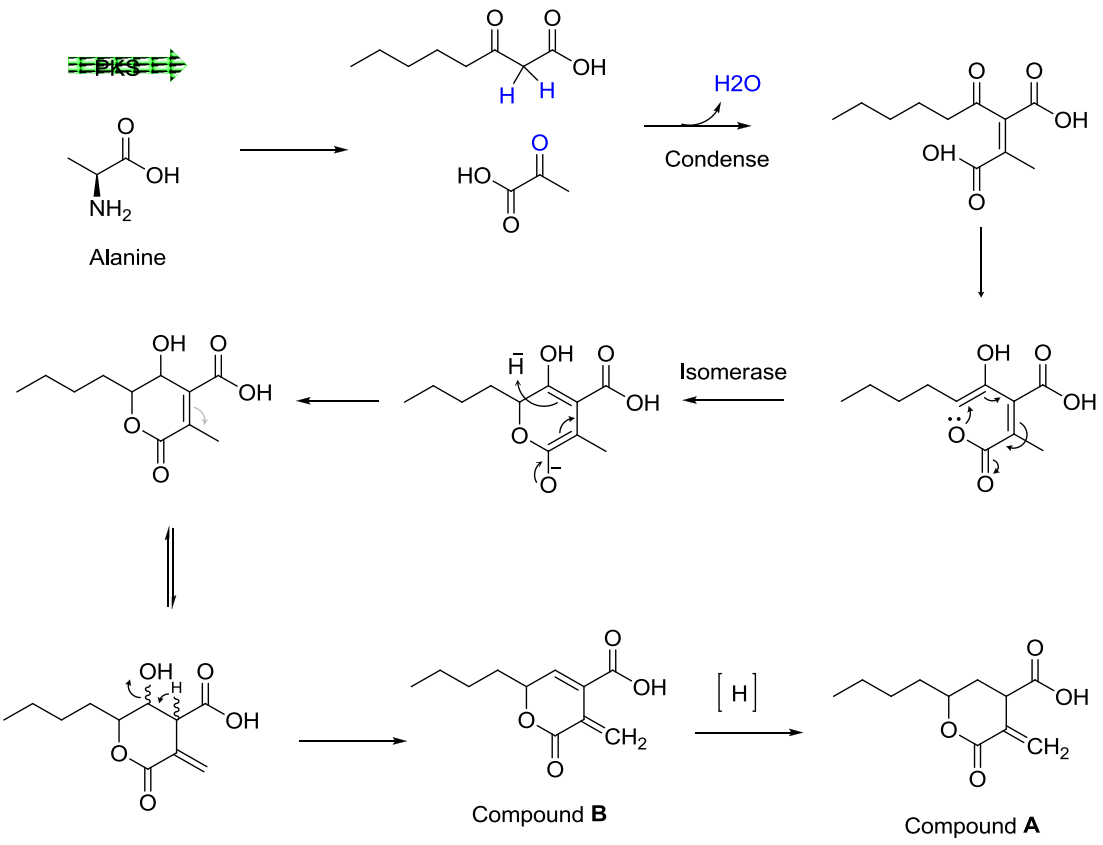
In filamentous fungi, the secondary metabolites are produced by the polyketide pathway because it is the only major route for the formation of biologically important metabolites; however it is challenging to isolate new and biologically potent metabolite from the putative sources but still, the living organisms are the rich source of potential metabolites.

Aromatic polyketides consist of a significant and structurally diverse group of natural products produced by fungi and plants. This family of metabolites is consisting a huge number pharmaceutical components, while on the other hand, some of them are highly toxic to both the human and/or plant. The high pharmacological properties, both the beneficial and harmful, have attracted the interest of researchers for the biosynthesis and discovery of the important natural products.

To make clear picture of the mechanisms of intermediate synthesis in the polyketide biosynthesis, there are several methods that play a vital role in the discovery of the biosynthesis. These includes: the time course production experiments, to find the right time for the feeding of isotopic labelled precursor; classical labelling techniques, and the estimation of incorporation by $^{13}C$ NMR spectroscopy; and the use of knockout experiments for the confirmation of thebiosynthetic pathways of the desire metabolites. Isotopic labelled acetate is one of the key precursors in determining the polyketides biosynthesis.

## 6.1 Proposed biosynthetic pathways of the new metabolites (41 and 42)

The skeleton of the two new compounds (**41** and **42**) from *Aspergillus oryzae* (*tenS.PKS/dmbC*) seem that they have been produced by some other PKS, because the architecture is not dependent on the genes incorporated for the production of straight chain polyketide metabolite. Therefore, it is suggested that both the metabolites are the product of a reaction between polyketide with alanine. The proposed biosynthetic transmit is given in scheme 6.1. However; as we can estimate the inducement of alanine on polyketides skeleton, it is necessary to work out with feeding acetate as precursor to prove the idea that acetate are the building blocks of polyketides. After then we can proceed for the feeding of alanine to know the role of alanine in the biosynthesis of these metabolites.




**Scheme 6.1 Proposed biosynthesis routes for compounds (41 and 42).**

## 6.2 Time course production of the targeted metabolites

It is interesting to know that time course production experiment is necessary for feeding the isotopic labelled precursor, because the time course production curve is essential to give an idea for the feeding of precursor on the right time. Therefore, in time course production experiment over a specific time period the target metabolites (**A** and **B**) yield in the form of peak integration was monitored by LCMS/HPLC in liquid culture. The yield will be compare with time of incubation. This experiment is very important for the feeding of labelled precursors and for further molecular experiments such as biosynthetic genes determination which is responsible for the production of metabolites.

### 6.2.1 Time course production of the targeted metabolite (A–41)

The time course production was measured in CGSB, there was no production in the first three days and then an unusual production of the targeted metabolite was observed. The metabolite were started to produce on day 3, which have shown in figure 6.1 and then a gradual increase till day 5 and then decrease, however from the curve it is was estimated to feed the precursor on three different days.

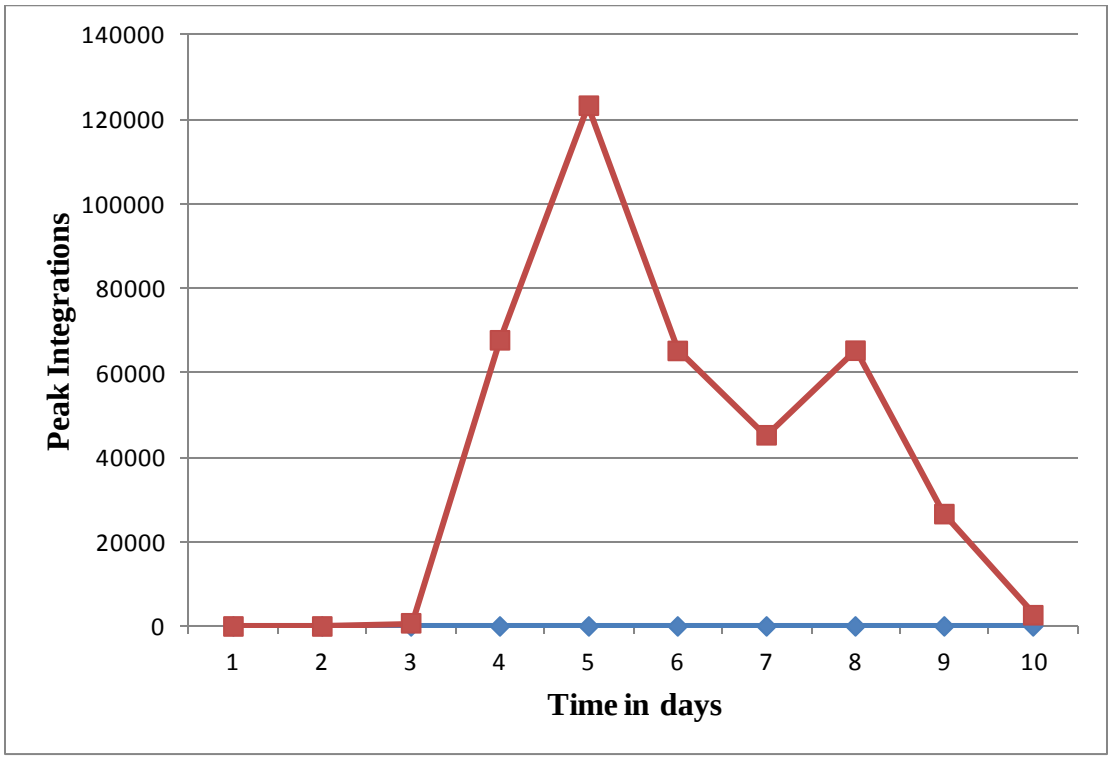



**Figure 6. 1 Time course for compound (41) *A. oryzae* in CGSB media.**

### 6.2.2 Time course production of the targeted metabolite (B–42)

As it clear from the previous chapters that both the metabolites are produced by *Aspergillus oryzae* therefore the time course production was measured in CGSB, however there was little difference in their productions, no production in the first three days like metabolite (**41**) and then a rapid increase in day 4 of the targeted metabolite was observed. The metabolite were started to produce on day 3 to day 4 and then decreased, which have shown in figure 6.2, however from the curve it is was estimated to feed the precursor on three different days, because it was necessary for both the metabolites.

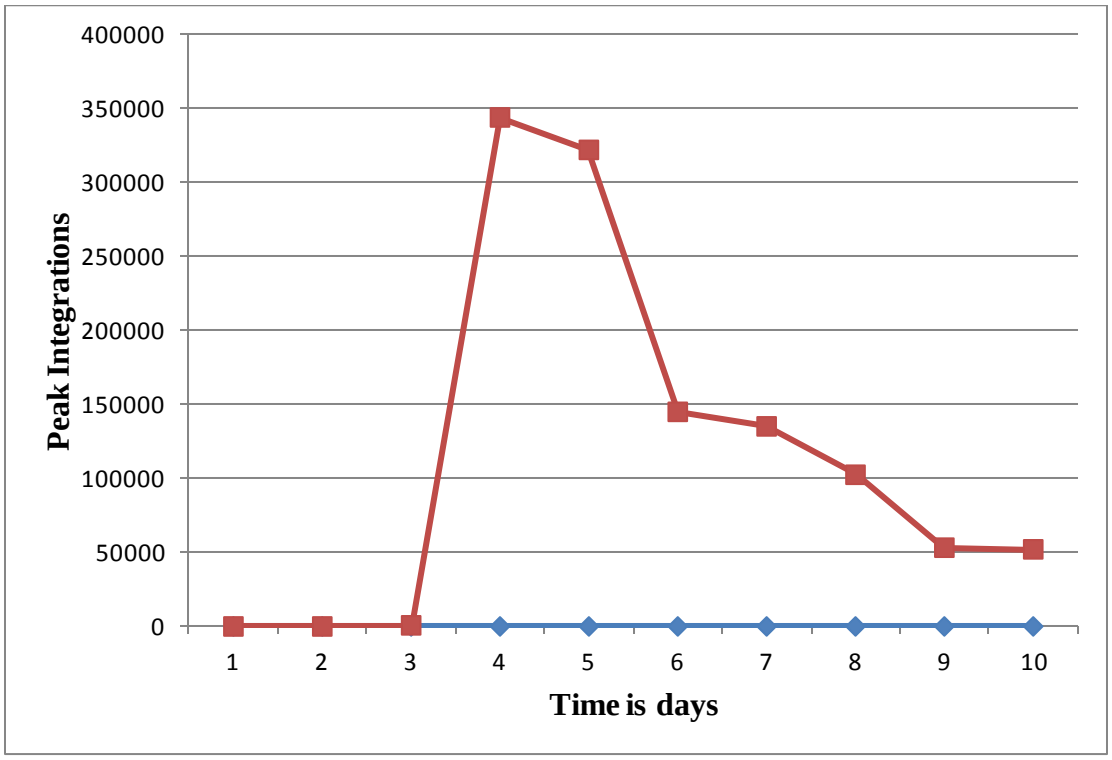



**Figure 6. 2 Time course for compound (42) *A. oryzae* in CGSB media.**

Generally the isotopic labelled precursors were fed after 3rd day, when the targeted metabolites begin to produce and reach to the highest level. For example during the elucidation of biosynthetic pathway for the two new compounds (**41** and **42**) the acetate were fed from 3rd day to 5th day. Because it is clear both the compounds begin to be produced from 3rd day and is going to its maximum on 4th and 5th day for compound (**41** and **42**) respectively. However, care must be taken to avoid the contaminations of culture; otherwise it will be the loss of labelled precursors.

A time course production study is also necessary to draw a map for the molecular studies such as to isolate and characterize the biosynthetic genes which are responsible for the production of the targeted metabolite.

## 6.3 Feeding of precursor containing stable isotope

Stable isotopes with single labelling have been utilized in many biosynthetic studies successfully. For example to determine the biosynthesis of our newly isolated compounds from *A. oryzae* (*tenS.PKS/dmbC*) i.e., compounds (**41** and **42**), combined incorporation experiments were performed because both the metabolites are from single fungi, in which the proposed labelled precursors [1–$^{13}$C]–acetate were fed to the shake flask cultures of *A. oryzae* (*tenS.PKS/dmbC*).

### 6.3.1 Feeding of [1–$^{13}$C]–acetate to (A–41)

The target metabolites (**41**) were isolated from the fermentations of labelled precursors by HPLC as shown in figure 6.3, by the same program as used for the normal isolation and then the incorporation was analyzed by $^{13}$C VNMR (500 MHz) spectroscopy. All [1–$^{13}$C]–enriched sites were indicated in table 6.1 for compound (**41**).

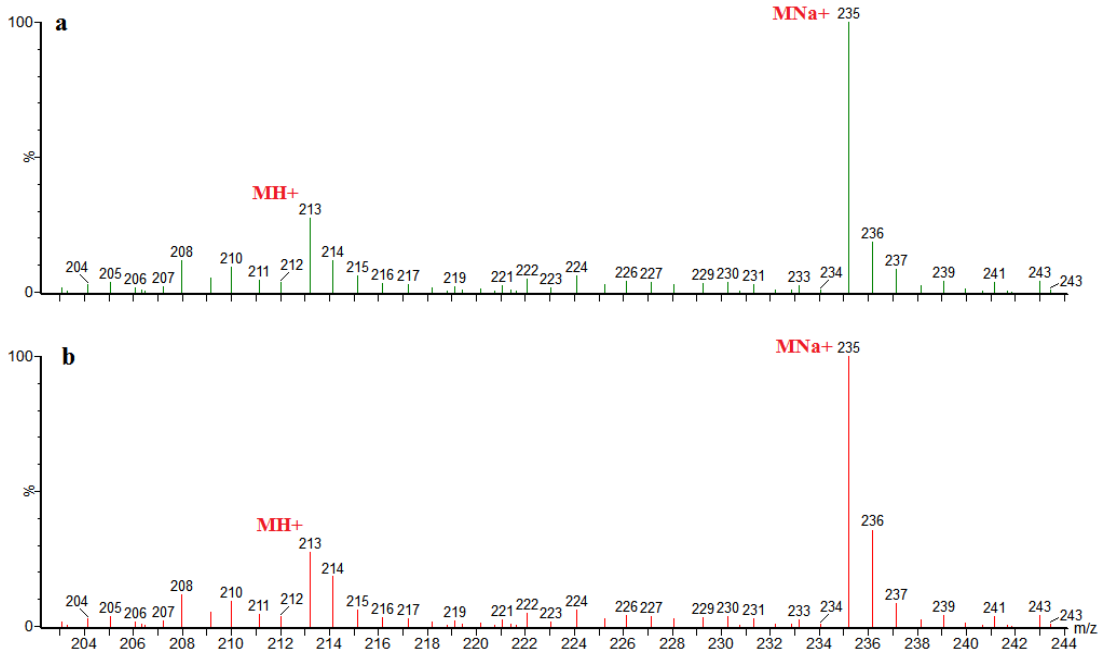



**Figure 6. 3 Comparison of the ES$^+$ of compound (41) *A. oryzae*, natural (trace a) and feeded with [1–$^{13}$C]–acetate (trace b).**

**Table 6.1** $^{13}$C NMR analysis of Compound (A) after feeding with [1–$^{13}$C] sodium acetate precursor,

| Carbon Number | Chemical shift (ppm) | Enrichment ratio* |
|---|---|---|
| **1** | 14.27 | 0.90 |
| **2** | 23.54 | **1.00** |
| **3** | 28.80 | 0.88 |
| **4** | 36.22 | **1.27** |
| **5** | 80.99 | 0.93 |
| **6** | 36.14 | **1.09** |
| **7** | 46.74 | 0.93 |
| **8** | 138.59 | 0.81 |
| **9** | 168.31 | **1.50** |
| **10** | 130.06 | 0.92 |
| **11** | 179.11 | **1.28** |

* **$^{13}$C signal intensity of each peak in the labelled (41) divided by that of the corresponding signal in the unlabeled, normalized to give an enrichment ratio of (A) for enriched peak (C 2 were taken as reference for [1–$^{13}$C] sodium acetate labelling).**

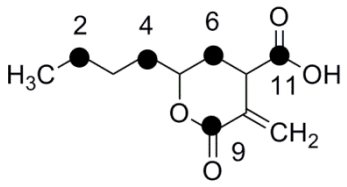



**Figure 6. 4 Compound showing the incorporation of 1–$^{13}$C acetate.**

### 6.3.2 Feeding of [1–$^{13}$C]–acetate to (B–42)

The target metabolites (**42**) were isolated from the fermentations of labelled precursors by HPLC as shown in figure 6.4, by the same program as used for the normal isolation and then the incorporation was analyzed by $^{13}$C VNMR (500 MHz) spectroscopy. All [1–$^{13}$C]–enriched sites were indicated in table 6.2 for compound (**42**).

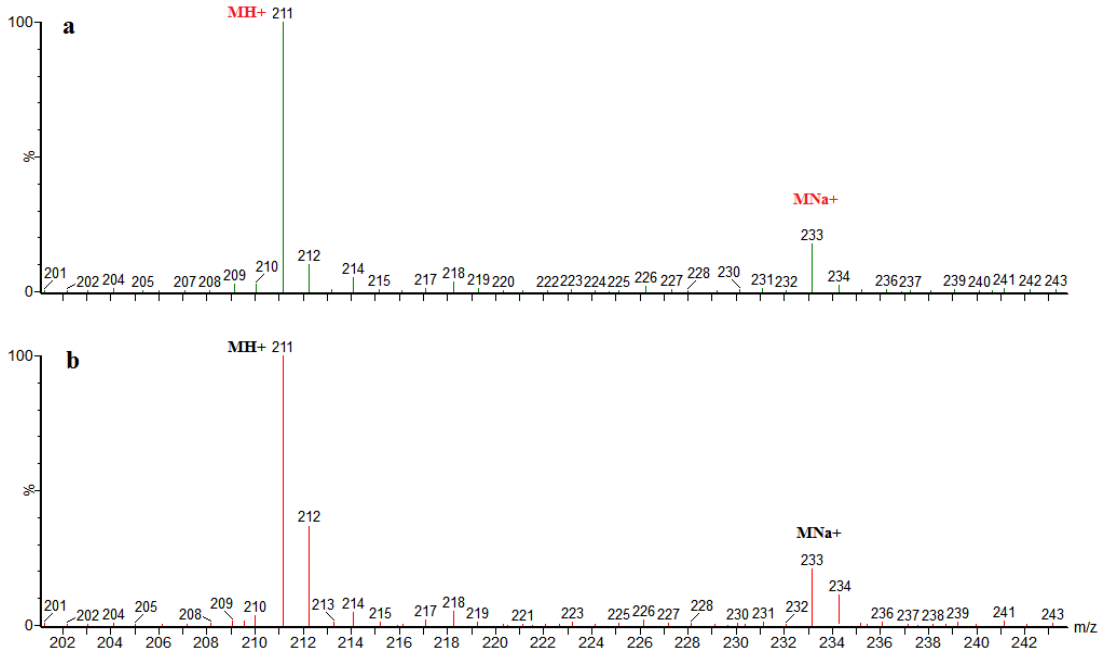



**Figure 6. 5 Comparison of the ES$^+$ of compound (42) from *A. oryzae*, natural (trace a) and feeded with [1–$^{13}$C]–acetate (trace b).**

**Table 6.2** $^{13}$C NMR analysis of Compound (B) after feeding with [1–$^{13}$C] sodium acetate precursor

| Carbon Number | Chemical shift (ppm) | Enrichment ratio* |
|---|---|---|
| **1** | 14.20 | 0.11 |
| **2** | 23.90 | **1.00** |
| **3** | 28.23 | 0.81 |
| **4** | 33.94 | **1.52** |
| **5** | 82.37 | 0.89 |
| **6** | 155.40 | **1.10** |
| **7** | 126.57 | 0.68 |
| **8** | 131.59 | 0.43 |
| **9** | 168.50 | 0.69 |
| **10** | 130.41 | 0.68 |
| **11** | 173.73 | **2.30** |

*** $^{13}$C signal intensity of each peak in the labelled (42) divided by that of the corresponding signal in the unlabeled, normalized to give an enrichment ratio of (B) for enriched peak (C 2 were taken as reference for [1–$^{13}$C] sodium acetate labelling)**

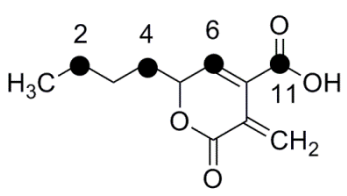



**Figure 6.6 Compound showing the incorporation of 1–$^{13}$C acetate.**

# CHAPTER 7

# DISCUSSIONS

# 7 DISCUSSIONS

## 7.1 General

About fifty percents (50%) of all the deaths are due to dangerous diseases in the developing countries, caused by several fundamental agents, like, *Bacillus subtilis, Escherichia coli, Klebsiella pneumonia* and *Staphylococcus aureus*. There are still a lot of therapeutic agents for their inhibition either from natural origin or synthetic [142]. However the misuse of therapeutic agents may cause resistance of the pathogens. Therefore, hunt for a new, safe and more potent biologically active secondary metabolite (s) is an urgent need for the curing the diseases in the developing areas [143]. About three quarter population of the world depends on indigenous plants and their materials for diseases [144-145] and about fifty six percents (56%) of lower income population of the world use plants medicine for their major health care [146]. However; no one can use fungi for their domestic health care, even though fungi are good producer of biologically active metabolites, because some of the fungi are toxic in nature. Majority of the human and animal therapeutic drugs have been produced by direct fungal fermentation, or by modification of the product through the process of fermentation [147]. This biodiversity favors the isolation of new antibiotics for example penicillin. Beside this fungi also produces variety of metabolites with high remedial value as antibiotics, cytotoxins, pesticides etc. [148].

While the fungi will resist to the production of same and equal amount of metabolites due to stressing in the in vitro conditions therefore, the exploitation of the epigenome of fungi is imagined to be a useful method for the production of natural products from their silent genes. To determine the effect of micro–molecule inhibitors on overturning the transcriptional silence of biosynthetic genes which are involved in biosynthesis of polyketide (PKS), non–ribosomal peptide (NRPS), and hybrid PKS–

NRPS (HPN). Examination of expressed sequence tag (ESTs) libraries from *Aspergillus niger* demonstrated that more than seventy percents (70%) of its PKS, NRPS and HPN encoding gene bunch were transcriptionally silenced in in–vitro culture conditions [149].

Transcriptional suppression of polyketide synthase (PKS), non–ribosomal peptide synthase (NRPS) and hybrid PKS–NRPS (HPN) genes clusters has dissatisfied the efforts of natural products chemists to fully explore the secondary metabolises of fungi. The full impact of the gene cluster silencing problem was recently illustrated with the soil–dwelling fungus, *Aspergillus niger*, showing that less than thirty percents (30%) of its thirty one (31) PKS, fifteen (15) NRPS and nine (09) HPN biosynthetic gene bunch were transcribed at a variety of standard laboratory conditions [150].

## 7.2 Isolation and identification of fungi

Fungi are the second most diverse group (organism) after plants which are still less investigated for biologically active secondary metabolites. From about 1.5 Million estimated known fungal species only about 5% have been explained and reported to produce secondary metabolites [151-152]. However; there are about 9.9 million species of fungi present on the planet, if so then this percentage will be even lower than five (5%) if we used 9.9 Million as the total number of fungi. There are different numbers of estimated fungal species in the whole world, varying from 0.5 to 9.9 Million; therefore we can roughly estimate that only 5% have been explored for biologically active metabolites [153].

Perhaps; the proportion of the investigated fungi is higher than the earlier report, because plenty of literatures reported secondary metabolites isolated from

unidentified fungi, therefore, the identification along with the isolation of fungi and their secondary metabolites is very much essential. But in realty after their identification, the fungi are still less explored for their metabolites and offer variety of novel natural products to be discovered. The little level of knowledge about fungi is due to some of the major factors described as: the majority of fungi are microscopic, numerous create their fruit bodies quickly and unreliably [154], to overcome all these shortcomings and explore a comprehensive literature survey regarding the importance of their secondary metabolites, will encourage the new coming scientist for the exploitation of fungi.

Identification and classifications of fungi in the perspective of their natural products (secondary metabolites) is necessary. However, the fungal identification will show the way of co–relation of specific metabolites with the class of fungi, and hence it will be an easiest way to isolate a certain groups of natural products, particularly novel compounds. In simple way the identification and classification of fungi is useful in de–replication purposes of isolated natural products. Thus, new isolated metabolites of the already identified fungi can be determined easily based on the existing or available data of known natural metabolites. These data will also be helpful to map out the presence of new compounds resulted from metabolic manufacturing.

Fungal species are mostly determined according to the reproductive structure, namely spore because of its stability in form and sizes [155]. This conventional method is currently complemented by molecular method. Nevertheless, fungal experts are still relying on spores to identify the fungi. Therefore, in this work 12 fungal strains have been successfully isolated from different area of KPK, Pakistan, which mostly belong to mould groups. Based on the fact that some moulds as *Penicillium* sp., *Aspergillus* sp. and *Cladosporium* sp. have been well investigated, thus the

present study focused on the exploitations of four (4) fungi, *Aspergillus* sp. and *Cladosporium* sp. were observed to produce biologically active secondary metabolites.

### 7.3 Nutrients and fungal growth

Fungi are heterotrophic and eukaryotic organisms that absorb their food so called "osmotrophic" [156]. Although, all the fungi depend on organic compounds, mainly on carbon which is a major part of living plants, animals and microbes. Carbon in the form of structural materials such as cellulose, lignin, chitin and keratin are normally difficult to degrade. However, fungi have the ability to degrade all these molecules [157]. In view of the fact, fungi can degrade structurally complex organic compounds but they are "non–diazotrophic", therefore, they need Nitrogen as a source in nutrients, they need to be supplied with nitrogen–containing compounds, either in in–organic or organic form [158].

Therefore, in the present study, we supplied different Carbon and Nitrogen sources by the use of five different media (Czapek–dox Broth (CB), Czapek Yeast–extract Broth (CYB), Yeast Extract Sucrose (YES), Potato Dextrose Broth (PDB) and Czapek–dox (supplemented with glucose & starch) Broth (CGSB)) as the basal testing medium. The Czapek–dox (supplemented with glucose & starch) Broth (CGSB) was chosen the best optimum media according to the previous studies in our groups. The results illustrated in chapter 4, section 4.1 optimization of media and biological activities, that this medium was suitable for both the growth and production of secondary metabolites, similar like Hagem Medium (HM) is the most suitable media for the growth as well as for bioactive metabolites production of marine fungi. Moreover, it is considered that that (HM) is complex and complete medium which is

consists of glucose, malt–extract and ammonium succinate, therefore glucose and maltose serves as carbon while ammonium succinate serves as nitrogen source, which is probably suitable for almost all kind of fungi to grow well and produce secondary metabolites [159].

After the logical solution of the food stuff for fungi, and further manipulation regarding the growth and metabolites productions, the type of cultivations are also important, therefore, it has been reported that the cultivation of fungi in the shaking cultures/conditions produce more active compounds than those from still cultures/conditions [139, 160]. However, still culture/conditions may also begin the production of bioactive metabolites, because the secondary metabolites are linked with the growth of fungi. The fungi cultivated in still/static culture/conditions undergo two different conditions. The surface layer is aerobic but has poor contact with the medium, whereas; the bottom of the media is anaerobic. This situation may result in growth inhibition and excessive production of secondary metabolites, depending on the characteristics of the fungus [161]. We have grown our fungus in both the conditions and the shaking culture produced more metabolites, however, it is still required to observe the metabolites of other fungi, as a comparison in the still culture in future.

### 7.4 Biological assays

In search of bioactive compounds, a total of twelve (12) extracts from four (4) fungal species were tested for antimicrobial, antifungal, cytotoxic and phytotoxic activities. From each fungal specie three (3) extracts i.e. ethyl acetate, *n*–Hexane and aqueous fractions were obtained from both the culture and mycelia. The results of biological activities explained that all the ethyl acetate extracts were active against

almost all the test organisms. However, the activities of the *Aspergillus oryzae* (tenS.PKS/dmbC) and *Cladosporium carrionii* (F–2) were relatively higher than those of *Aspergillus carbonarius* (NRRL–369) and *Cladosporium resinae* (NRRL–6437).

Beside this, in some cases the aqueous extract while, in rare cases the *n*–Hexane fractions also showed inhibitions. These results were compared with mostly bioactive compounds reported in the research papers isolated from fungal broth culture in several reviews [92, 162]. Therefore, it was concluded by comparing the results of biological activities of our extracts with those of reported in literatures that marine–derived fungi most probably produces extracellular metabolites.

The term extra cellular metabolites refers to the presence of biologically active secondary metabolites in the culture medium which is probably linked with the self–defense mechanisms of the fungi. The mechanisms are suggested to prevent suicide due to its own secondary metabolites. Various mechanisms in the fungi and other micro–organisms have been presumed, as an example metabolic shielding mechanism. In this metabolic shielding mechanism, antibiotics are produced and secreted as inert pro–drugs and subsequently will be activated during or following export [163].

As secondary metabolites in the fungi serve as antibiosis a “competitive weapons” against other microorganisms, it can be concluded that biologically active secondary metabolites are normally secreted as extracellular metabolites by fungi [164]. It has been observed that the strong biological activities especially antimicrobial activities of the secondary metabolites are due to flavonoids, because it has been shown to be the most effective against MRSA [165-166]. The flavonoids are biologically active because of its complex formation can disrupt the bacterial membranes cell wall [167-168]. The antifungal properties in the extract are due to the

presence of thiophenes which have been reported from the extract of sunflower and marigold [169]. It is not necessary that all the biologically potent metabolites will be developed in to antibiotics; however agrochemicals are also essential for plants to protect them from diseases and promising for high yielding [170]. Homodestruxin–B is natural compound produced by *Alternaria brassicae*, that possess phytotoxic properties [171].

## 7.5 Bioactive compounds of fungi

Fungi are the living organisms which can live anywhere in the environment, while the fungi living in marine environments are normally associated with other organisms. In general, existence of fungi in marine environment is classified into three main groups, i.e., parasitic, saprophytic and symbiotic fungi.

Now the production of secondary metabolites is often associated to their role in the environment. As an example, (Schulz *et al.*,) reported that endophytic fungi which were isolated from healthy algae and plants produced natural products in higher proportion compared to the soil isolates [172]. Based on this condition, it was assumed that the presence of the fungi in seaweeds or plants as their hosts affected the creativity of the fungi to produce bioactive metabolites.

We did not proceeded for the marine or endophytic fungi, however from the present study were found that four fungal isolates, i.e., *A. carbonarius* (NRRL–369), *A. oryzae* (tenS.PKS/dmbC), *C. carrionii* (F–2) and *C. resinae* (NRRL–6437), exhibited considerable bioactivity, in which *A. oryzae* (tenS.PKS/dmbC) and *C. carrionii* (F–2) were remarkably active against pathogenic bacteria and fungi.

In the present study, varying of treatments and culture conditions were applied to induce the fungal cultures to produce bioactive secondary metabolites. However,

the inductions were not always followed by production of more potent metabolites. Miller stated that the production of secondary metabolites of fungi in more quantity in the laboratory is a difficult problem, since very limited understanding of secondary metabolites exists for filamentous fungi. Certain conditions are required to optimize for fungi to produce secondary metabolites [173].

Bioactive secondary metabolites production in fungi is influenced by some factors such as the nature of the fungus, its substratum and stage of development. Nutritional modifications such as carbon and nitrogen sources can affect productivity of marine fungi as well as terrestrial fungi to produce active metabolites. For example, 2.0% starch and 0.24% potassium nitrate as a carbon and an inorganic nitrogen sources respectively, supported the highest level of antimicrobial production by *Penicillium viridicatum* [174].

*Penicillium* and *Aspergillus* are fungi that have been intensively investigated in searching for bioactive secondary metabolites, both terrestrial and marine origin while *Cladosporium* have not been investigated that much. Thus, there has been a common philosophy to avoid common genera like *Penicillium* and *Aspergillus* due to the risk of rediscoveries. However, they are continuously showing to produce novel and biologically active compounds. *Penicillium* and *Aspergillus* are among the genera with the highest index of creativity in the Kingdom of Fungi [175]. It can be found from some recent literatures which reported new compounds isolated from both fungi [176-179]. Therefore, *Aspergillus oryzae* (tenS.PKS/dmbC), *Cladosporium carrionii* (F–2) were exploited in this project for the production of new biologically active secondary metabolites, and hence; we obtained two new metabolites from *A. oryzae* and one new from *Cladosporium*.

## 7.6 Estimation of polyketide biosynthesis

There are different approaches for the estimation of polyketides biosynthesis such as feeding of stable isotopic labelled precursor, gene knockout [180]. Biotechnologist and natural products chemist deals with the identification, isolation, structure elucidation studies and the biosynthetic pathways of substances produced by living organisms especially micro–organisms [181]. The isolation of new metabolites from natural sources are challenging job but still, micro–organisms are the important source of potential natural products. The emergence of biotechnology and genetics has now transformed the basis of natural products discovery and the recent focus of research is to understand the ability of living machinery to synthesize complex chemical by manipulation in their biosynthetic pathways to produce new compounds for the drug discovery strategies.

To determine the mechanisms of intermediate events in biosynthesis of secondary metabolites, several techniques have been utilized. These include: time course production experiments; classical labelling techniques; utilization of enzyme inhibitors; and use of knockout mutants of the biosynthetic pathways.

## 7.7 Time course production of target metabolites

In time experiment the desire metabolites [$MH^+$ 211 and 213] were monitored over a specific time period in liquid culture. The yield is then compared with the time of incubation and a curve is drawn known as a time course production curve. This experiment is very much essential for further biosynthetic studies including feeding of labelled precursors. An accurate curve of the production of metabolites and time course curve is important for the incorporation studies. Because this will determines

the best time of feeding the labelled precursors to the cell cultures of selected organism for optimum uptake.

Usually feeding of putative precursors is performed just after when the target metabolites begin to be produced and before it reaches a maximum level. Time course production studies also give interesting insight into the biosynthesis of target metabolites. After identification of the parent compound, transformations to the downstream related metabolites and their inter conversions during incubation can be determined from accurate time production experiments. A detailed analysis of a fermentation time course of the producing organism and in vivo conversions of the parent product detected in the biosynthetic sequence can sometimes result in a complete picture of the biosynthetic relationships of the members of the same family of metabolites.

## 7.8 Feeding of stable isotope

The use of stable isotope labelling methods is important to study the biosynthetic pathways of secondary metabolites, labelled acetate has been successfully demonstrated in fungi for explaining the studies of biosynthetic routs of polyketides and terpenoids types secondary metabolites [70]. Single labelled stable isotopes have been utilized in many biosynthetic studies successfully. For example to determine the origin of polyketides, therefore, we fed [1–$^{13}$C] to the shaking flask cultures of *A. oryzae*. The targeted metabolites [$MH^+$ 211 and 213] were isolated from the fermentations of labelled precursors and then the metabolites were analyzed by high field $^{13}$C NMR spectroscopy. All the acetate [1–$^{13}$C] enriched sites were indicated by NMR at levels from 5–10% enhancement over the average background

level of natural $^{13}C$ at 1.1%. This showed that all the proposed precursors were accumulated into the two new metabolites by *A. oryzae*.

# CHAPTER 8

# CONCLUSIONS

# 8 CONCLUSIONS

Natural products have been produced by living organisms (fungi and plants) as secondary metabolites. The secondary metabolites have been analysed to assess their biological significance. They are very diverse; structurally and functionally and their diversity have led them active against various chronic diseases, therefore they are important for the welfare of human being [35]. Plants have been used as medicine since a long time ago (4500 to 1600 BC) and are important for the health of human and other animals, however, fungi have been estimated to produced probably more potent metabolites, whereas they have not been explored [179].

Currently, we have explored four fungal species including *A. carbonarius*, *A. oryzae*, *C. carrionii* and *C. resinae* in order to obtain some biologically potent secondary metabolites. Therefore nutrient media were optimized for the growth and production of secondary metabolites, *Aspergillus* species produced relatively more metabolites in Czapek–dox (Glucose and Starch) broth media (CGSB). Whereas; *Cladosporium* species produced relatively more metabolites in Czapek yeast extracts broth (CYB). Two additional chemicals (suberoyl anilide hydroxamic acid; SAHA and 5–azacytidine; 5–AZA) were also used as chemical inducers for all fungi except *C. carrionii*. Both the chemical compounds resulted in higher secondary metabolites production from all the species.

Secondary metabolites produced by all the fungi were subjected for their biological activities, such as antibacterial, antifungal, cytotoxic and phytotoxic assays and as result two species i.e. *A. oryzae* and *C. carrionii* were selected for their secondary metabolites production and as result three new and two known metabolites were isolated. Two new metabolites were isolated from *A. oryzae* while one new and two known metabolites were isolated from *C. carrionii* using preparative High

Performance Liquid Chromatography (HPLC) and column chromatography techniques. The new metabolites were 6–butyl–3–methylene–2–oxotetrahydro–2H–pyran–4–carboxylic acid (**A–41**), 6–butyl–3–methylene–2–oxo–3,6–dihydro–2H–pyran–4–carboxylic acid (**A–42**) and (3S,6S)–3–allyl–6–benzylpiperazine–2,5–dione (**D–44**) whereas, the known metabolites were 5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one (**C–43**), 6–(3–methylbut–2–enyl)–1H–indole–3–carboxylic acid (**45**). Furthermore; *A. oryzae* was studied for biosynthesis studies using [1–$^{13}$C] labelled acetate.

Now from the results it is clear that the fungi offers an extensive ground for the research to conduct further in this area, while the methodology used, could provide base and encouragement for the research work and to explore the fungi on scientific grounds.

# CHAPTER 9

# FUTURE RECOMENDATIIONS OR DIRECTIONS

# 9 FUTURE RECOMENDATIIONS OR DIRECTIONS

In this research, four fungal species including *A. carbonarius*, *A. oryzae*, *C. carrionii* and *C. resinae* were explored for their biological activities, and as result two species i.e. *A. oryzae* and *C. carrionii* were selected for their secondary metabolites production. Furthermore; *A. oryzae* was studied for biosynthesis studies. Therefore, it is believed that this secondary metabolites production work could be further enhanced by future studies in the following areas.

- Exploitation of *A. carbonarius* for secondary metabolites
- Exploitation of *C. resinae* for secondary metabolites
- Feeding of the isotopic precursor for estimation of the biosynthetic pathways of the new metabolite (**C**) isolated from *C. carrionii*
- Use of double labelled acetate to provide idea to the biosynthetic chemists about the structural changes of the two new metabolites **A–41**and **B–42** from *A. oryzae*.
- Alanine feeding to the two new metabolites **A–41** and **B–42** isolated from *A.oryzae* for confirmation of the role of alanine in the biosynthesis of these metabolites
- Gene knockout experiment for the confirmation of the gene (s) involved in the biosynthesis of the targeted and/or isolated metabolites.

# CHAPTER 10

# REFERENCES

# 10 REFERENCES

1. Turner G, (2000). Exploitation of fungal secondary metabolites old and new. Microbiology Today. 27: p. 118-121

2. Forbes B, D Sahm and A Weissfeld, (1998). Laboratory methods for detection of antibacterial resistance. Baily & Scotts Diagnostic Microbiology. 11: p. 229-50

3. Fox E M and B J Howlett, (2008). Secondary metabolism: regulation and role in fungal biology. Current opinion in microbiology. 11 (6): p. 481-487

4. Gao P and J Martin, (2002). Volatile metabolites produced by three strains of Stachybotrys chartarum cultivated on rice and gypsum board. Applied occupational and environmental hygiene. 17 (6): p. 430-436

5. Hertweck C, A Luzhetskyy, Y Rebets and A Bechthold, (2007). Type II polyketide synthases: gaining a deeper insight into enzymatic teamwork. Natural product reports. 24 (01): p. 162-190

6. Fleming A, (1929). On theantibacterial action of cultures of a pénicillium, with special reference to their use in the isolation of B. influenzae. But. I. Exper. Path. 10: p. 226-236

7. Zhao J, L Zhou, J Wang, T Shan, L Zhong, X Liu, and X Gao, (2010). Endophytic fungi for producing bioactive compounds originally from their host plants. Curr Res, Technol Educ Trop Appl Microbiol Microbial Biotechnol. 1: p. 567-576

8. John M, K Krohn, U Flörke, H J Aust, S Draeger, and B Schulz, (1999). Biologically Active Secondary Metabolites from Fungi. 12. 1 Oidiolactones AF, Labdane Diterpene Derivatives Isolated from Oidiodendron truncata §. Journal of Natural Products. 62 (9): p. 1218-1221

9. Strobel G A, R V Miller, C Martinez-Miller, M M Condron, D B Teplow, and W Hess, (1999). Cryptocandin, a potent antimycotic from the endophytic fungus Cryptosporiopsis cf. quercina. Microbiology. 145 (8): p. 1919-1926

10. Singh M, J E Janso, S Luckman, S F Brady, J Clardy, M Greenstein, and W Maiese, (2000). Biological activity of guanacastepene, a novel diterpenoid antibiotic produced by an unidentified fungus CR115. ChemInform. 31 (31): p. no-no

11. Yamada T, M Iritani, M Doi, K Minoura, T Ito, and A Numata, (2001). Absolute stereostructures of cell-adhesion inhibitors, macrosphelides C, E–G and I, produced by a Periconia species separated from an Aplysia sea hare. Journal of the Chemical Society, Perkin Transactions 1. (22): p. 3046-3053

12. Blunt J W, B R Copp, W P Hu, M H G Munro, P T Northcote, and M R Prinsep, (2008). Marine natural products. ChemInform. 39 (20): p. no-no

13. Banerjee S, Z Wang, M Mohammad, F H Sarkar and R M Mohammad, (2008). Efficacy of Selected Natural Products as Therapeutic Agents against Cancer⊥. Journal of Natural Products. 71 (3): p. 492-496

14. Fremlin L J, A M Piggott, E Lacey and R J Capon, (2009). Cottoquinazoline A and cotteslosins A and B, metabolites from an australian marine-derived strain of Aspergillus versicolor. Journal of Natural Products. 72 (4): p. 666-670

15. Lee Y M, J K Hong and K S Bae, (2007). Polyketides from a sponge-derived fungus, Aspergillus versicolor. Natural Product Sciences. 13 (1): p. 90-96

16. Wicklow D, P Dowd, A Alfatafta and J Gloer, (1996). Ochratoxin A: an antiinsectan metabolite from the sclerotia of Aspergillus carbonarius NRRL 369. Canadian journal of microbiology. 42 (11): p. 1100-1103

17. Crous P, U Braun, K Schubert and J Z Groenewald, (2007). Delimiting Cladosporium from morphologically similar genera. Studies in Mycology. 58 (1): p. 33-56

18. Rivas S and C M Thomas, (2005). Molecular interactions between tomato and the leaf mold pathogen Cladosporium fulvum. Annu. Rev. Phytopathol. 43: p. 395-436

19. Henry L and A Ross, (1967). Chromoblastomycosis (possibly Cladosporium) of the breast in an English woman. Journal of Clinical Pathology. 20 (2): p. 124-127

20. Stierle A, G Strobel and D Stierle, (1993). Taxol and taxane production by Taxomyces andreanae, an endophytic fungus of Pacific yew. Science (New York, NY). 260 (5105): p. 214

21. Bultman T L and J C Murphy, (2000). Do fungal endophytes mediate wound-induced resistance. Microbial Endophytes. p. 421-452

22. Berdy J. Are actinomycetes exhausted as source of secondary metabolites? in Proceedings of the 9th International Symposium on the Biology of Actinomycetes. 1995.

23. Strohl W R, (1997) Biotechnology of antibiotics. Vol. 82: M. Dekker.

24. Bentley R and J Bennett, (1999). Constructing polyketides: from Collie to combinatorial biosynthesis. Annual Reviews in Microbiology. 53 (1): p. 411-446

25. Hopwood D A and D H Sherman, (1990). Molecular genetics of polyketides and its comparison to fatty acid biosynthesis. Annual review of genetics. 24 (1): p. 37-62

26. Bergmann S, J Schümann, K Scherlach, C Lange, A Brakhage, and C Hertweck, (2007). Genomics-driven discovery of PKS-NRPS hybrid metabolites from Aspergillus nidulans. Nature chemical biology. 3 (4): p. 213-217

27. Ahmad B and A A. Khan, (2013). Antibacterial, antifungal and phytotoxic activities of Luffa cylindrica and Momordica charantia. Journal of Medicinal Plants Research. 07 (22): p. 1593-1599

28. Abid Ali Khan, (2009). Phytochemical Evaluation, Bioassay Screening and Standardization of Luffa cylindrica and Momordica charantia. M Phill Thesis (Biotechnology). p. 1-117

29. Moore D and L N Frazer, (2002) Essential fungal genetics: Springer.

30. Cox R J, (2007). Polyketides, proteins and genes in fungi: programmed nano-machines begin to reveal their secrets. Organic & biomolecular chemistry. 05 (13): p. 2010-2026

31. Ou S H, (1985). Blast. Rice diseases. 2nd ed. Wallingford, UK: CAB International. p. 109-201

32. Griffin D H, (1996) Fungal physiology: Wiley-Liss.

33. Goffeau A, B G Barrell, H Bussey, R W Davis, B Dujon, H Feldmann, F Galibert, J D Hoheisel, C Jacq, and M Johnston, (1996). Life with 6000 genes. Science. 274 (5287): p. 546-567

34. Mewes H W, K Albermann, M Bahr, D Frishman, A Gleissner, J Hani, K Heumann, K Kleine, A Maierl, and S G Oliver, (1997). Overview of the yeast genome. Nature. 387 (6632): p. 7-8

35. Zerikly M and G L Challis, (2009). Strategies for the discovery of new natural products by genome mining. ChemBioChem. 10 (04): p. 625-633

36. Newman D J, G M Cragg and K M Snader, (2003). Natural products as sources of new drugs over the period 1981-2002. Journal of Natural Products. 66 (07): p. 1022-1037

37. Lopez S N, I Ramallo, M G Sierra, S Zacchino and R L E Furlan, (2007). Chemically engineered extracts as an alternative source of bioactive natural product-like compounds. Proceedings of the National Academy of Sciences. 104 (02): p. 441-444

38. Bills G F, G Platas, A Fillola, M R Jimenez, J Collado, F Vicente, J Martin, A Gonzalez, J Bur Zimmermann, and J R Tormo, (2008). Enhancement of antibiotic and secondary metabolite detection from filamentous fungi by growth on nutritional arrays. Journal of applied microbiology. 104 (06): p. 1644-1658

39. Munchhof M J and A Meyers, (1995). An Asymmetric Route to Chiral, Nonracemic 2-Substituted Piperidines. Synthesis of (-)-Pipecoline,(+)-Coniine, and (-)-Coniceine. The Journal of Organic Chemistry. 60 (22): p. 7084-7085

40. Hanson D, P L Traynor, K M Ditz and D Reicosky, (1981). Gramine in Barley Forage - Effects of Genotype and Environment1. Crop Sci. 21 (5): p. 726-730

41. Virag R, (1982). Intracavernous injection of papaverine for erectile failure. The Lancet. 320 (8304): p. 938

42. Brodie B B and S Udenfriend, (1943). The estimation of quinine in human plasma with a note on the estimation of quinidine. Journal of Pharmacology and Experimental Therapeutics. 78 (2): p. 154-158

43. Gershenzon J and N Dudareva, (2007). The function of terpene natural products in the natural world. Nature chemical biology. 03 (07): p. 408-414

44. Sacchettini J C and C D Poulter, (1997). Creating isoprenoid diversity. Science. 277 (5333): p. 1788-1789

45. Barkovich R and J C Liao, (2001). Review: metabolic engineering of isoprenoids. Metabolic engineering. 3 (1): p. 27-39

46. Banthorpe D and D Baxendale, (1970). Terpene biosynthesis. Part III. Biosynthesis of (+)-and (–)-camphor in Artemisia, Salvia, and Chrysanthemum species. J. Chem. Soc. c. (19): p. 2694-2696

47. Bouwmeester H J, J Gershenzon, M C J M Konings and R Croteau, (1998). Biosynthesis of the Monoterpenes Limonene and Carvone in the Fruit of Caraway I. Demonstration of Enzyme Activities and Their Changes with Development. Plant Physiology. 117 (3): p. 901-912

48. Hughes P R, (1974). Myrcene: A precursor of pheromones in Ips beetles. Journal of Insect Physiology. 20 (7): p. 1271-1275

49. Wolf G, (1996). A history of vitamin A and retinoids. The FASEB Journal. 10 (9): p. 1102-1107

50. Govindachari T, P Mohamed and P Parthasarathy, (1970). Ishwarane and aristolochene, two new sesquiterpene hydrocarbons from Aristolochia indica. Tetrahedron. 26 (02): p. 615-619

51. Kawaide H, T Sassa and Y Kamiya, (1995). Plant-like biosynthesis of gibberellin A in the fungus Phaeosphaeria sp. L487. Phytochemistry. 39 (2): p. 305-310

52. Finking R and M a Marahiel, (2004). Biosynthesis of nonribosomal peptides 1. Annu. Rev. Microbiol. 58: p. 453-488

53. Sieber S A and M Marahiel, (2005). Molecular mechanisms underlying nonribosomal peptide synthesis: approaches to new antibiotics. Chem Rev. 105 (2): p. 715-38

54. Mootz H D, D Schwarzer and M Marahiel, (2002). Ways of assembling complex natural products on modular nonribosomal peptide synthetases. ChemBioChem. 3 (6): p. 490-504

55. Umezawa H, K Maeda, T Takeuchi and Y Okami, (1966). New antibiotics, bleomycin A and B. The Journal of antibiotics. 19 (05): p. 200-209

56. Weber G, K Schörgendorfer, E Schneider-Scherzer and E Leitner, (1994). The peptide synthetase catalyzing cyclosporine production in Tolypocladium niveum is encoded by a giant 45.8-kilobase open reading frame. Current genetics. 26 (2): p. 120-125

57. Stachelhaus T M M, (1995). Modular structure of peptide synthetases revealed by dissection of the multifunctional enzyme GrsA. J. Biol. Chem. 270: p. 6163-6169

58. Mootz Hd M M, (1997). The tyrocidine biosynthesis operon of Bacillus brevis: complete nucleotide sequence and biochemical characterization of functional internal denylation domains. J. Bacteriol. 179: p. 6843-6850

59. Conti E, Stachelhaus T, Marahiel M.A and Brick P, (1997). Structural basis for the activation of phenylalanine in the nonribosomal biosynthesis of gramicidin S. EMBO J. 16 (4174-4183): p.

60. Stachelhaus T, Mootz Hd, Bergendahl V and M Ma., (1998). Peptide bond formation in nonribosomal peptide biosynthesis. Catalytic role of the condensation domain. J. Biol. Chem. 273: p. 22773-22781

61. Stachelhaus T, Huser A and M Ma., (1996). Biochemical characterization of peptidyl carrier protein (PCP), the thiolation domain of multifunctional peptide synthetases. Chem. Biol. 3: p. 913-921

62. Kejing T, L Du, X Sun, M Cai, T Zhu, X Zhou, Q Gu, and Y Zhang, (2009). Biosynthesis of aspergiolide A, a novel antitumor compound by a marine-derived fungus Aspergillus glaucus via the polyketide pathway. Tetrahedron Letters. 50 (00): p. 1082–1085

63. Staunton J and K J Weissman, (2001). Polyketide biosynthesis: a millennium review. Natural product reports. 18 (04): p. 380-416

64. Chan Y A, A M Podevels, B M Kevany and M G Thomas, (2009). Biosynthesis of polyketide synthase extender units. Natural product reports. 26 (01): p. 90-114

65. Shen B, (2003). Polyketide biosynthesis beyond the type I, II and III polyketide synthase paradigms. Current opinion in chemical biology. 07 (02): p. 285-295

66. Hopwood D A, (1997). Genetic contributions to understanding polyketide synthases. Chemical reviews. 97 (07): p. 2465-2498

67. Crump M P, J Crosby, C E Dempsey, J Parkinson, M Murray, D Hopwood, and T J Simpson, (1997). Solution structure of the actinorhodin polyketide synthase acyl carrier protein from Streptomyces coelicolor A3 (2). Biochemistry. 36 (20): p. 6000-6008

68. Birch A, R Massy-Westropp and C Moye, (1955). Studies in relation to biosynthesis. VII. 2-Hydroxy-6-methylbenzoic acid in Penicillium griseofulvum Dierckx. Australian Journal of Chemistry. 8 (4): p. 539-544

69. Shoolingin-Jordan P and I Campuzano, (1999). Biosynthesis of 6-methylsalicylic acid. Comprehensive Natural Products Chemistry. 1: p. 345-365

70. Simpson T J, (1998) Application of isotopic methods to secondary metabolic pathways, in Biosynthesis. Springer. p. 1-48.

71. Simpson T J, (1987). Applications of multinuclear NMR to structural and biosynthetic studies of polyketide microbial metabolites. Chem. Soc. Rev. 16: p.

72. Vaidya I and A S Rabba, (1993). Fungi in folk medicine. Folklore. 100: p. 102-106

73. Simpson T, (1987). Applications of multinuclear NMR to structural and biosynthetic studies of polyketide microbial metabolites. Chemical Society Reviews. 16: p. 123-160

74. Pachlatko J, (1998). Natural products in crop protection. Chimia. 52: p. 29-47

75. Fox T, M Fertleman, P Cahill and R D Palmer, (2003). Medical slang in British hospitals. Ethics & Behavior. 13: p. 173-189

76. Amann R I, R Ludwig and K Schleifer, (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiological Reviews. 59: p. 143-169

77. Kaeberlein T, Lewis, K. And Epstein, S. S., (2002). Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment. Science, . 296: p. 1127-1129

78. Handelsman J, (2004). Metagenomics: Applications of genomics to uncultured microorganisms. Microbiology and Molecular Biology Reviews. 68: p. 669-685

79. Daniel R, (2004). The soil metagenome – a rich resource for the discovery of novel natural products. Current Opinion in Biotechnology. 15: p. 199-204

80. Bode H B and R Muller, (2005). The impact of bacterial genomics on natural product research. Angewandte Chemie International Edition. 44: p. 6828-6846

81. Pelzer S, A Vente and A Bechthold, (2005). Novel natural compounds obtained by genome-based screening and genetic engineering Current Opinion in Drug Discovery and Development. 8: p. 228-238.

82. Knight V, J J Sanglier, D Ditullio, S Braccili, P Bonner, J Waters, Hughes, D., and L Zhang, (2003). Diversifying microbial natural products for drug discovery Applied Microbiology and Biotechnology. 62: p. 446-458

83. Bode H B, Bethe, B., Hofs, R. And Zeeck, A., (2002). Big effects from small changes: Possible ways to explore nature's chemical diversity ChemBioChem. 03: p. 619-627

84. Strobel G and B Daisy, (2003). Bioprospecting for microbial endophytes and their natural products. Microbiology and Molecular Biology Reviews. 67 (4): p. 491-502

85. Demain a L, (2006). From natural products discovery to commercialization: a success story. Journal of industrial microbiology & biotechnology. 33 (7): p. 486-495

86. Gullo V P, J Mcalpine, K S Lam, D Baker and F Petersen, (2006). Drug discovery from natural products. Journal of industrial microbiology & biotechnology. 33 (7): p. 523-531

87. Demain a L, (2000). Microbial biotechnology. Trends in Biotechnology. 18 (1): p. 26-31

88. Pinto a C, D H S Silva, V D S Bolzani, N P Lopes and R D a Epifanio, (2002). Produtos naturais: atualidade, desafios e perspectivas. Química nova. 25 (1): p. 45-61

89. Deacon H, (2006). Towards a sustainable theory of health-related stigma: lessons from the HIV/AIDS literature. Journal of community & applied social psychology. 16 (6): p. 418-425

90. Demain A, (1999). Pharmaceutically active secondary metabolites of microorganisms. Applied microbiology and biotechnology. 52 (4): p. 455-463

91. Ebel R, ( 2010). Natural product diversity from marine fungi. In: Mander L, Liu H-W, eds. Comprehensive Natural Products II: Chemistry and Biology. Oxford, Elsevier. 2: p. 223-262.

92. Gibson D G, J I Glass, C Lartigue, V N Noskov, R-Y Chuang, M a Algire, G a Benders, M G Montague, L Ma, and M M Moodie, (2010). Creation of a bacterial cell controlled by a chemically synthesized genome. Science. 329 (5987): p. 52-56

93. Muller H, E Carlson and A Schalet, (1961). Mutation by alteration of the already existing gene. Genetics. 46 (2): p. 213-226

94. Kilbey B, (1995). Charlotte Auerbach (1899-1994). Genetics. 141 (1): p. 1-5

95. Jackson D A, R H Symons and P Berg, (1972). Biochemical method for inserting new genetic information into DNA of Simian Virus 40: circular SV40 DNA molecules containing lambda phage genes and the galactose operon of Escherichia coli. Proceedings of the National Academy of Sciences. 69 (10): p. 2904-2909

96. Goeddel D V, D G Kleid, F Bolivar, H L Heyneker, D G Yansura, R Crea, T Hirose, A Kraszewski, K Itakura, and A D Riggs, (1979). Expression in

Escherichia coli of chemically synthesized genes for human insulin. Proceedings of the National Academy of Sciences. 76 (1): p. 106-110

97. Meyer V, (2008). Genetic engineering of filamentous fungi—progress, obstacles and future trends. Biotechnology advances. 26 (2): p. 177-185

98. Ruiz-Díez B, (2002). Strategies for the transformation of filamentous fungi. Journal of applied microbiology. 92 (2): p. 189-195

99. Fincham J, (1989). Transformation in fungi. Microbiological reviews. 53 (1): p. 148-170

100. Gouka R J, C Gerk, P J Hooykaas, P Bundock, W Musters, C T Verrips, and M J De Groot, (1999). Transformation of Aspergillus awamori by Agrobacterium tumefaciens–mediated homologous recombination. Nature Biotechnology. 17 (6): p. 598-601

101. Michielse C B, P J Hooykaas, C a Van Den Hondel and A F Ram, (2005). Agrobacterium-mediated transformation as a tool for functional genomics in fungi. Current genetics. 48 (1): p. 1-17

102. Haber J E, (1999). DNA repair: Gatekeepers of recombination. Nature. 398 (6729): p. 665-667

103. Timberlake W E and A M Marshall, (1989). Genetic engineering of filamentous fungi. Science. 244 (4910): p. 1313-1317

104. Jacobs D I, M Olsthoorn, I Maillet, M Akeroyd, S Breestraat, S Donkers, R Van Der Hoeven, C Van Den Hondel, R Kooistra, and T Lapointe, (2009). Effective lead selection for improved protein production in Aspergillus niger based on integrated genomics. Fungal Genetics and Biology. 46 (1): p. S141-S152

105. Meyer V, D Mueller, T Strowig and U Stahl, (2003). Comparison of different transformation methods for Aspergillus giganteus. Current genetics. 43 (5): p. 371-377

106. Chi J-T, H Y Chang, N N Wang, D S Chang, N Dunphy, and P O Brown, (2003). Genomewide view of gene silencing by small interfering RNAs. Proceedings of the National Academy of Sciences. 100 (11): p. 6343-6346

107. Meister G and T Tuschl, (2004). Mechanisms of gene silencing by double-stranded RNA. Nature. 431 (7006): p. 343-349

108. Agrawal N, P Malhotra and R K Bhatnagar, (2004). siRNA-directed silencing of transgene expressed in cultured insect cells. Biochemical and biophysical research communications. 320 (2): p. 428-434

109. Heneghan M N, A M Costa, M P Challen, P R Mills, A Bailey, and G D Foster, (2007). A comparison of methods for successful triggering of gene silencing in Coprinus cinereus. Molecular biotechnology. 35 (3): p. 283-296

110. Zheng X, Y Kobayashi and M Takeuchi, (1998). Construction of a low-serine-type-carboxypeptidase-producing mutant of Aspergillus oryzae by the expression of antisense RNA and its use as a host for heterologous protein secretion. Applied microbiology and biotechnology. 49 (1): p. 39-44

111. Nakayashiki H, (2005). RNA silencing in fungi: mechanisms and applications. FEBS letters. 579 (26): p. 5950-5957

112. Berdy J, (2005). Bioactive microbial metabolites. The Journal of antibiotics. 58 (1): p. 1-26

113. Karlsson J, M Saloheimo, M Siika-Aho, M Tenkanen, M Penttilä, and F Tjerneld, (2001). Homologous expression and characterization of Cel61A (EG IV) of Trichoderma reesei. European Journal of Biochemistry. 268 (24): p. 6498-6507

114. Sims J W and E W Schmidt, (2008). Thioesterase-like role for fungal PKS-NRPS hybrid reductive domains. Journal of the American Chemical Society. 130 (33): p. 11149-11155

115. Collemare J, A Billard, H U Bohnert and M H Lebrun, (2008). Biosynthesis of secondary metabolites in the rice blast fungus Magnaporthe grisea: the role of hybrid PKS-NRPS in pathogenicity. Mycological research. 112 (02): p. 207-215

116. Weinacker A, A Chen, M Agrez, R Cone, S Nishimura, E Wayner, R Pytela, and D Sheppard, (1994). Role of the integrin alpha v beta 6 in cell attachment to fibronectin. Heterologous expression of intact and secreted forms of the receptor. Journal of Biological Chemistry. 269 (9): p. 6940-6948

117. Sweeney H, A J Straceski, L a Leinwand, B a Tikunov and L Faust, (1994). Heterologous expression of a cardiomyopathic myosin that is defective in its actin interaction. Journal of Biological Chemistry. 269 (3): p. 1603-1605

118. Pickens L B, Y Tang and Y-H Chooi, (2011). Metabolic engineering for the production of natural products. Annual review of chemical and biomolecular engineering. 2: p. 211-236

119. Pfeifer B A and C Khosla, (2001). Biosynthesis of polyketides in heterologous hosts. Microbiology and Molecular Biology Reviews. 65 (1): p. 106-118

120. Tang L, S Shah, L Chung, J Carney, L Katz, C Khosla, and B Julien, (2000). Cloning and heterologous expression of the epothilone gene cluster. Science. 287 (5453): p. 640-642

121. Ahmed S, S Riaz and A Jamil, (2009). Molecular cloning of fungal xylanases: an overview. Applied microbiology and biotechnology. 84 (1): p. 19-35

122. Bacha N, (2009). CHARACTERIZATION polyketones SYNTHASES INVOLVED IN BIOSYNTHESIS OF OCHRATOXIN, penicillic ACID, AND OF ASPERLACTONE On isoasperlactone AT Aspergillus westerdijkiae. Ph D Thesis (Microbiology & Industrial Biocatalysis). p. 1-255

123. L M Halo, M N Heneghan, A a Yakasai, Z Song, K Williams, A M Bailey, R J Cox, C M Lazarus, and T J Simpson, (2008). Late stage oxidations during the biosynthesis of the 2-pyridone tenellin in the entomopathogenic fungus Beauveria bassiana. Journal of the American Chemical Society. 130 (52): p. 17988-17996

124. Halo L M, J W Marshall, A a Yakasai, Z Song, C P Butts, M P Crump, M Heneghan, A M Bailey, T J Simpson, C M Lazarus, and R J Cox, (2008). Authentic heterologous expression of the tenellin iterative polyketide synthase nonribosomal peptide synthetase requires coexpression with an enoyl reductase. ChemBioChem. 9 (4): p. 585-94

125. Bakeer W I M M, (2011). Overexpression of Secondary Metabolism Genes from Magnaporthe grisea and Beauveria bassiana. Ph D Thesis (Fungal Biotechnology) p. 01-196

126. Davet P and F Rouxel, (1997) Detecting and isolating soil fungi: Institut National de la Recherche Agronomique (INRA).

127. Chesters C G C and R H Thornton, (1956). A comparison of techniques for isolating soil fungi. Transactions of the British Mycological Society. 39 (3): p. 301-313

128. Cox R J and D O'hagan, (1991). Synthesis of isotopically labelled 3-amino-2-phenylpropionic acid and its role as a precursor in the biosynthesis of tenellin and tropic acid. J. Chem. Soc., Perkin Trans. 1. (10): p. 2537-2540

129. Benkeblia N, (2004). Antimicrobial activity of essential oil extracts of various onions (Allium cepa) and garlic (Allium sativum). LWT-Food Science and Technology. 37 (2): p. 263-268

130. Gulluce M, M Sokmen, D Daferera, G Agar, H Ozkan, N Kartal, M Polissiou, A Sokmen, and F Sahin, (2003). In vitro antibacterial, antifungal, and antioxidant activities of the essential oil and methanol extracts of herbal parts and callus cultures of Satureja hortensis L. Journal of agricultural and food chemistry. 51 (14): p. 3958-3965

131. Atta-Ur-Rahman, M I Choudhary and J Thomsen. Manual of bioassay techniques for natural product research. 1999, Harward Academic Press, Amsterdam.

132. Meyer Bn, Ferrigni Nr, J L Putnam Je, Nichols De and M Jl, (1982). Brine shrimp: a convenient general biossay for active plant constituents. Plant. Med. 45: p. 31-34

133. Mclaughlin J L, (1988). Brine shrimp, crown gall tumors: simple bioassays for the discovery of plant antitumor agents. Proceedings of NIH Workshop, Bioassay For Discovery of Antitumoral, Antiviral Agents from Natural Sources,. Bethesda. 18 (19): p. 22

134. De Almeida L F, F Rolim Frei, E D Mancini, L Martino and V De Feo, (2010). Phytotoxic activities of Mediterranean essential oils. Molecules. 15 (6): p. 4309-4323

135. Gerwick W H, P J Proteau, D G Nagle, E Hamel, A Blokhin, and D L Slate, (1994). Structure of curacin A, a novel antimitotic, antiproliferative and brine shrimp toxic natural product from the marine cyanobacterium Lyngbya majuscula. The Journal of Organic Chemistry. 59 (6): p. 1243-1245

136. Hostettmann K, A Marston and M Hostettmann, (1997) Preparative chromatography techniques: applications in natural product isolation: Springer.

137. Heneghan M N, A a Yakasai, K Williams, K a Kadir, Z Wasil, W Bakeer, K M Fisch, A M Bailey, T J Simpson, and R J Cox, (2011). The programming role of trans-acting enoyl reductases during the biosynthesis of highly reduced fungal polyketides. Chemical Science. 2 (5): p. 972-979

138. Lu H, W X Zou, J C Meng, J Hu and R X Tan, (2000). New bioactive metabolites produced by< i> Colletotrichum</i> sp., an endophytic fungus in< i> Artemisia annua</i>. Plant Science. 151 (1): p. 67-73

139. Helmholz H, P Etoundi and U Lindequist, (1999). Cultivation of the marine basidiomycete Nia vibrissa (Moore & Meyers). J. Biotechnol. 70: p. 203-206

140. Choi P J, J Sperry and M a Brimble, (2010). Heteroatom-Directed Reverse Wacker Oxidations. Synthesis of the Reported Structure of (−)-Herbaric Acid. The Journal of Organic Chemistry. 75 (21): p. 7388-7392

141. Holler U, J.B. Gloer and D. T. Wicklow., (2002). Biologically active polyketide metabolites from an undetermined fungicolous hyphomycete resembling Cladosporium. J. Nat. Prod. . 65: p. 876-882.

142. Lauro Figueroa Valverde, Francisco Díaz Cedillo, Guillermo Ceballos Reyes and M L Ramos, (2008). Synthesis and Antibacterial Activity of Pregnenolone-Vitamin B1 Conjugate. J. Mex. Chem. Soc. 52 (2): p. 103-135

143. Hadi Mehrgan F M, Shima Pakdaman and Mahshid Poursaeed, (2008). Antibacterial Activity of Thymus pubescens Methanolic Extract. Iranian Journal of Pharmaceutical Research. 7 (4): p. 291-295

144. Chanda J P a S, (2008). In vitro antifungal activity of methanol extracts of some Indian medicinal plants against pathogenic yeast and moulds African Journal of Biotechnology. 7 (23): p. 4349-4353

145. Sanjesh G Rathi V H B a P G P, (2010). Antifungal Activity of Embelia Ribes Plant Extracts. International Journal on Pharmaceutical and Biological Research. 1 (1): p. 6-10

146. Pirzada a J, Shaikh, W., Usmanghani, K and Mohiuddin E., (2010). Antifungal Activity of Dodonaea Viscosa Jacq Extract on Pathogenic Fungi Isolated From Super Ficial Skin Infection. Pak. J. Pharm. Sci.,. 23 (3): p. 337-340

147. Raquel Carvalho Montenegro P C J, Ro Mulo Augusto Feio Farias, Manoel Andrade-Neto, Franciglauber Silva Bezerra, Maria Elisabete A. Moraes, Manoel Odorico De Moraes, Cla´Udia Pessoa and Letı´Cia V. Costa-Lotufo,, (2004). Cytotoxic Activity of Pisosterol, a Triterpene Isolated from Pisolithus tinctorius (Mich.: Pers.) Coker & Couch, 1928. Z. Naturforsch. 59c (1): p. 519-522

148. Siddhardha B P P, Suryanarayana M. and V Y Upadyayula, (2009). Isolation and Biological Evaluation of Two Bioactive Metabolites from Aspergillus gorakhpurensis. Rec. Nat. Prod. . 3 (3): p. 161-164

149. Fisch. K. M G a F, Gipson. M, , H a R Henrikson. J. C, Jackson. L, and W H a C R H Najar. F. Z, (2009). Chemical induction of silent biosynthetic pathway transcription in Aspergillus niger. J Ind Microbiol Biotechnol 36: p. 1199-1213

150. Jon C. Henrikson a R H, P. Matthew Joyner and Robert H. Cichewicz, (2009). A chemical epigenetics approach for engineering the in situ biosynthesis of a cryptic natural product from Aspergillus niger. Org. Biomol. Chem. 7: p. 435–438

151. Hawksworth D L, (1991). The fungal dimension of biodiversity: magnitude, significance, and conservation. Mycol. Res. 95 (6): p. 641-655.

152. Hawksworth D L, (2001). The magnitude of fungal diversity: the 1.5 million species estimate revisited. Mycol. Res. . 105 (12): p. 1422-1432

153. Mueller G M and J P Schmit, (2007). Fungal biodiversity: What do we know? What can we predict? . Biodivers. Conserv.: p. 16, 1-5

154. Cannon P F, (1997). Strategies for rapid assessment of fungal diversity. . Biodivers. Conserv. 6: p. 669-680

155. Kohlmeyer J and E Kohlmeyer, ( 1979). Marine Mycology: The Higher Fungi;. Academic Press; New York. p.

156. Kendrick B, (2000). The Fifth Kingdom, 3rd ed.; . Focus Publ.:USA.: p.

157. Jennings D H and G Lysek, (1999). Fungal Biology: Understanding the Fungal Lifestyle. 2nd ed. . p.

158. Walker G M and N a White, ( 2005). Introduction to Fungal Physiology. In Fungi: Biology and Applications. Kavanagh, K. ed.; Wiley: West Sussex.: p.

159. Kreisel H and F Schauer, (1987). Methoden des mykologischen Laboratoriums. Veb Gustav Fisher Verlag, Jena.: p.

160. Shushni M a M, R Mentel, U Lindequist, R Jansen and A-F Balticols, (2009). , naphthalenone derivatives with antiviral activity, from an ascomycetous fungus. Chem. Biodivers. . 6. 127-137: p.

161. Griffin D H, (1994). Fungal Physiology, 2nd ed.; Wiley-Liss Inc.: New York.: p.

162. Debbab A, A H Aly, W H Lin and P Proksch, (2010). Bioactive compounds from marine bacteria and fungi. . Microb. Biotechnol.: p.

163. Cundliffe E and A L Demain, (2010). Avoidance of suicide in antibiotic-producing microbes. J. Ind. Microbiol. Biotechnol. . 37: p. 643-672

164. Vining L C, (1990). Functions of secondary metabolites. Annu. Rev. Microbiol. 44: p. 395-427

165. Sato Y, H Shibata, N Arakaki and T Higuti, (2004). 6, 7-dihydroxyflavone dramatically intensifies the susceptibility of methicillin-resistant or -sensitive Staphylococcus aureus to beta-lactams. Antimicrob. Agents Chemother. 48: p. 1357-1360

166. Hx X and L Sf, (2001). Activity of plant flavonoids against antibiotic-resistant bacteria. Phytother. Res. 15: p. 39-43

167. Mehrgana H, F Mojabb, S Pakdamanc and M Poursaeed, (2008). Antibacterial Activity of Thymus pubescens Methanolic Extract. Iranian Journal of Pharmaceutical Research. 7 (4): p. 291-295

168. Tsuchiya H, M Sato and T Miyazaki, (1996). Comparative study on antibacterial activity of phythochemical flavanones against methicillin-resistant Sthaphylococcus aureus. J. Ethnopharmacol. 50: p. 27-34

169. Gomez-Rodríguez O, E Zavaleta-Mejia, V Gonzalez-Hernandez, M Livera-Munoz and E Cardenas-Soriano, (2003). Allelopathy and microclimatic modification of intercropping with marigold on tomato early blight disease development. Field Crops Res. 83: p. 27-34

170. Mancini F, A J Termorshuizen , J L S Jiggins and A H C Van Bruggen, (2008). Increasing the environmental and social sustainability of cotton farming through farmer education in Andhra Pradesh, India. Agric. Systems. 96: p. 16-25

171. Bains P S, J P Tewari and W a Ayer, (1993). A note on phytotoxicity of homodestruxin B – a compound produced by Alternaria brassicae. Phytoprotection. 3: p. 157-160

172. Schulz B, C Boyle, S Draeger, A-K Römmert and K Krohn, (2002). Endophytic fungi: a source of novel biologically active secondary metabolites. . Mycol. Res. 106 (9): p. 996-1004

173. Miller J D, (1986). Secondary Metabolites in Lignicolous Marine Fungi. In The Biology of Marine Fungi. Moss, S.T.; Ed.: . Cambridge University Press; Cambridge.: p. 61-68

174. Kansoh a L, O H Khattab, Z M Abd-Elrazek and H M Motawea, (2010). Broad spectrum antimicrobial agent from a local isolate of marine fungus strain. J. Appl. Sci. Res. . 6 (6): p. 580-588

175. Rømer-Rassing B and H Gürtler, (2002). In Integration of Modern Taxonomic Methods for Penicillium and Aspergillus Classification. Samson, R.A.; Pitt, J.I., eds. . Harwood Academic Publisher. Australia. p.

176. Zhao W Y, T J Zhu, G T Fan, H B Liu, Z C Fang, Q Q Gu, and W M Zhu, (2010). Three new dioxopiperazine metabolites from a marine-derived fungus Aspergillus fumigatus Fres. . Nat. Prod. Res. . 24 (107): p. 953-95

177. Lee Y M, H Li, J Hong, H Y Cho, K S Bae, M a Kim, D-K Kim, and J H Jung, (2010). Bioactive metabolites from the sponge-derived fungus Aspergillus versicolor. Arch. Pharm. Res. 33 (2): p. 231-235

178. Wei M Y, C Y Wang, Q a Liu, C L Shao, Z G She, and Z C Lin, (2010). Five sesqui-terpenoids from a marine-derived fungus Aspergillus sp. isolated from a gorgonian Dichotella gemmacea. . Mar. Drugs. 8: p. 941-949

179. Oliyiowola A, (1984). W.H.O Chronicle. 38: p. 76

180. Schroeckh V, K Scherlach, H-W Nützmann, E Shelest, W Schmidt-Heck, J Schuemann, K Martin, C Hertweck, and A a Brakhage, (2009). Intimate bacterial–fungal interaction triggers biosynthesis of archetypal polyketides in Aspergillus nidulans. Proceedings of the National Academy of Sciences. 106 (34): p. 14558-14563

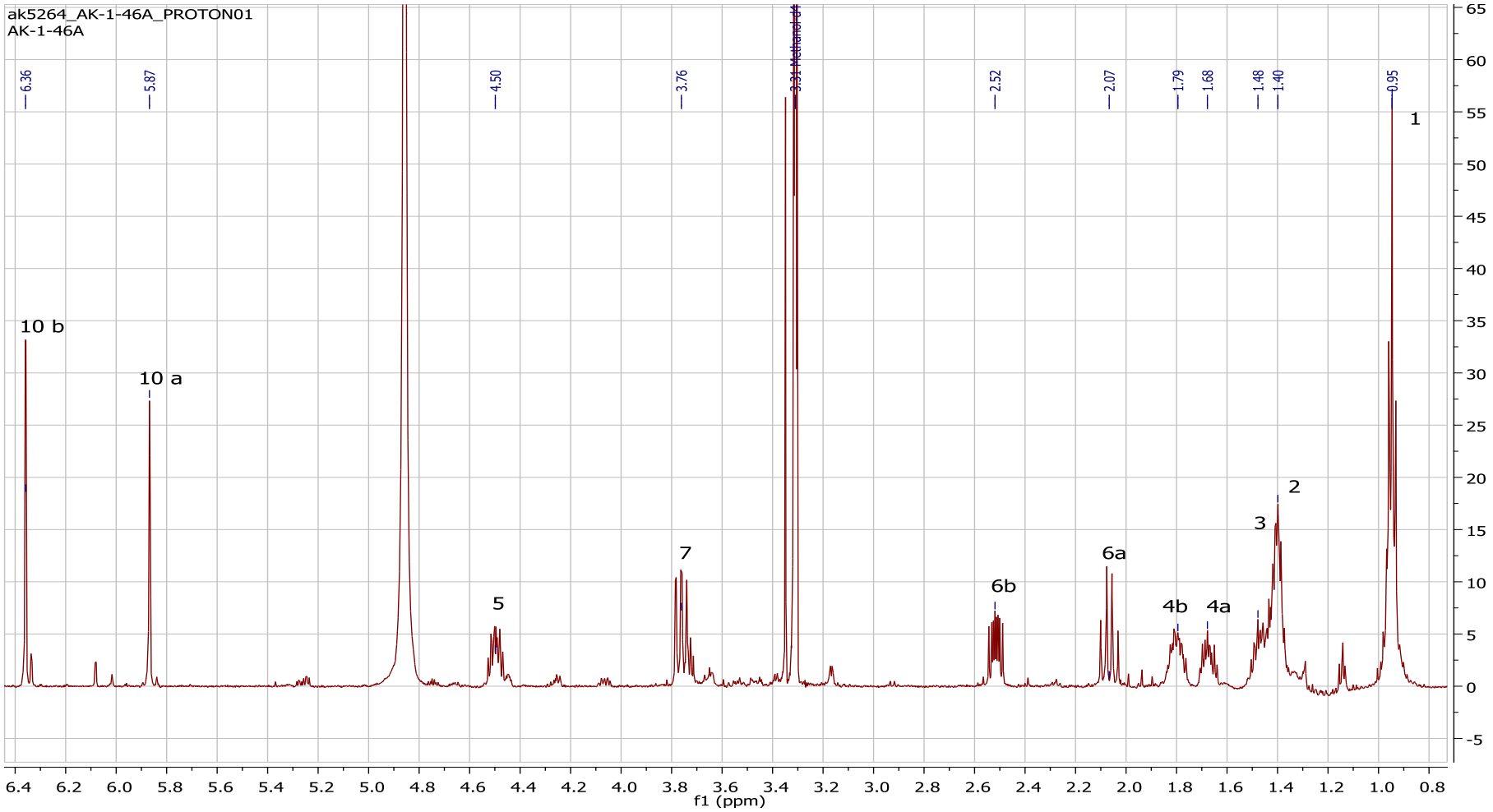
181. Bergamo D C B, M J Kato, V S Bolzani and M Furlan, (2005). Biosynthetic origins of the isoprene units of 4-nerolidylcatechol in Potomorphe umbellata. Journal of the Brazilian Chemical Society. 16 (6B): p. 1406-1409.

# APPENDICES

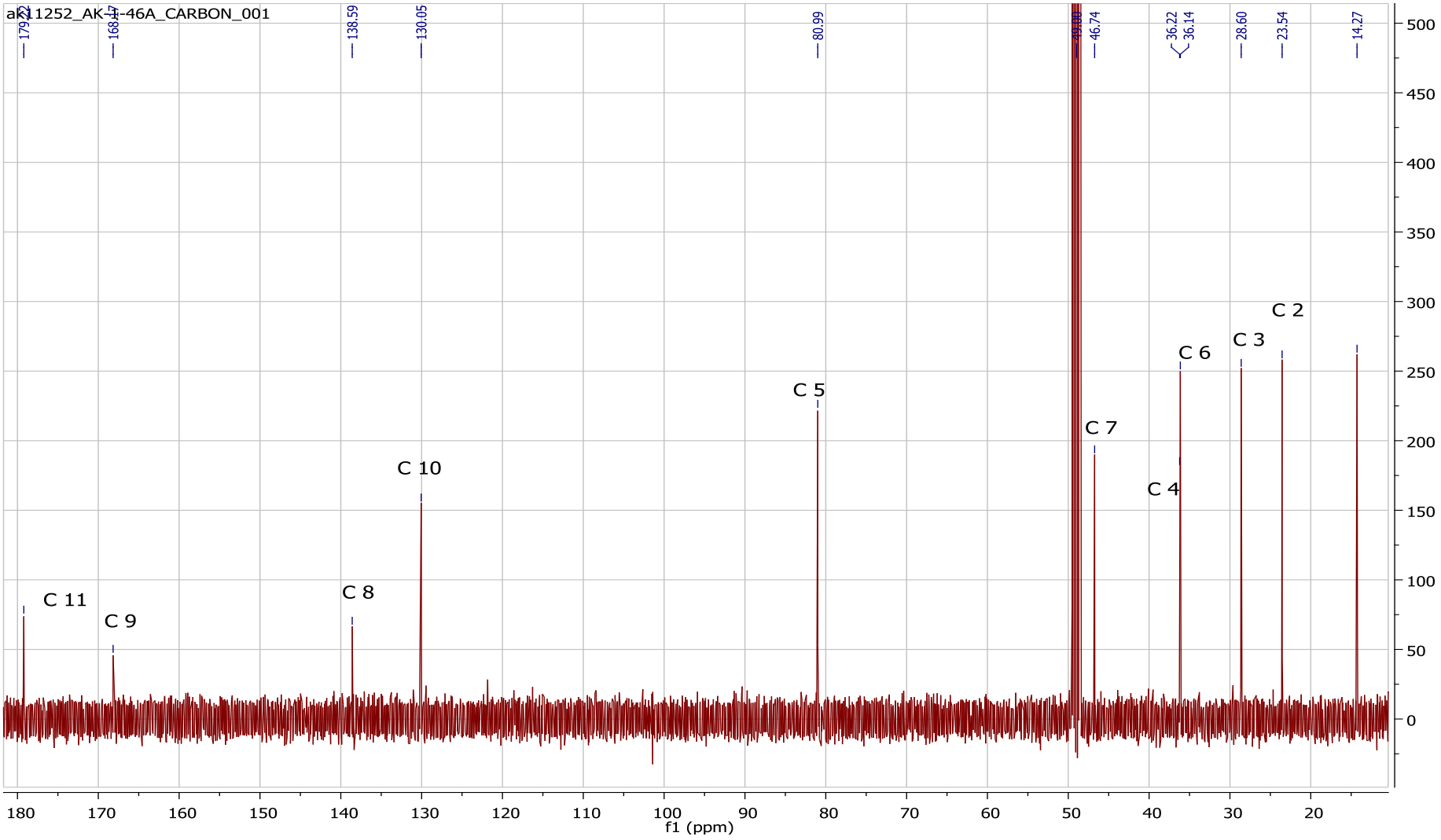
## SPECTRA OF THE NEW COMPOUNDS

# APPENDICES: SPECTRA OF THE NEW COMPOUNDS

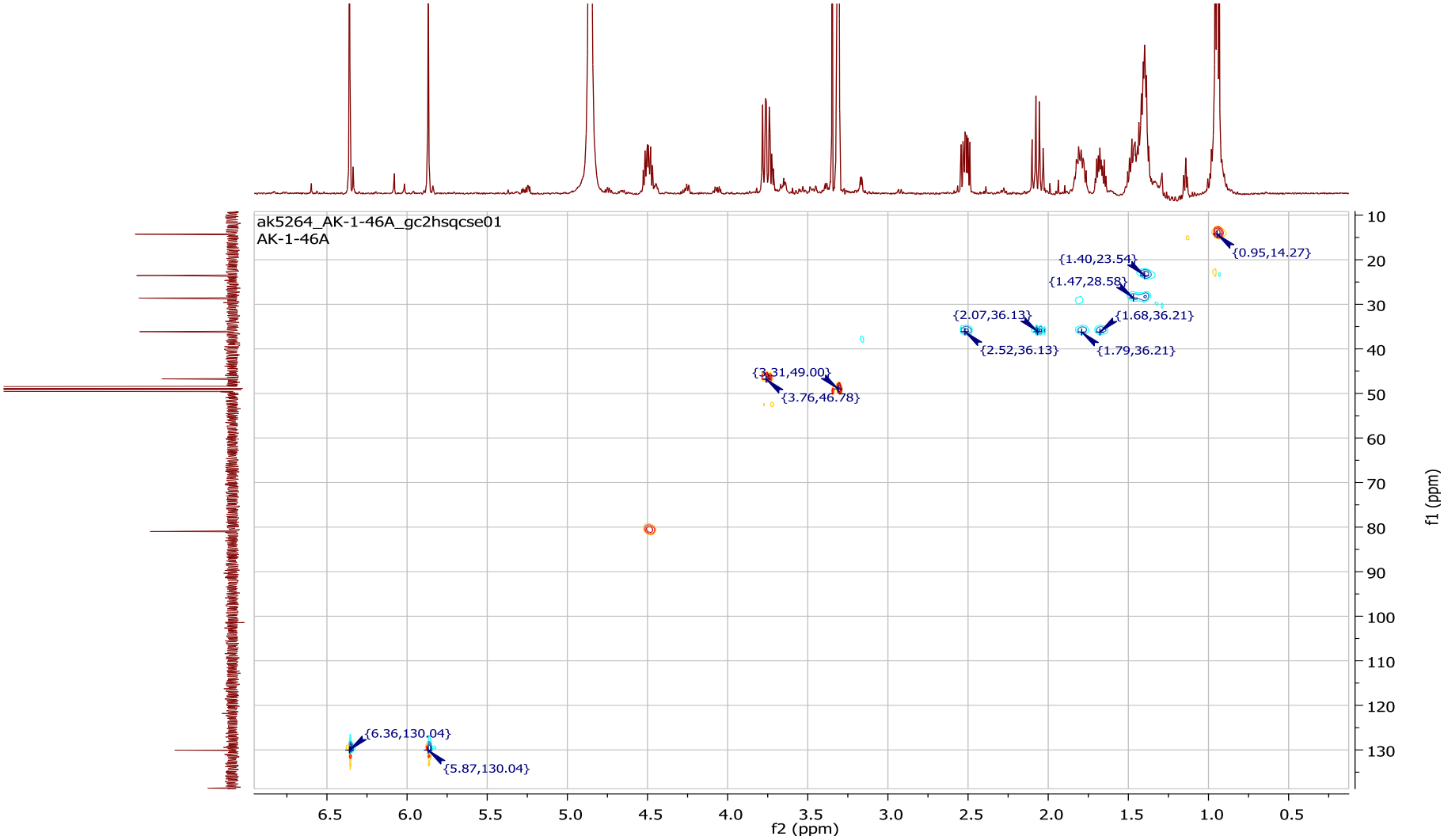
## APPENDIX I: $^{+}$H NMR of New Compound (A-41)



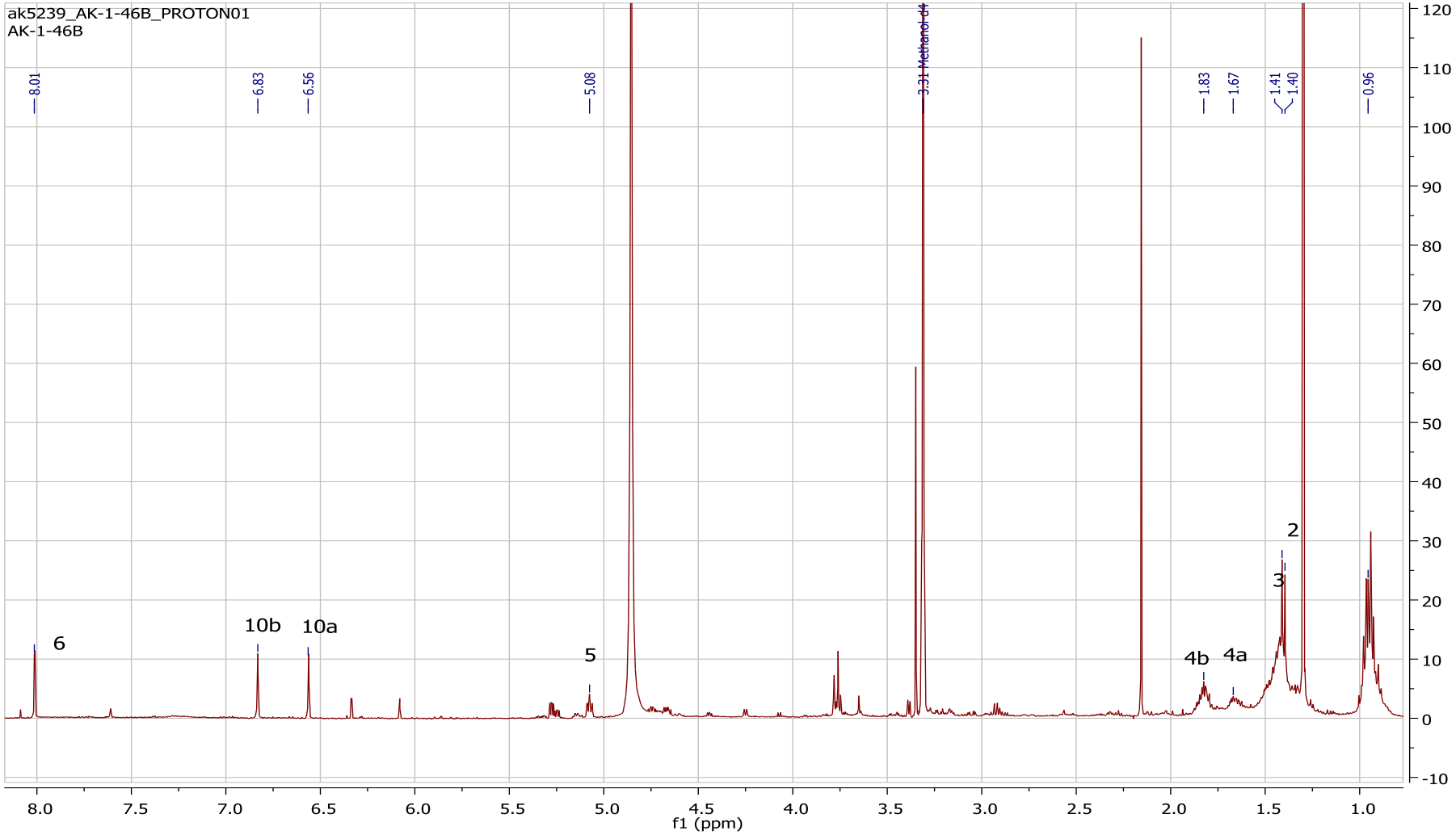
**APPENDIX II:** **$^{13}$C NMR of New Compound (A-41)**



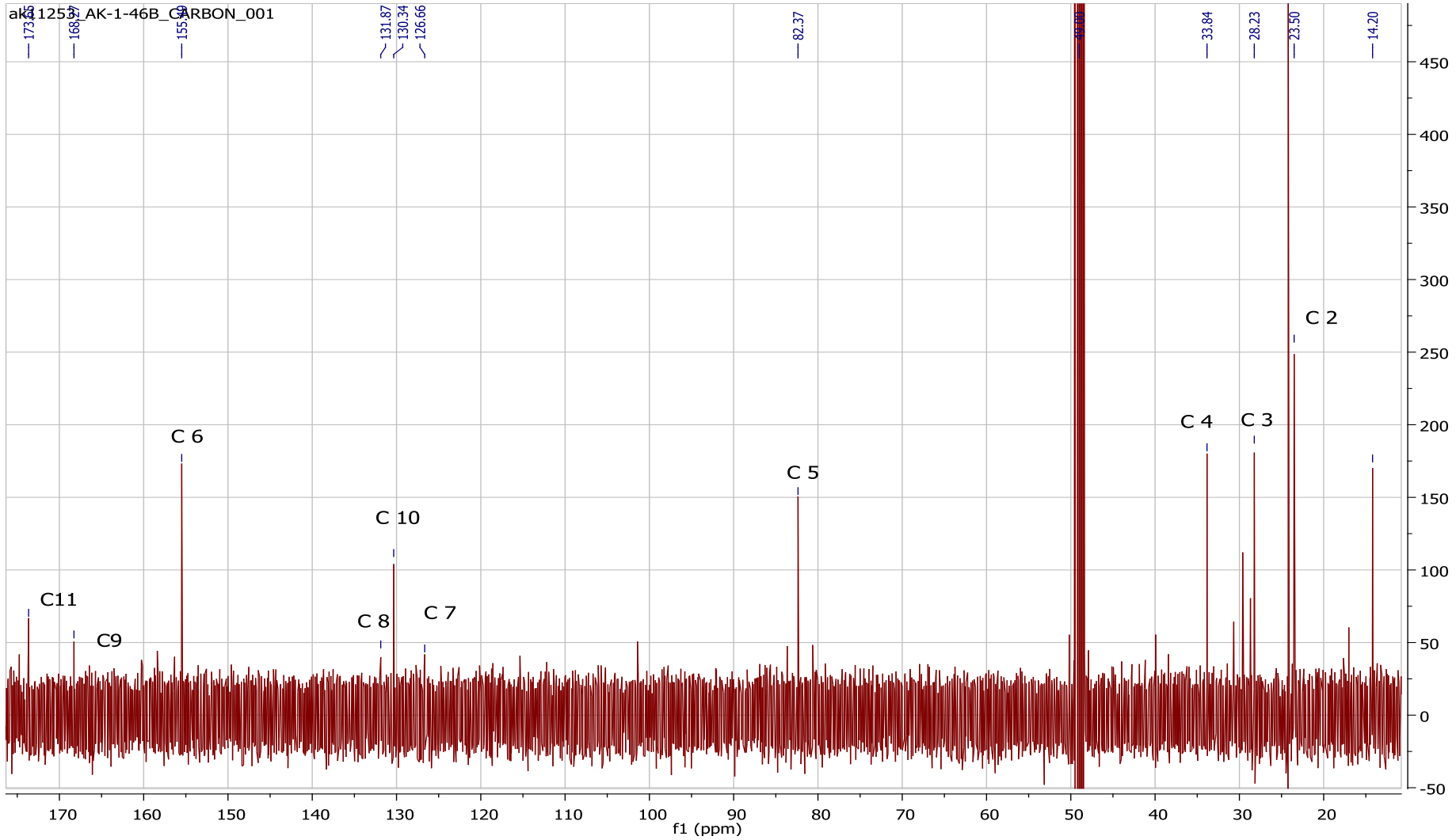
**APPENDIX III: HSQC 2D-NMR of New Compound (A-41)**



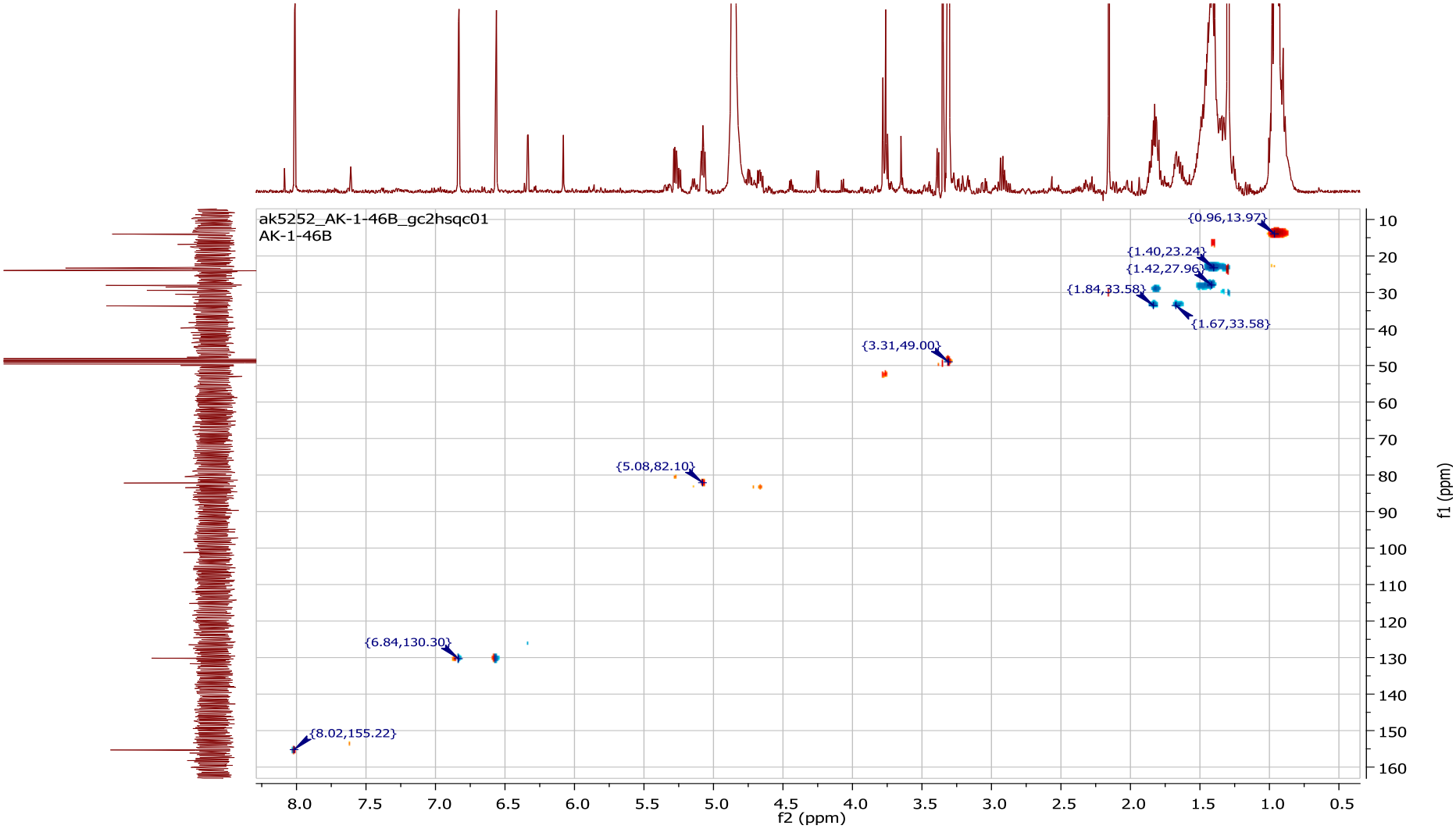
**APPENDIX IV: $^{+}$H NMR of New Compound (B-42)**



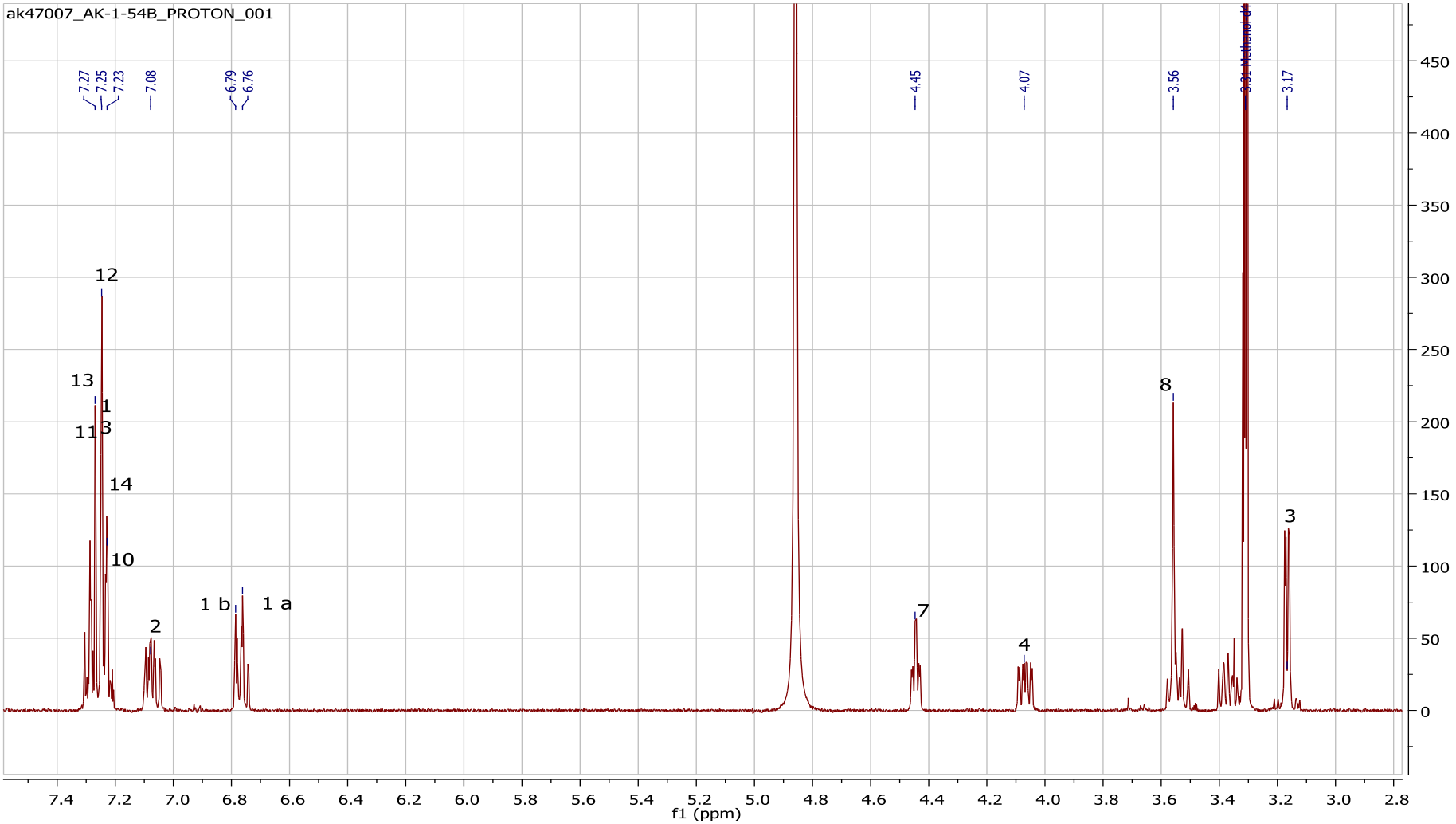
**APPENDIX V:** **$^{13}$C NMR of New Compound (B-42)**



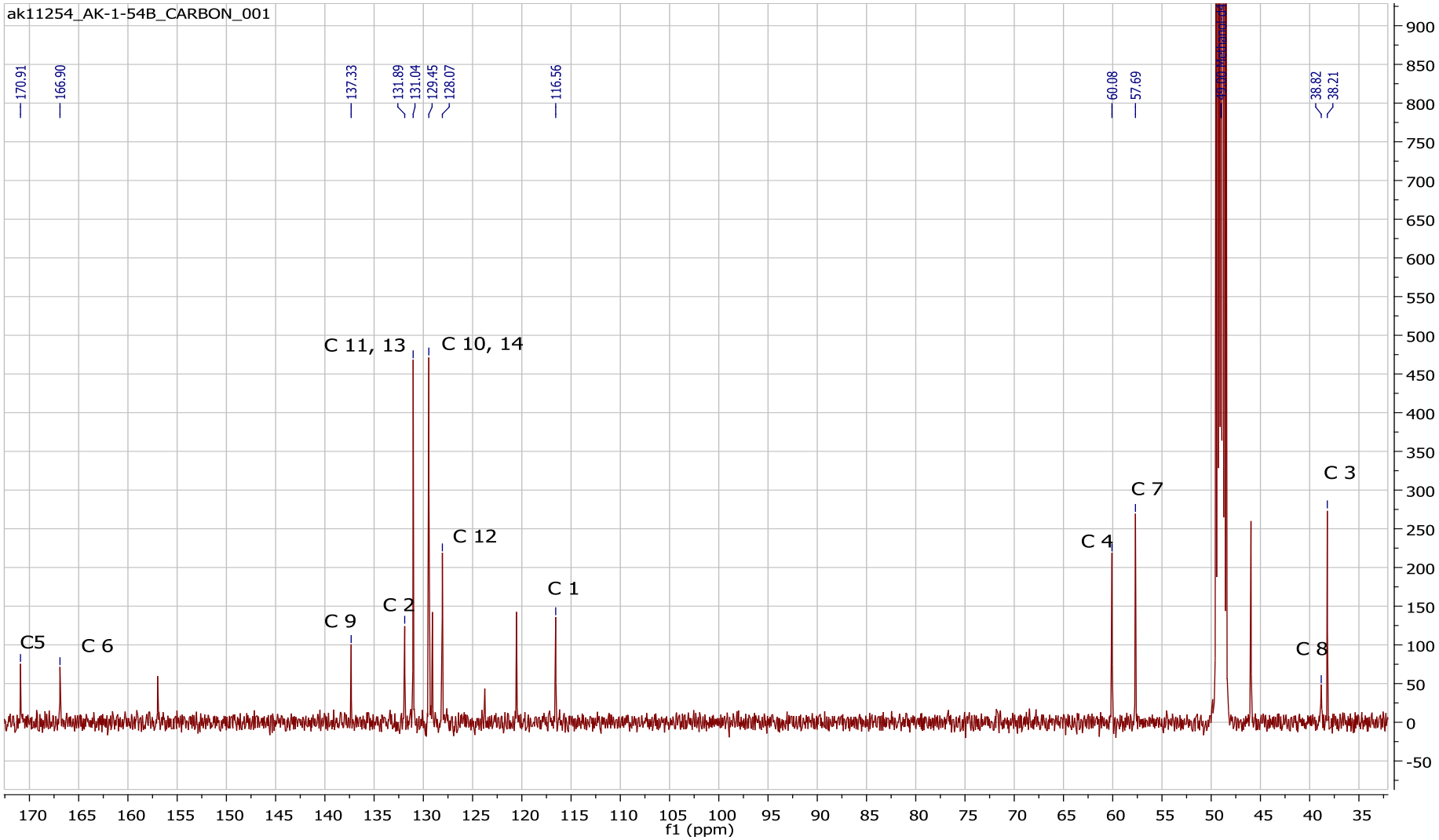
**APPENDIX VI: HSQC 2D-NMR of New Compound (B-42)**



**APPENDIX VII: $^{+}$H NMR of New Compound (D-44)**



**APPENDIX VIII: $^{13}$C NMR of New Compound (D-44)**



**APPENDIX IX: HSQC 2D-NMR of New Compound (D-44)**